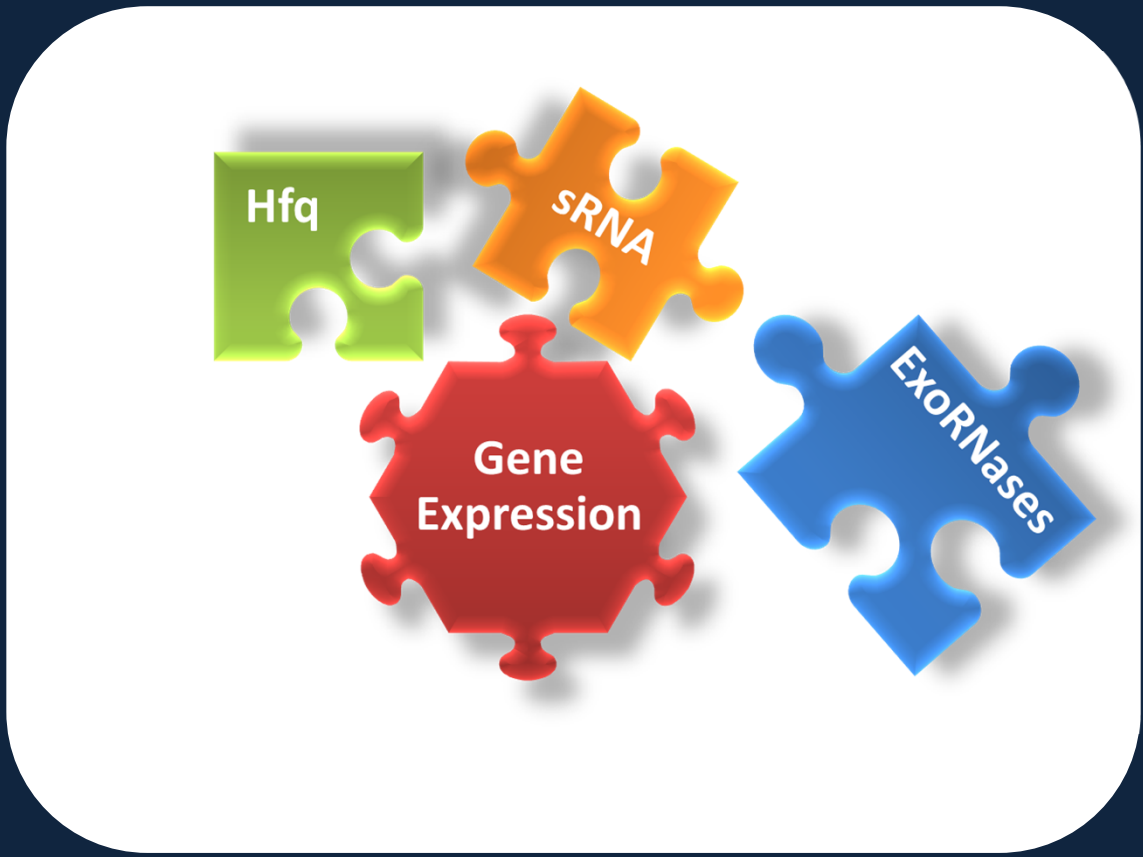
Interplay of exoribonucleases, Hfq and small RNAs structural determinants in the control of gene expression

Vânia Sofia Fidalgo Pobre



Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras, December, 2012

INSTITUTO DE TECNOLOGIA QUÍMICA E BIOLÓGICA /UNL

Knowledge Creation

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To my mother,
my father and
my brother

“If we knew what it was we were doing, it would not be called research, would it?”

— Albert Einstein

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Table of Contents

Abstract

Abstract

Small non-coding RNAs (sRNAs) are regulatory molecules that typically are not translated into proteins. These molecules are often highly structured and very stable and can affect many genetic pathways in all domains of life. Bacterial small regulatory RNAs (sRNAs) parallel microRNAs in their ability to control multiple targets. Small RNAs can bind to proteins or to mRNA targets. The sRNAs that act by an antisense mechanism can have full (*cis*-encoded) or partial complementarity (*trans*-encoded) with their targets. Most of the *trans*-encoded sRNAs studied so far in *Escherichia coli* bind the RNA chaperone Hfq. The 5' end of antisense RNAs is usually found to be critical for the interaction with targets, generally inhibiting translation and promoting mRNA decay. RNases are key elements in the control of RNA levels in the cell and not surprisingly are also critical in the regulation of sRNAs. In *E. coli* there are three 3'-5'exoribonucleases that accomplish most of the mRNA exodegradative activity: ribonuclease II (RNase II), ribonuclease R (RNase R) and polynucleotide phosphorylase (PNPase).

The main goal of this Doctoral work was to study the degradation pathways of sRNAs. It was already known that 3'-5' exonucleolytic degradation was a major regulatory pathway controlling the levels of the small non-coding MicA RNA, an important regulator of outer membrane protein expression. Besides ribonucleases there are other factors involved in the decay of sRNAs. In this work we addressed some of these factors and their functions in the degradation of sRNAs.

Hfq promotes sRNA-mRNA duplex formation and is important to stabilize sRNAs. However, the transient existence of sRNAs free from Hfq binding is part of the normal dynamic lifecycle of a sRNA. In the first part of this work, we studied the degradation pathways of sRNAs in the absence of Hfq. We have found that PNPase is the main ribonuclease involved in the rapid degradation of sRNAs, especially those that are not bound to Hfq. In Hfq^{-} cells the inactivation of PNPase

leads to increased levels of the sRNAs MicA, GlmY, RyhB and SgrS. We also found that in the absence of Hfq all sRNAs are trimmed at their 3'-end resulting in slightly shorter transcripts than their full-length species.

The turnover of Hfq-free sRNAs is growth-phase regulated and PNPase activity is particularly important in stationary phase. In fact, the contribution of PNPase to degradation of sRNAs is far greater than RNase E, which was commonly believed to be the main enzyme in the initiation of decay of sRNAs. The lack of poly(A) polymerase I (PAP I) also affects the degradation of Hfq-free sRNAs, although to a minor extent.

Small RNAs are not "innocent" molecules waiting to be degraded. The sequence and structural features of the small RNAs influence their degradation. In the second part of this work, we characterised the RNA determinants involved in the stability of the sRNA MicA and further analysed how this may influence the regulation of its targets. Based on MicA sequence and secondary structure we predicted the following MicA domains: a linear 5' end sequence; a structured module harbouring two stem loops, an internal A/U-rich sequence that is the predicted Hfq binding site and a transcriptional terminator with a U-rich linear 3' end. Mutations were introduced and designed to affect certain domains, but not the global secondary structure of the MicA.

Our results showed that besides the 5´domain of MicA, the stem loops and the 3´poly(U) tail are also important in target binding. *In vivo* and *in vitro* experiments showed that not only the AU-rich sequence but also the transcriptional terminator are critical for stability and Hfq-binding. The different MicA modules confer different stabilities and once again, PNPase was shown to be the most important exoribonuclease involved in MicA degradation. The specific MicA modules differentially affect the expression of the targets. Disruption of the 5´ region of MicA significantly affects all mRNA targets analysed. STEM2 was

found to be more important for the *in vivo* repression of both ompA and ecnB mRNAs while STEM1 was critical for regulation of tsx mRNA levels. Disruption of the 3´U-rich sequence greatly affects all the targets analysed. In conclusion, we found that MicA RNA can use different modules to regulate its targets.

In the third part of this work, we analysed the entire RNA content of the cell. To investigate the roles of the three main exoribonucleases we used a whole transcriptome sequencing approach (RNA-seq). We used cufflinks algorithm to determine the relative abundance of the transcripts and cuffdiff algorithm to find significant changes in transcript expression when comparing two samples. After this step, we clustered the differentially expressed transcripts into different functional categories using the program GeneCodis to retrieve gene ontology terms and integrate the diverse biological information.

We started by comparing the transcriptome changes that occur when cells go from exponential to stationary phase. We identified more than 1000 transcripts that were significantly different between the exponential and stationary wild-type samples. Most of these transcripts are somehow connected to the *E. coli* membrane and transport. We found that the three exoribonucleases have different roles depending on the growth phase. However, there is some overlap between PNPase, RNase II and RNase R functions in both exponential and stationary phases.

In exponential phase, RNase II significantly affected 187 transcripts. The majority of these transcripts belong to flagellar assembly and motility functional categories suggesting that RNase II mutant may present defects in motility. On the other hand, RNase R affected 202 transcripts of which the most interesting ones seems to link RNase R to anaerobic respiration. PNPase was the exoribonuclease whose mutation affected most transcripts, to a total of 226. Many of these

transcripts are stable RNAs (rRNAs, tRNAs and sRNAs) suggesting that PNPase might have a very important role in their metabolism.

Regarding stationary phase, RNase R seems to be the most important enzyme in RNA degradation. In a Δrnr mutant there are almost 700 transcripts that are differentially expressed, while Δrnb and Δpnp mutants only significantly affect 117 and 228 transcripts, respectively. On the other hand PNPase seems to be the most important exoribonuclease involved in the degradation of sRNAs. In the Δpnp mutant 41% of the *E. coli* sRNAs are up-regulated.

In summary, the work on this dissertation contributed to expand our knowledge not only on the small RNA degradation and mRNA targets control but also on the role of the exoribonucleases in RNA metabolism.

Resumo

Resumo

Pequenos RNAs não codificantes (sRNAs) são moléculas reguladoras que normalmente não são traduzidas em proteínas. Estas moléculas são na sua maioria muito estruturadas, muito estáveis e podem afectar múltiplas vias genéticas em todos os domínios da vida. Os pequenos RNAs reguladores (sRNAs) bacterianos são similares aos microRNAs na sua capacidade de controlar múltiplos alvos. Pequenos RNAs pode ligar-se a proteínas ou ao mRNA alvo. Os sRNAs que atuam por um mecanismo de antisense pode ter complementaridade completa (transcrito na mesma região mas em sentido contrário – cis) ou parcial (transcrito noutra região da sequência mas produzido em sentido contrário – trans) com os seus alvos. A maioria dos sRNAs trans-codificados estudados até agora em *Escherichia coli* ligam-se ao chaperone de RNA Hfq. A extremidade 5' dos sRNAs é geralmente considerada crítica para a interacção com os alvos, e geralmente inibem a tradução e promovem a degradação do mRNA. RNases são elementos chave no controle dos níveis de RNA na célula e também são fundamentais na regulação dos sRNAs. Em *E. coli*, há três 3'-5'exoribonucleases que realizam a maior parte da actividade degradativa do RNA: Ribonuclease II (RNase II), Ribonuclease R (RNase R) e “polynucleotide phosphorylase” (PNPase).

O objetivo principal deste trabalho de Doutoramento foi estudar as vias de degradação de sRNAs. Já se sabia que a degradação exonucleolítica 3'-5 ' era uma importante via reguladora no controlo dos níveis do pequeno RNA não-codificante MicA, um importante regulador da expressão das proteínas da membrana externa. Além das ribonucleases há outros fatores envolvidos na degradação de sRNAs. Neste trabalho abordamos alguns desses fatores e suas funções na degradação de sRNAs.

A proteína Hfq promove a formação de sRNA-mRNA duplexes e é importante para estabilizar os sRNAs. No entanto, a existência transitória de

sRNAs livres da ligação ao Hfq faz parte do ciclo de vida normal e dinâmico de um sRNA. Na primeira parte deste trabalho, foram estudados os processos de degradação dos sRNAs na ausência de Hfq. Nós descobrimos que a PNPase é a principal ribonuclease envolvida na rápida degradação de sRNAs, especialmente aqueles que não estão ligados ao Hfq. Em células sem Hfq a inactivação da PNPase conduz ao aumento dos níveis dos sRNAs MicA, GlmY, RyhB e SgrS. Também descobrimos que na ausência de Hfq todos os sRNAs são cortados na sua extremidade 3', resultando em transcritos ligeiramente mais curtos do que os transcritos de comprimento normal.

A degradação dos sRNAs não ligados ao Hfq é regulada de acordo com a fase de crescimento e a actividade da PNPase é particularmente importante na fase estacionária. De facto, a contribuição da PNPase na degradação de sRNAs é muito maior do que a da RNase E, a qual foi geralmente reconhecida como a principal enzima involvida na degradação de sRNAs. A deleção da “poly(A) polimerase I” (PAP I) afecta também a degradação de sRNAs não ligados ao Hfq, mas em menor grau.

Pequenos RNAs não são "inocentes" moléculas à espera de serem degradadas. A sequência e as características estruturais dos pequenos RNAs influenciam a sua degradação. Na segunda parte deste trabalho, foram caracterizados os determinantes do RNA envolvidos na estabilidade do sRNA MicA e ainda foi analisada a sua influência na expressão dos alvos do MicA. Com base na sequência e na estrutura secundária do MicA prevem-se os seguintes domínios no MicA: uma sequência linear na extremidade 5', um módulo estruturado com dois stem loops, uma sequência interna rica em A/U que é o local previsto para a ligação do Hfq e um terminador de transcrição com uma extremidade linear 3' rica em Uridinas. As mutações foram introduzidas e concebidas para afectar certos domínios, mas não a estrutura secundária global do MicA.

Os nossos resultados mostraram que, além do domínio 5' do MicA, os stem loops e a cauda 3'poly (U) também são importantes na ligação aos alvos. As experiências *in vivo* e *in vitro* mostraram que não só a sequência rica em A/Us, mas também o terminador de transcrição são críticos para a estabilidade e ligação com o Hfq. Os diferentes módulos do MicA conferem diferentes estabilidades e, mais uma vez, a PNPase mostrou ser a exoribonuclease mais importante envolvida na degradação do MicA. Os módulos específicos do MicA afetam diferencialmente a expressão dos alvos. Perturbação da região 5' do MicA não afecta significativamente o alvo *lam*B mRNA, no entanto os níveis de *omp*A e *ecn*B são dramaticamente aumentados. Em contraste, mutações nos stem loops aumentam fortemente os níveis do mRNA *lam*B, mas quase não afetam os mRNAs *omp*A e *ecn*B. Apenas interrupção da seqüência 3' rica em Us afeta muito todos os alvos analisados.

Na terceira parte deste trabalho, analisamos todo o RNA da célula. Para investigar os papéis das três principais exoribonucleases usamos uma abordagem de sequenciamento de todo o transcriptoma (RNA-seq). Usou-se o algoritmo cufflinks para determinar a abundância relativa dos transcritos e o algoritmo cuffdiff para encontrar mudanças significativas na expressão dos transcritos ao comparar duas amostras. Após este passo, agruparam-se os transcritos diferencialmente expressos em diferentes categorias funcionais, utilizando o programa GeneCodis para obter os termos da ontologia de genes e integrar a diversificada informação biológica.

Começámos por comparar as alterações que ocorrem no transcriptoma quando as células passam da fase exponencial para a fase estacionária. Foram identificados mais de 1000 transcritos significativamente diferentes entre as amostras wild-type em exponencial e em estacionária. A maioria desses transcritos estão de alguma forma ligado à membrana e ao transporte em *E. coli*.

Nós descobrimos que as três exoribonucleases têm papéis diferentes, dependendo da fase de crescimento. No entanto, existe alguma sobreposição entre os papéis da PNPase, da RNase II e da RNase R em ambas as fases exponencial e estacionária.

Na fase exponencial, a RNase II alterava significativamente 187 transcritos. A maioria destes transcritos pertencem ás categorias funcionais de montagem de flagelos e mobilidade sugerindo que o mutante da RNase II pode apresentar defeitos na mobilidade. Por outro lado, a RNase R alterava 202 transcritos, dos quais os mais interessantes parece ligar a RNase R com a respiração anaeróbica. A PNPase foi a exoribonuclease cuja mutação alterou mais transcritos, num total de 226. Muitos desses transcritos são RNAs estáveis (rRNAs, tRNAs e sRNAs) sugerindo que a PNPase pode ter um papel muito importante no seu metabolismo.

No que diz respeito fase estacionária, a RNase R parece ser a enzima mais importante na degradação de RNA. No mutante Δrnr há quase 700 transcritos que são diferencialmente expressos, enquanto que os mutantes Δrnb e Δpnp apenas afectam significativamente 117 e 228 transcritos, respectivamente. Por outro lado, a PNPase parece ser a mais importante exoribonuclease envolvida na degradação de sRNAs. No mutante Δpnp os níveis de 41% dos sRNAs de *E. coli* estão aumentados.

Em resumo, o trabalho nesta Dissertação contribuiu para expandir o nosso conhecimento, não só sobre a degradação de pequenos RNAs e controlo dos mRNA alvos, mas também sobre o papel das exoribonucleases no metabolismo do RNA.

List of Publications

The work presented in this Dissertation contributed to the following publications:

Papers in international scientific periodicals with referees:

Andrade J.M., **Pobre V**., Silva I.J., Domingues S., and Arraiano C.M. (2009). The role of 3´-5´exonucleases in RNA degradation. Progress in Nucl. Acids Res. and Molecular Biology Review, 85:187-229.

Arraiano C.M., Andrade J.M., Domingues S., Guinote I.B., Malecki M., Matos R.G., Moreira R.N., **Pobre V**., Reis F.P., Saramago M., Silva I.J. and Viegas S.C. (2010). The critical role of RNA processing and degradation in the control of gene expression. FEMS Microbiol Rev., Review, 34(5): 883-923.

Andrade J.M., **Pobre V**., Matos A.M. and Arraiano C.M. (2012). The crucial role of PNPase in the degradation of small RNAs that are not associated with Hfq. RNA 18: 844-855.

Matos RG, Bárria C, **Pobre V**, Andrade JM and Arraiano CM (2012). Exoribonucleases as modulators of virulence in pathogenic bacteria. Front. Cell. Inf. Microbio. 2:65. doi: 10.3389/fcimb.2012.00065

Pobre V, Andrade J.M. And Arraiano C.M. (2012). Small RNA modules confer different stabilities and selectively choose among different targets. Accepted with modifications.

Reis F, **Pobre V**, Silva IJ, Malecki M, Arraiano CM (2012). The RNB Family of Exonucleases – Putting the 'Dis' in Disease. Accepted in WIREs RNA

Chapters in books:

Matos R.G., **Pobre V**., Reis F.P., Malecki M., Andrade J.M. and Arraiano C.M. (2011) Structure and Degradation Mechanisms of 3' to 5' Exoribonucleases, Chapter 8 in Ribonucleases, Nucleic Acids and Molecular Biology Series. Allen W. Nicholson (Editor), Springer-Verlag Berlin Heidelberg.

Thesis Outline

Thesis outline

This dissertation is divided into five main chapters

Chapter 1 is a general introduction relating RNA degradation mechanisms and some factors involved in RNA. This introduction focuses mainly on the degradative ribonucleases, small RNAs, Hfq and their importance for RNA degradation.

The results of this Doctoral work are presented in the in the chapters 2, 3 and 4. Each of these chapters has its own Introduction, Results, Discussion, Materials and Methods and References sections.

Chapter 2 explores the degradation pathways of small RNAs in the absence of the RNA chaperone Hfq. It is shown that PNPase has a crucial role in the degradation of small RNAs especially if they are not associated with Hfq. It was demonstrated that under these conditions the PNPase contribution for sRNAs decay is even higher than RNase E, which had been considered the main ribonuclease involved in sRNA decay.

Chapter 3 analyses the role of the small RNA MicA sequence and structure in the stability of this sRNA and their function in target selectivity. It is shown that the different MicA modules confer different stabilities and that PNPase is the main exoribonuclease involved in the degradation of MicA. The 5´domain of MicA, the stem loops and the 3´poly(U) tail are also important in target-binding and the different MicA modules differentially affect the expression of the targets.

Chapter 4 studies the global roles of the 3'-5'exoribonucleases in the transcriptome of *Escherichia coli* by RNA-Seq. This advanced technology was used to identify the transcripts affected by RNase II, RNase R and PNPase in exponential and stationary cells. The three exoribonucleases have different roles depending on the growth phase. Some of their functions overlap. A deletion of both RNase II and RNase R is somehow compensated by the cell. PNPase is the main enzyme involved in small RNA degradation.

Chapter 5 is the final discussion based on the results from the previous chapters and connects the main results from this Dissertation. This chapter also includes future perspectives.

Chapter 1

Introduction

Keywords: RNA degradation, Ribonucleases, RNase E, RNase III, RNase II, RNase R, PNPase, small non-coding RNAs, Hfq.

This chapter was based on:

Arraiano CM, Andrade JM, Domingues S, Guinote IB, Malecki M, Matos RG, Moreira RN, **Pobre V**, Reis FP, Saramago M, Silva IJ, Viegas SC. 2010. The critical role of RNA processing and degradation in the control of gene expression. FEMS Microbiol Rev 34:883-923.

Andrade JM, **Pobre V**, Silva IJ, Domingues S, Arraiano CM. 2009. The role of 3'-5' exoribonucleases in RNA degradation. Prog Mol Biol Transl Sci 85:187-229.

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1 General Introduction

The RNA levels in the cell depend on the efficiency of the transcription, translation and the rate of degradation. Although transcription and translation are important to determine RNA steady state levels, the processing and degradation of RNA are also key factors in the regulation of gene expression. During this Dissertation the main focus will be on RNA degradation. Ribonucleases (RNases) are the enzymes that are able to process and degrade RNA. RNases are present in all domains of life, and play a central role in the control of gene expression by determining the levels of functional RNAs in the cell (Régnier & Arraiano, 2000; Arraiano & Maquat, 2003; Parker & Song, 2004). Many of the RNases in the cell are essential and others have overlapping functions (Régnier & Arraiano, 2000). They are also involved in the quality control of all types of RNAs, allowing the recycling of the ribonucleotides in the cell (Li et al., 2002; Silva et al., 2011). This introduction will focus on the ribonucleases involved in RNA degradation in *Escherichia coli*, which was the model organism throughout this Dissertation.

2 RNA Degradation pathways in *E. coli*

In order to degrade RNAs, ribonucleases can act alone or they can be part of RNA degradation complexes. Ribonucleases can be divided into endoribonucleases (which cleave the RNA molecules internally) and exoribonucleases (which degrade the RNA by removing terminal nucleotides from the 3' end of the RNA molecules). Exoribonucleases can act hydrolytically, releasing nucleotide monophosphates, or phosphorolytically, if they use inorganic phosphate to cleave the molecules releasing nucleotide diphosphates (Zuo & Deutscher, 2001). Exoribonucleases cooperate in RNA degradation even if they

can compete for access to the same substrate. In *E. coli* there are two endoribonucleases (RNase E and RNase III) and four exoribonucleases (RNase II, RNase R, PNPase and oligoribonuclease) involved in mRNA degradation (Table 1).

Table 1. Ribonuclease involved in RNA degradation in *E. coli*

Ribonuclease	Gene	Family	Comments
RNase E	*rne*	RNase E	Cleaves single stranded RNA.
RNase III	*rnc*	RNase III	Cleaves double stranded RNA.
RNase II	*rnb*	RNase II	Sensitive to RNA secondary structures. Can also protect RNA from degradation.
RNase R	*rnr*	RNase II	Highly effective against RNA duplexes. Stress induced protein. Growth-phase regulated.
PNPase	*pnp*	PDX	Degrades double stranded RNA when in multiprotein complexes.
Oligoribonuclease	*orn*	DEDD	Essential enzyme. Degrades the short oligoribonucleotides released from the degradative action of other exoribonucleases.

The RNA degradation pathways are not universal (Grunberg-Manago, 1999), and there are different mechanisms in bacteria (gram-positive versus gram-negative) and eukaryotes (Arraiano et al., 2010). However in all systems the intrinsic characteristics of both available enzymes and RNA seem to control the degradation of individual RNAs. Still, some common characteristics arise from the analysis of different RNA degradation pathways. Perturbation of RNA structural features may also work as an efficient degradation signal. Relaxation of secondary structures may result in an easier accessibility of RNases, namely exposing the 3' RNA end to exoribonucleolytic attack.

In *E. coli* the decay of the majority of transcripts starts with an endoribonucleolytic cleavage by RNase E (Figure 1). This endoribonuclease prefers a monophosphorylated 5' end but not in a strict way, and several RNAs that do not follow this rule have been described (Kime et al., 2010). RNase III is another enzyme responsible for the initial endoribonucleolytic cleavage of structured

RNAs. However, unlike RNase E (that only cleaves single-stranded RNAs) RNase III cleaves double-stranded RNAs.

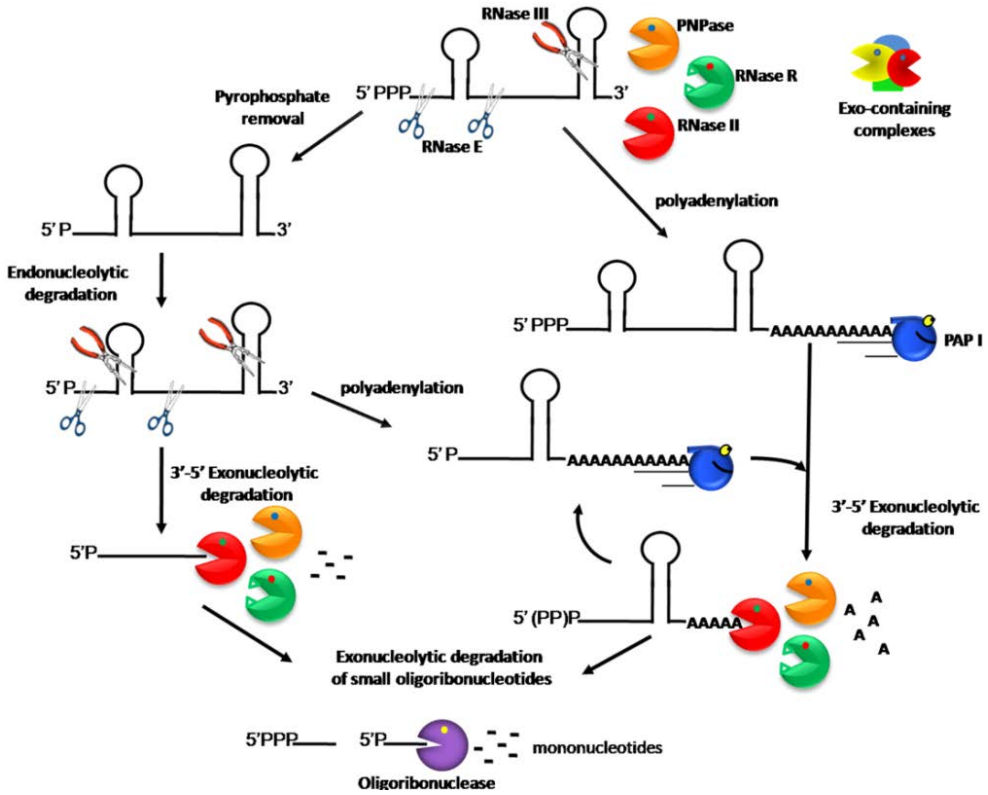

Figure 1. Model of RNA degradation pathways in *E. coli*.
The decay of the majority of transcripts starts with an endoribonucleolytic cleavage by RNase E (single stranded RNA) or RNase III (double stranded RNA). After endoribonucleolytic cleavages, the linear transcripts are rapidly degraded by the 3'–5' degradative exoribonucleases, RNase II, RNase R, and PNPase. RNase R, unlike RNase II and PNPase, is efficient against highly structured RNAs. PNPase in association with other proteins, namely RNA helicases, can also unwind RNA duplexes. A minor pathway in the cell is the exoribonucleolytic degradation of full-length transcripts. Poly (A) polymerase (PAP I) adds a poly (A) tail to the short 3' overhang. These tails provide a toehold to which exoribonucleases can bind. Cycles of polyadenylation and exoribonucleolytic digestion can overcome RNA secondary structures. The small oligoribonucleotides (2–5 nucleotides) released by exoribonucleases are finally degraded to mononucleotides by oligoribonuclease. Adapted from (Arraiano et al., 2010).

After endoribonucleolytic cleavages, the linear transcripts are rapidly degraded by the 3'–5' degradative exoribonucleases, RNase II, RNase R, and PNPase. RNase II and PNPase are sensitive to secondary structures while RNase R is the only exoribonuclease efficient against highly structured RNAs. However, PNPase can associate with other proteins, namely RNA helicases, to unwind RNA duplexes and consequently degrade structured RNAs (Andrade et al., 2009b). A smaller pathway in the cell does not require the initial endoribonucleolytic cleavage, instead polyadenylation emerges as important factor controlling the exoribonucleolytic activity (Dreyfus & Régnier, 2002). It extends the 3' linear region providing a toehold that exoribonucleases can use to bind and initiate degradation. Interestingly, this mechanism was conserved through evolution and destabilizing poly(A) tails also promote exonucleolytic RNA degradation in eukaryotes (LaCava et al., 2005; Vanácová et al., 2005; Wyers et al., 2005).

3 RNase E

RNase E, encoded by *rne* gene, was first identified by a temperature-sensitive mutation (*rne-3071*) (Apirion & Lassar, 1978) and was initially described as an activity required for the processing of *E. coli* 9S rRNA (Ghora & Apirion, 1978). The *ams* (*a*ltered *m*RNA *s*tability) locus was identified by a temperature-sensitive mutation (*ams-1*) (Ono & Kuwano, 1980) and was shown to have an important role in *E. coli* turnover (Ono & Kuwano, 1979). Later it was shown that these two previously identified genes, *rne* and *ams* were actually different mutant alleles of the same gene encoding RNase E (Mudd et al., 1990; Babitzke & Kushner, 1991; Melefors & von Gabain, 1991; Taraseviciene et al., 1991). This important endoribonuclease is essential for cell growth, and inactivation of temperature-sensitive mutants inhibits processing and prolongs the lifetime of bulk mRNA

(Apirion & Lassar, 1978; Ono & Kuwano, 1979; Arraiano et al., 1988; Mudd et al., 1990; Babitzke & Kushner, 1991; Melefors & von Gabain, 1991; Taraseviciene et al., 1991). It has been described that RNase E plays a central role in the processing of precursors of 5S ribosomal RNA (Apirion & Lassar, 1978; Misra & Apirion, 1979), 16S ribosomal RNA (Li et al., 1999), tRNAs (Ow & Kushner, 2002), tmRNA (Lin-Chao et al., 1999) and the M1 RNA component of the RNase P ribozyme (Lundberg & Altman, 1995; Ko et al., 2008). Homologous of RNase E have been identified in more than 50 bacteria, archaea, and plants (Lee & Cohen, 2003).

3.1 RNase E structure and function

E. coli RNase E is a 1061 residue enzyme composed of two distinct functional regions. The amino-terminal half forms the catalytic domain (residues 1–529) and is relatively conserved among prokaryotes (Marcaida et al., 2006). The carboxy-terminal half of RNase E (residues 530-1061) is a non-catalytic region, largely unstructured and poorly conserved (Callaghan et al., 2004). Segment-A is located between residues 565 and 582 and is responsible for binding of the full length RNase E to the inner cytoplasmic membrane (Khemici et al., 2008). However, segment-A is not necessary for membrane interaction of the catalytically active segment (Murashko et al., 2012). Residues 601–700 form an arginine-rich segment that binds RNA *in vitro* and that is believed to enhance the activity of RNase E in mRNA degradation *in vivo* (Lopez et al., 1999; Ow et al., 2000). Residues 701– 1061 form a scaffold for interactions between RNase E and the other major components of the degradosome, a protein complex involved in mRNA decay (Kaberdin et al., 1998; Vanzo et al., 1998).

The first crystal structure for a member of the RNase E family has been determined at 2.9 Å, and it reveals that the catalytic domain of RNase E forms a

homotetramer with a molecular mass of roughly 260 kDa, organized as a dimer of dimers (Callaghan et al., 2005). Each protomer is composed of two globular portions, the large and small domains. The large domain consists of several subdomains including the 5'-sensor as well as subdomains structurally similar to protein folds found in S1, DNase I, and RNase H. In isolation each protomer appears elongated, with a large domain comprising the subdomains (S1, 5'-sensor, RNase H and DNase I), an elongated linker region (Zn-link) and then the small domain. The dimer-dimer interface is formed by the small domains. At the junction point there is a zinc binding site (Callaghan et al., 2005). The arrangement of the domains within each dimer resembles the blades and handles of an open pair of scissors. The positively charged surface within RNase H, 5' sensor and DNase I subdomains mediate the interaction of the catalytic domain of RNase E with the membrane (Murashko et al., 2012).

E. coli RNase E is a single-stranded, nonspecific endoribonuclease with a preference for cleaving A/U-rich sequences (Mackie, 1992; McDowall et al., 1995). *In vitro* experiments have shown that purified *E. coli* RNase E prefers to cleave RNAs that are monophosphorylated at the 5' end (Mackie, 1998). It was shown that RppH (RNA pyrophosphohydrolase) converts the 5' terminus of primary transcripts from a triphosphate to a monophosphate (Celesnik et al., 2007; Deana et al., 2008). However, some structured substrates can be cleaved independently of its state of phosphorylation by RNase E even if the 5' end forms a secondary structure (Baker & Mackie, 2003; Hankins et al., 2007). This indicates that while 5'-monophosphate-dependent pathway makes a significant contribution to mRNA degradation (Mackie, 1998, 2000), there is another pathway of initial substrate recognition by RNase E termed 'bypass' or 'internal entry' (Baker & Mackie, 2003; Kime et al., 2010). The requirements for this pathway seems to be only the existence of multiple single stranded segments in a conformation that allows interaction with RNase E (Kime et al., 2010).

The crystal structure explains some features of the protein and suggests a mechanism of RNA recognition and cleavage. A pocket is formed between the 5' sensor and the RNase H subdomains and can bind a monophosphate group at a 5' end (Callaghan et al., 2005). The catalytic site is physically separated of the 5' sensing site. It contains conserved residues on the surface of the DNase I subdomain of RNase E and coordinate a magnesium ion implicated in catalysis. A 'mouse-trap' model for communication between the 5' sensing pocket and the site of catalysis has been suggested. S1 and 5' sensing domains move together as one body to clamp down the substrate (Koslover et al., 2008). This conformational change suggests a mechanism of RNA recognition and catalysis that explains the enzyme's preference for substrates with a 5'-monophosphate over a 5'-triphosphate and 5'-hydroxy RNA. It was also observed substantial flexibility at one of the dimer-dimer interfaces, a deformation that may be essential to accommodate structured RNA for processing by internal entry.

3.2 Control of RNase E expression

The cellular level and activity of RNase E are subject to complex regulation. First, the enzyme concentration in the cell is regulated by a feedback loop in which RNase E modulates decay of its own mRNA maintaining the level of the enzyme within a narrow range (Mudd & Higgins, 1993; Jain & Belasco, 1995; Diwa et al., 2000; Sousa et al., 2001; Ow et al., 2002). Recently it was shown that the 5' sensor domain is essential for efficient autoregulation of RNase E (Garrey & Mackie, 2011). Second, the efficiency of RNase E cleavage depends on the structure of the substrates and the accessibility of putative cleavage sites. A 5' monophosphate in substrate RNAs serves as an allosteric activator of RNase E activity (Mackie, 1998; Jiang & Belasco, 2004). Third, interactions of mRNA targets with Hfq and small RNAs exert an important role on the cleavage of certain

mRNAs by RNase E (Wagner et al., 2002). Fourth, the activity of RNase E is globally affected by protein inhibitors, namely L4 ribosomal protein, RraA and RraB (*r*egulator of *r*ibonuclease *a*ctivity A and B, respectively) that interact with RNase E and inhibit RNase E endonucleolytic cleavages of a selective group of transcripts (Lee et al., 2003; Gao et al., 2006). Fifth, the membrane localization of RNase E and its association with the bacterial cytoskeleton may affect its function through various mechanisms (Liou et al., 2001; Khemici et al., 2008; Taghbalout & Rothfield, 2008).

3.3 Relating RNase E and RNase G

RNase G is a paralogue of RNase E (McDowall et al., 1993), belonging to the RNase E/G family, and is also involved in the degradation and processing of RNA (Carpousis et al., 2009). *E. coli* RNase G was initially identified by its role in chromosome segregation and cell division (Okada et al., 1994). RNase G was subsequently shown to exhibit endoribonuclease activity both *in vivo* (Li et al., 1999; Wachi et al., 1999; Umitsuki et al., 2001) and *in vitro* (Jiang et al., 2000; Tock et al., 2000). A strong resemblance has been identified between RNase G and the amino-terminal portion of *E. coli* RNase E, sharing a high level of sequence identity (35%) and similarity (50%) (McDowall et al., 1993). Purified RNase G has *in vitro* properties similar to RNase E and both enzymes are required for a two-step sequential reaction of 5' maturation of the 16S rRNA gene (Li et al., 1999; Wachi et al., 1999). Residues of RNase E that can contact a 5'-monophosphorylated end and coordinate the catalytic magnesium ion are conserved in RNase G (McDowall et al., 1993; Callaghan et al., 2005). The precise cleavage sites of RNase E and RNase G are not strictly conserved (Li et al., 1999; Tock et al., 2000). The 5'-monophosphate end, which stimulates RNase G, is generated by RppH (Deana et al., 2008) or by other endoribonucleases (Lee et al., 2002). Recently it was shown

that RNase G interaction with a single-stranded segment, linked physically to a 5'-monophosphorylated-end, is an important determinant of the overall affinity of RNA binding (Jourdan et al., 2010). Moreover, it was demonstrated that the sequence of a site bound by RNase G can moderate the maximal cleavage rate (Jourdan et al., 2010). RNase G is a paralogue of RNase E but up to now most of the research on RNA degradation has been focusing on RNase E.

3.4 RNase E in other organisms

Some variants of RNase E can be found in α-Proteobacteria*, Synechocystis sp.* and in the high G+C Gram-positive bacteria (Condon & Putzer, 2002). In *Rhodobacter capsulatus*, RNase E is the responsible enzyme for the majority of the endonucleolytic cleavages. In this organism RNase E has 118 KDa with a conserved N-terminal region (Jager et al., 2001) and a C-terminal portion, probably involved in the scaffold of degradosome assembly. It was purified in two different complexes, one where it is associated with a helicase and an unidentified protein, while in the other one was coupled with a helicase, the transcription terminator Rho and an unidentified protein (Jager et al., 2001). Moreover, in *R. capsulatus,* this enzyme is involved in the endonucleolytic process and stabilization of *cspA* mRNA (Jager et al., 2004). Similarly to *R. capsulatus, Pseudomonas syringae*, a psychrophilic bacterium, has also an RNase E which is associated with RNase R and the DEAD-box helicase RhlE in a degradosome (Purusharth et al., 2005).

4 Ribonuclease III

Ribonuclease III (RNase III) was originally identified by Robertson and co-workers in extracts of *E. coli* as the first specific double-stranded RNA (dsRNAs) endoribonuclease (Robertson et al., 1968). Members of RNase III family are widely distributed among prokaryotic and eukaryotic organisms, sharing structural and functional features (Lamontagne et al., 2001). However, until now homologues of RNase III have not been found in the genomes of archaea (Condon & Putzer, 2002).

4.1 RNase III family of enzymes

The RNase III family comprises four classes, according to their polypeptide structure. The class I members of the RNase III family are ubiquitously found in bacteria, bacteriophages and some fungi (MacRae & Doudna, 2007).

The Class II is exemplified by the eukaryotic Drosha protein while the class III is represented by the eukaryotic Dicer (MacRae & Doudna, 2007). The nucleases Drosha and Dicer have very important roles in RNA interference. Finally, the Class IV is represented by the Mini-RNase III of *Bacillus subtilis* (Redko et al., 2008). Taken together, the functional and evolutionary conservation of RNase III family in bacteria and higher organisms is indicative of their biological relevance in RNA maturation and degradation. Despite the fact that RNase E is considered the major ribonuclease that catalyses the initial rate-determining cleavage of several transcripts, RNase III family of enzymes has emerged as one of the most important group of endoribonucleases in the control of RNA stability (Jaskiewicz & Filipowicz, 2008).

4.2 RNase III structure and substrate recognition

E. coli RNase III has served as the prototypical member of the family. In this model microorganism, RNase III is encoded by the *rnc* gene, and is active as a 52 kDa homodimer (Li & Nicholson, 1996). Each monomer contains a C-terminal dsRBD, located in the last 74 amino acids, which is responsible for substrate recognition and adopts a tertiary fold with the characteristic α_1-β_1-β_2-β_3-α_2-structure that is conserved throughout the RNase III family (Blaszczyk et al., 2001). Additionally, each monomer is also composed by an N-terminal NucD. When the two monomers are combined (RNase III homodimer), they form a single processing center in the subunit interface, in which each monomer contributes to the hydrolysis of one RNA strand of the duplex substrate. Ji and collaborators (Blaszczyk et al., 2004; Gan et al., 2006) solved the structure of the hyperthermophilic bacteria *Aquifex aeolicus* RNase III and the data has revealed two functional forms of dsRNA binding by RNase III: a catalytic form, functioning as a dsRNA-processing enzyme, cleaving both natural and synthetic dsRNA; and a non-catalytic form, in which RNase III has a role of dsRNA binding protein (without cleaving). The later activity is in agreement with previous studies in which this enzyme binds certain substrates in order to influence gene expression, affecting RNA structures (Calin-Jageman & Nicholson, 2003), (Court, 1993; Oppenheim et al., 1993; Dasgupta et al., 1998). Magnesium (Mg^{2+}) is the preferred co-factor. Recent data are indicative that each active site contains two divalent cations during substrate hydrolysis (Meng & Nicholson, 2008).

The RNase III substrate selection consists in a combination of structural determinants and sequence elements referred as reactivity epitopes, such as the helix length, the strength of base-pairing or the occurrence of specific nucleotide pairs (termed proximal and distal boxes) located at defined positions related to the cleavage site. In addition, there are also two classes of double-helical elements that can function as negative determinants, which can either inhibit the

recognition of this endoribonuclease or suppress the cleavage (without affecting recognition) (Zhang & Nicholson, 1997; Pertzev & Nicholson, 2006).

4.3 RNase III activity and function

All enzymes of this family are hydrolytic and have specificity for dsRNAs, generating 5' monophosphate and 3' hydroxyl termini with a two base overhang at the 3' end (Meng & Nicholson, 2008). RNase III in *E. coli* is not essential, however it was observed that mutants for this endoribonuclease have a slow-growth phenotype (Nicholson, 1999). This enzyme was initially identified due to its role in the maturation of tRNA precursors and rRNA. Regarding maturation of rRNA, RNase III is involved in the processing of 16S and 23S from a 30S rRNA precursor (Babitzke *et al*. 1993). In *Salmonella* and other members of α-proteobacteria, RNase III is also responsible for the cleavage of intervening sequences (IVS) found in their 23S rRNA (Evguenieva-Hackenberg & Klug, 2000). RNase III is also involved in the decay of several mRNA species (Condon & Putzer, 2002; Calin-Jageman & Nicholson, 2003). For example, in *E. coli*, this enzyme participates in the first step of the decay of *pnp* mRNA (Régnier & Portier, 1986), the gene encoding Polynucleotide Phosphorylase (PNPase), downregulating its synthesis (Régnier & Grunberg-Manago, 1990; Robert-Le Meur & Portier, 1992; Jarrige et al., 2001). Interestingly, this endoribonuclease has also the ability to regulate its own synthesis with a specific cleavage near the 5' end of its own mRNA that removes a stem-loop, which acts as a degradation barrier (Bardwell et al., 1989; Matsunaga et al., 1996; Lioliou et al., 2012). A recent work show that in *Staphylococcus aureus* RNase III is involved in rRNA and tRNA maturation and regulates the turnover of mRNAs and non-coding RNAs (Lasa et al., 2011; Lioliou et al., 2012).

RNase III has been seen to work as a stress response modulator, controlling the steady state levels of genes involved in cellular adaptation to stress (Santos et al., 1997; Freire et al., 2006; Sim et al., 2010). It was seen in *Salmonella typhimurium* that RNase III regulates the levels of the small RNA (sRNA) MicA (Viegas et al., 2007), a main regulator of the abundant outer membrane protein OmpA that has an important structural role in the cell and is involved in pathogenesis (Guillier et al., 2006). The enzyme is also involved in the decay of sRNA/mRNA complexes upon translational silencing (Vogel et al., 2004; Afonyushkin et al., 2005), (Huntzinger et al., 2005; Kaberdin & Blasi, 2006). In this way, cleavage by RNase III within the sRNA/mRNA duplex and the resulting subsequent decay of the mRNA intermediate by the *E. coli* RNA decay machinery could resemble the RNA interference (RNAi) in the eukaryotic cells (Agrawal et al., 2003). RNAi is an evolutionary conserved phenomenon that functions as a safeguard for the maintenance of genomic integrity. This phenomenon permits the selective post-transcriptional downregulation of target genes in the cells, in which RNase III-like enzymes dictate the degradation of dsRNA molecules (Jagannath & Wood, 2007; Ma et al., 2007; Jinek & Doudna, 2009). Accordingly, RNase III family has been associated with gene expression regulation, potential antivirus agent, and tumor suppressor (Lamontagne et al., 2001).

5 RNase II

E. coli RNase II is a 3'-5' exoribonucleases and is the prototype of the RNase II family of enzymes (Mian, 1997; Mitchell et al., 1997; Zuo & Deutscher, 2001; Grossman & van Hoof, 2006). RNase II-like proteins are widespread among the three domains of life and in eukaryotes they are the catalytic component of the exosome (Liu et al., 2006; Dziembowski et al., 2007). The exosome is a multi-

protein complex involved in RNA degradation in eukaryotes. In *Saccharomyces cerevisiae* Rrp44/Dis3 is the RNase II family member. Rrp44 has some extra domains at the N-terminal (CR3 and Pin) and can work also as an endoribonuclease (Schaeffer et al., 2009). In *Schizosaccharomyces pombe* and mammals there are several important members of this family such as Dis3, Dis3L and Dis3L2 (Tomecki et al., 2010; Malecki et al., 2012).

5.1 Control of RNase II expression

RNase II is encoded by the *rnb* gene that can be transcribed from two promoters P1 and P2 and terminates in a Rho-independent terminator 10 nucleotides downstream of *rnb* stop codon (Zilhão et al., 1993; Zilhão et al., 1995a; Zilhão et al., 1996). PNPase regulates RNase II expression by degrading the *rnb* mRNA (Zilhão et al., 1996). RNase III and RNase E endoribonucleases are also involved in the control of RNase II expression at the post-transcriptional level. RNase III does not affect *rnb* mRNA directly, but affects PNPase levels and RNase E is directly involved in the *rnb* mRNA degradation (Zilhão et al., 1995b).

RNase II is also post-translationally regulated at level of protein stability and its levels are also adjusted according to growth conditions. *gmr* (Gene Modulating RNase II) is located downstream of *rnb* and is involved in the modulation of stability of RNase II (Cairrão et al., 2001). Gmr has a PAS domain which can act as an environmental sensor detecting changes in growth conditions.

5.2 RNase II activity and RNA degradation

E. coli RNase II is a sequence-independent hydrolytic exoribonuclease that processively degrades RNA in the 3'-5' direction, yielding 5'-nucleoside

monophosphates. However, the processive degradation of an RNA molecule by RNase II is easily blocked by secondary structures, and the enzyme is known to stall around seven nucleotides before it reaches a double-stranded region (Cannistraro & Kennell, 1999; Spickler & Mackie, 2000). In *E. coli* RNase II is the major hydrolytic enzyme and participates in the terminal stages of mRNA degradation (Deutscher & Reuven, 1991). However the enzyme is not essential for *E. coli* growth unless PNPase is also missing (Donovan & Kushner, 1986; Zilhão et al., 1995a). Although RNase II degrading activity is sequence-independent, its favourite substrate is the homopolymer poly(A). Since the presence of a poly(A) tail is often needed for the RNA degradative process, the rapid degradation of polyadenylated stretches by RNase II can paradoxically protect some RNAs by impairing the access of other exoribonucleases (Hajnsdorf et al., 1994; Pepe et al., 1994; Coburn & Mackie, 1996; Marujo et al., 2000; Mohanty & Kushner, 2000; Folichon et al., 2005b). Indeed, in the absence of RNase II a large number (31%) of *E. coli* mRNAs are decreased, especially ribosomal protein genes, suggesting a major function for this enzyme in the protection of specific mRNAs through poly(A) tail removal (Mohanty & Kushner, 2003).

5.3 RNase II structure and function

The structure of *E. coli* RNase II and its RNA-bound complex was determined (Frazão et al., 2006). This was the first structure of an exoribonuclease from the RNase II family that has been solved (Frazão et al., 2006). The overall X-ray crystallographic structure of the wild-type enzyme (Frazão et al., 2006; Zuo et al., 2006) revealed four domains, as previously predicted by Amblar *et al.* (Amblar et al., 2006). Three RNA binding domains have been identified: two cold shock domains (CSD1 and CSD2) in the N-terminal region and an S1 RNA-binding domain at the C-terminus. The catalytic site resides in the

central RNB domain, whose structure has shown an unprecedented fold characteristic of this family. This domain contains four highly conserved sequence motifs (I-IV) with some invariant carboxylate residues (Mian, 1997). The RNA-binding domains (CSD1, CSD2 and S1) are grouped together on one side of the structure, while the active site is on the other side of the molecule (Frazão et al., 2006).

Elimination of the N-terminal CSD1 resulted in an increase of the RNA-binding affinity of the enzyme for poly(A), suggesting that this domain may have a role in controlling the movement of the enzyme on the poly(A) chain (Amblar et al., 2006; Arraiano et al., 2008). Interestingly, without all the RNA-binding domains the enzyme is still able to degrade RNA, although with much less efficiency than the wild-type enzyme (Matos et al., 2009; Vincent & Deutscher, 2009).

The structure of the RNA-bound enzyme revealed that the RNA fragment interacts with the protein at two non-contiguous regions, the "anchor" and catalytic regions (Cannistraro & Kennell, 1994; Frazão et al., 2006). Nucleotides 1-5, at the 5'-end of the RNA fragment, are located in the "anchor" region in a deep cleft between the two CSDs and the S1 domain. The final nucleotides 9-13 are located in a cavity deep within the RNB domain, stacked and "clamped" between the conserved residues Phe358 and Tyr253. A 10-nucleotide fragment is the shortest RNA able to retain contacts with both anchor and catalytic regions. This fact explains why RNase II is processive on long RNA molecules but becomes distributive on substrates shorter than 10-15 nucleotides. When the RNA molecule is shorter than five nucleotides, the required packing of the bases can no longer occur, preventing the translocation of the RNA and a final end product of four nucleotides is released (Frazão et al., 2006). Tyr-253 has been identified as the responsible residue for setting the RNase II end-product, and its substitution

was shown to change the smallest end product of degradation from 4 to 10 nucleotides (Barbas et al., 2008). This mutation has been proposed to cause loosening of the RNA substrate at the catalytic site and, as a consequence, binding at the anchor region would be essential to keep the RNA attached to the protein and allow cleavage. Molecules shorter than 10 nucleotides are too small to be simultaneously bound at both sites meaning that they would have to be degraded distributivety (Barbas et al., 2008).

The access to the catalytic pocket is restricted to single-stranded RNA by steric hindrance, which explains the inability of RNase II to degrade double-stranded RNA. DNA is not a substrate because there is a specific interaction between the protein and the ribose rings of nucleotides that directly contact the enzyme (Frazão et al., 2006). Residues Tyr-313 and Glu-390 have been demonstrated to be responsible for the discrimination of cleavage of RNA *versus* DNA (Barbas et al., 2009).

Several residues in the catalytic region are important for catalysis (Amblar & Arraiano, 2005; Frazão et al., 2006). Asp-201 and Asp-210 substitution led to a significant loss of RNase II activity and Arg-500 has also been shown to be crucial for RNA cleavage (Frazão et al., 2006; Barbas et al., 2008, 2009). However, Asp-209 is the only essential residue for RNA degradation (Amblar & Arraiano, 2005; Barbas et al., 2008). The conserved residue Glu-542 has been proposed to facilitate the elimination of the leaving nucleotide upon phosphodiester cleavage (Frazão et al., 2006). Interestingly its substitution by alanine rendered the mutant RNase II much more active than the wild-type and significantly increased the RNA-binding ability. 3D modelling of the mutant enzyme indicated that the substitution induced a subtle conformational change in the RNB domain. This resulted in a reorganization of the RNA-binding interface that turned the RNase II into the so-

called “super-enzyme”, an enzyme with extraordinary catalysis and binding abilities (Barbas et al., 2009).

6 RNase R

RNase R encoded by the *rnr* gene (previously *vac*B) is a 3’-5’ hydrolytic exoribonuclease from the RNase II family of exoribonucleases (Cheng & Deutscher, 2002; Vincent & Deutscher, 2006). The *rnr* gene is second in an operon together with *nsr*R (a transcriptional regulator), *rlm*B (rRNA methyltransferase), and *yjf*I (unknown function). Transcription is driven from a possible σ^{70} promoter upstream of *nsr*R (Cairrão et al., 2003). *rnr* mRNAs are post-transcriptional regulated by RNase E, although RNase G may also participate (Cairrão & Arraiano, 2006). RNase R is a processive and sequence independent enzyme, with a wide impact in RNA metabolism (Cairrão et al., 2003; Cheng & Deutscher, 2005; Oussenko et al., 2005; Andrade et al., 2006; Purusharth et al., 2007; Andrade et al., 2009a). It is unique amongst the RNA degradative exoribonucleases present in *E. coli* as it can easily degrade highly structured RNAs (Cheng & Deutscher, 2002, 2003; Awano et al., 2010). RNase R is able to degrade a RNA duplex provided there is a single stranded 3’ overhang (Cheng & Deutscher, 2002; Vincent & Deutscher, 2006). In fact, RNase R was shown to be a key enzyme involved in the degradation of polyadenylated RNA (Andrade et al., 2009a).

6.1 RNase R modular organization and function

RNase R shows a modular organization of RNA binding domains (CSD1 and CSD2 located at the N-terminus and a C-terminal S1 domain) flanking the central

catalytic RNB domain, typically found on RNase II-family members. A three-dimensional model of RNase R has been proposed based on the structure of its paralogue RNase II (Barbas et al., 2008). Mutational analysis identified important residues located in the active centre: D272, D278 and D280 (Matos et al., 2009; Vincent & Deutscher, 2009). A D280N mutant showed no exonucleolytic activity, analogous to what was reported with the D209N mutant in RNase II (Amblar & Arraiano, 2005; Matos et al., 2009; Awano et al., 2010). RNase R degradation is processive and unlike RNase II, the final end-product of digestion is a dinucleotide. Tyrosine Y324 was found to be responsible for setting the final end-product of RNase R (Matos et al., 2009).

RNase R was shown to bind RNA more tightly within its catalytic channel than does RNase II. Surprisingly, a mutant expressing only the nuclease domain (RNB) is able to degrade a perfect double stranded RNA (Matos et al., 2009; Vincent & Deutscher, 2009). Probably the RNA binding domains "block" the entrance of dsRNA into the catalytic channel. Accordingly, it was proposed that RNA binding domains actually discriminate the substrates that can be targeted by RNase R, favouring the selection of RNA molecules harbouring a 3' linear tail. It has been suggested that RNase R can function both as an exoribonuclease as well as an RNA "helicase" (Awano et al., 2010). RNase R intrinsic "helicase" unwinding activity is dependent on RNA-binding regions (S1, CDS1 and most importantly CDS2). The double stranded RNA must have a 3' linear overhang in order to become a suitable substrate to RNase R helicase activity. Altogether, RNA binding domains of RNase R seem to be responsible for the selection of RNA substrates harbouring a 3' linear region, which can be provided by polyadenylation (Andrade et al., 2009a). Clearly, only the resolution of RNase R structure will allow the fully understanding of its remarkable modes of action.

6.2 RNase R role in RNA and protein quality control and mRNA decay

RNase R is critical in RNA quality control, namely in degradation of defective tRNAs (Vincent & Deutscher, 2006; Awano et al., 2010) and rRNA (Cheng & Deutscher, 2003). Together with PNPase, RNase R eliminates aberrant fragments of 16S and 23S rRNA whose accumulation potentially affects ribosome maturation and assembly. Furthermore, the importance of RNase R in the accuracy of gene expression is broadening with its role in protein quality control. In the absence of RNase R, the small stable SsrA/tmRNA is not properly processed, leading to defects in *trans*-translation and significant errors in protein tagging for proteolysis (Cairrão et al., 2003). RNase R has also emerged as an important novel contributor to mRNA degradation. The absence of both RNase R and PNPase results in the strong accumulation of REP-containing mRNA sequences (Cheng & Deutscher, 2005). However, the presence of only one of these exoribonucleases is sufficient to remove such transcripts, revealing again a functional overlap between these two enzymes. Remarkably, RNase R was also shown to degrade the *omp*A transcript in a growth-phase specific manner (Andrade et al., 2006). In the stationary phase of growth, the single inactivation of RNase R results in the accumulation of *omp*A mRNA and this correlated with increasing intracellular levels of OmpA protein. This work revealed a role for RNase R in control of gene expression that could not be replaced by any of the other exoribonucleases.

6.3 RNase R is a stress induced protein

The activity of RNase R is modulated according to the growth conditions of the cell and responds to environmental stimuli. RNase R is a general stress-induced protein, whose levels are increased 3-10 fold under several stresses,

namely in cold-shock, and stationary-phase of growth (Cairrão et al., 2003; Andrade et al., 2006). RNase R is a highly unstable protein in exponential phase, however this protein is stabilized in stationary phase and other stress conditions, leading to its relative increase (Chen & Deutscher, 2010). tmRNA and SmpB binding to the C-terminal region of RNase R is responsible for the unstability of RNase R in exponential phase (Liang & Deutscher, 2010). This binding is regulated by acetylation of RNase R by the Pka acetylating enzyme that is absent from the cell under stress conditions such as stationary phase and cold-shock (Liang & Deutscher, 2012).

6.4 RNase R in other organisms and its role in virulence

RNase R-like enzymes are widespread in most sequenced genomes. Although most of the knowledge on this protein came from work in *E. coli*, many RNase R from other bacterial species have been identified. Notably, RNase R has also been implicated in the establishment of virulence in a growing number of pathogens.

In *Shigella flexneri* RNase R was shown to be required for the expression of the invasion factors IpaB, IpaC, IpaD and VirG (Tobe et al., 1992). The disruption of VacB gene in other *Shigella spp*. and enteroinvasive *Escherichia coli* resulted in reduced expression of virulence phenotypes (Tobe et al., 1992). In *Legionella pneumophila* RNase R is the only hydrolytic exoribonuclease present. This protein is not essential for growth at optimal temperature, however, it is important for growth and viability at low temperatures and induces the competence development (Charpentier et al., 2008). To the date, only one exoribonuclease, RNase R (MgR), was identified in *Mycoplasma genitalium*, where is an essential protein (Hutchison et al., 1999). MgR shares some properties of both *E. coli* RNase

R and RNase II and can carry out a broad range of RNA processing and degradative functions (Lalonde et al., 2007). Similarly to what happens in *E. coli*, RNase R from *Aeromonas hydrophila* is also a cold-shock protein essential for the viability at lower temperatures and it absence leads to a reduction in *A. hydrophila* motility (Erova et al., 2008). The infection of mouse cells with Δ*rnr* strains shows that the virulence is attenuated, confirming the role of this enzyme in the pathogenesis of this organism (Erova et al., 2008). In *Streptococcus pneumoniae* there is a unique homolog of RNase II family of enzymes which was shown to be a RNase R-like protein (Domingues et al., 2009). Proteins isolated from different strains regarding their virulence ability (virulent *vs.* non-virulent) are different regarding their activity and RNA affinity (Domingues et al., 2009). Further studies are still necessary to confirm if the differences observed in RNase R protein are responsible for the virulence of these strains.

In *Pseudomonas syringae*, RNase R is the exoribonuclease present in the degradosome as opposed to most other systems where PNPase is part of such complexes (Purusharth et al., 2005). Like in *E. coli*, RNase R is also particularly important at low temperatures, since inactivation of the *rnr* gene inhibits growth of both *P. putida* (Reva et al., 2006) and *P. syringae* (Purusharth et al., 2007) at 4°C. In *P. syringae* RNase R is involved in 3'-end maturation of 16S and 5S rRNA, and in tmRNA turnover (Purusharth et al., 2007).

Overall, RNase R-deficient bacteria have been shown to be less virulent than the wild-type parental strains. However, how this is achieved is still not completely clear. This is probably related to critical RNA degradation pathways. The fact that RNase R was found to be essential in the degradation of small RNAs, namely the virulence regulator SsrA/tmRNA, opens the way to broaden its role in pathogenesis. Altogether, the available data suggests that bacterial RNase R may

be attractive as a potential therapeutic agent but clearly more studies are required.

7 PNPase

PNPase is a 3'-5' phosphorolytic exoribonuclease that belongs to the PDX family of exoribonucleases, which also includes RNase PH from bacteria, and the core of the exosome in archaea and eukaryotes (Mian, 1997; Zuo & Deutscher, 2001; Pruijn, 2005). The enzyme is involved in global mRNA decay, being widely conserved from bacteria to plants and metazoans (Zuo & Deutscher, 2001; Bermúdez-Cruz et al., 2005).

7.1 Control of PNPase expression

PNPase is encoded by the *pnp* gene and is transcribed from two promoters (Portier & Régnier, 1984). *pnp* expression is negatively autoregulated at the post-transcriptional level by the concerted action of PNPase and RNase III (Portier et al., 1987; Robert-Le Meur & Portier, 1992, 1994; Jarrige et al., 2001; Carzaniga et al., 2009). This autoregulation can be disrupted by Ribosomal protein S1 that binds to the *pnp* mRNA 5'-UTR (Briani et al., 2008). In an RNase III deficient strain there is a 10-fold increase of the PNPase levels (Portier*, et al.*, 1987). PNPase levels are also affected by polyadenylation. It is likely that polyadenylated transcripts titrate out the amount of PNPase available to carry out normal autoregulation (Mohanty & Kushner, 2002). PNPase and RNase II are cross-regulated (Zilhão et al., 1996). In the absence of RNase II, PNPase levels are increased and PNPase overexpression leads to a decrease in RNase II activity (Zilhão et al., 1996).

PNPase does not seem to be indispensable to *E. coli* at optimal temperature, unless either RNase II or RNase R is also missing (Donovan & Kushner, 1986; Cheng et al., 1998). However, PNPase is essential for *E. coli* growth at low temperatures (Luttinger et al., 1996; Piazza et al., 1996; Zangrossi et al., 2000) and certain mutations of the RNA binding domains have been shown to confer a cold-sensitive phenotype (García-Mena et al., 1999; Briani et al., 2007; Matus-Ortega et al., 2007). However, over-expression of RNase II could complement cold shock function of PNPase (Awano et al., 2008). PNPase was also shown to be involved in the long-term survival of *Campylobacter jejuni* at temperatures below 10°C (Haddad et al., 2009). In *E. coli,* cold temperature induction of *pnp* expression occurs at post-transcriptional levels including reversal of *pnp* autoregulation (Zangrossi et al., 2000; Beran & Simons, 2001; Mathy et al., 2001).

7.2 PNPase activities and RNA degradation

PNPase processively catalyses the 3'-5' phosphorolytic degradation of RNA, releasing nucleoside diphosphates. Although the degrading activity of *E. coli* PNPase is known to be blocked by double stranded RNA structures (Spickler & Mackie, 2000), PNPase can form complexes with other proteins allowing it to degrade through extensive structured RNA. The main multi-protein complex that integrates PNPase is the degradosome. To degrade certain double-stranded RNAs PNPase can form a complex ($\alpha_3\beta_2$) with the RhlB helicase (Lin & Lin-Chao, 2005), (Liou et al., 2002). PNPase also forms complexes with Hfq and PAP I (Mohanty et al., 2004). The enzyme was reported to degrade a stem-loop without the assistance of RhlB, but this could be related with the low thermodynamic stability of the stem-loop (Mohanty & Kushner, 2009). In the gram-negative bacteria *Thermus thermophilus*, the PNPase homologue (Tth PNPase) was shown to have

phosphorolytic activity at the optimal temperature 65ºC. Surprisingly, it is able to completely degrade RNAs with very stable intramolecular secondary structures (Falaleeva et al., 2008).

A minimal 3' overhang of 7-10 unpaired ribonucleotides is required for an RNA molecule to be bound by PNPase (Py et al., 1996; Cheng & Deutscher, 2005) and the action of the enzyme on folded RNAs is known to be stimulated by 3' polyadenylation (Xu & Cohen, 1995; Py et al., 1996; Carpousis et al., 1999; Spickler & Mackie, 2000). PNPase is also able to catalyse the polymerization of RNA from nucleoside diphosphates at low inorganic phosphate concentration (Godefroy, 1970; Littauer & Soreq, 1982; Sulewski et al., 1989). *In vivo*, PNPase is essentially devoted to the processive degradation of RNA, but is also responsible for adding the heteropolymeric tails observed in *E. coli* mutants devoid of the main polyadenylating enzyme PAP I (Mohanty & Kushner, 2000 ; Slomovic et al., 2008). In exponentially growing *E. coli*, more than 90% of the transcripts are polyadenylated and Rho-dependent transcription terminators were suggested to be modified by the polymerase activity of PNPase (Mohanty & Kushner, 2006). In spinach chloroplasts, Cyanobacteria and *Streptomyces coelicolor*, PNPase seems to be the main tail polymerizing enzyme (Yehudai-Resheff et al., 2001; Rott et al., 2003; Sohlberg et al., 2003). PNPase–dependent RNA tailing and degradation are believed to occur mainly at low ATP concentrations, since ATP has been shown to inhibit both activities (Del Favero et al., 2008). Recently, it was shown that *Bacillus Subtilis* PNPase, in the presence of Mn^{2+} and low-levels of inorganic phosphate (Pi) is also able to degrade ssDNA, while in the presence of Mg^{2+} and higher amounts of Pi it degrades RNA. This information suggests that PNPase degradation of RNA and ssDNA occur by mutually exclusive mechanisms (Cardenas et al., 2009). Due to the ability of PNPase to carry out several distinct activities, the enzyme can be considered as a multifunctional protein. It is a pleiotropic regulator, involved in a number of different pathways of RNA degradation. Indeed, it is the only

exoribonuclease in *Streptomyces* being an essential enzyme in these organisms (Bralley & Jones, 2003; Bralley et al., 2006). In *E. coli*, PNPase is now believed to play a greater role in mRNA degradation than previously thought and its inactivation increases the steady-state levels of many transcripts (Deutscher & Reuven, 1991; Mohanty & Kushner, 2003). The enzyme was also reported to play an important role in protecting *E. coli* cells under oxidative stress (Wu et al., 2009). In *B. subtilis* the RNA decay is primarily phosphorolytic and this major activity is attributed to the PNPase, which is the principal 3'-to-5' exoribonuclease in this organism. Deletion of PNPase in *B. subtilis* causes a number of phenotypes like competence deficiency, cold and tetracycline sensitivity, and filamentous growth (Hahn et al., 1996; Luttinger et al., 1996; Wang & Bechhofer, 1996). Recently, several studies demonstrated that PNPase activity is affected by several cellular compounds like the ppGpp (Guanosine pentaphosphate), citrate and the second messenger cyclic diguanylic acid (c-di-GMP) (Gatewood & Jones, 2010; Nurmohamed et al., 2011; Tuckerman et al., 2011). ppGpp was shown to inhibit the activity of PNPase in Streptomyces species, however *E. coli* PNPase activity was not affected by ppGpp (Gatewood & Jones, 2010). On the other hand, citrate can either inhibit PNPase or stimulate PNPase activity. Binding of metal-chelated citrate in the active site appears to inhibit enzyme activity. Contrarily, metal-free citrate binds at a vestigial active site and stimulates PNPase activity (Nurmohamed et al., 2011). C-di-GMP was also shown to enhance several PNPase activities in a dose-dependent manner (Tuckerman et al., 2011).

7.3 PNPase structure and function

X-ray crystal structures of *E. coli* and *Streptomyces antibioticus* PNPase reveal a homotrimeric subunit organization with a ring-like architecture (Figure 1) (Symmons et al., 2000; Shi et al., 2008; Nurmohamed et al., 2009). Each monomer

exhibits a five-domain arrangement: at the N-terminus two RNase PH domains (PH1 and PH2) are linked by an α-helical domain; two RNA-binding domains, KH and S1, are found in the C-terminal end. In the quaternary structure the KH and S1 domains are found together in one face of the trimer, while the active site is found in the opposite side.

PNPase mutants lacking either the S1 or KH domain retain phosphorolytic activity (Jarrige et al., 2002; Stickney et al., 2005; Matus-Ortega et al., 2007). However, the presence of both KH and S1 domains are required for a proper binding (Matus-Ortega et al., 2007) and their absence may lead to a severe decrease in the number of molecules processed (Stickney et al., 2005). The crystal structure of a KH/S1 deletion mutant along with biochemical and biophysical data strongly suggests that these domains are involved not only in RNA-binding, but also contribute to the formation of a more stable trimeric structure (Shi et al., 2008). Indeed, a previous study has shown that the S1 domain from PNPase was able to induce trimerization of a chimeric RNase II containing PNPase S1 (Amblar et al., 2007).

Association of the three subunits encloses a central channel. A properly constricted channel and the conserved basic residues located in the neck region have been shown to play critical roles in trapping RNA for processive degradation (Shi et al., 2008). Two constricted points have been identified in the channel and the structure of PNPase in complex with RNA clearly indicates that the pathway followed by the RNA molecule is along the central pore in the direction to the active site (Symmons et al., 2000; Shi et al., 2008; Nurmohamed et al., 2009). The ability of the aperture at the central channel and its neighbouring regions to undergo conformational changes is likely to be a key aspect of the dynamic translocation of RNA by PNPase (Nurmohamed et al., 2009). The crystal structure of *Caulobacter crescentus* PNPase helped explaining the RNA directionality

(Hardwick et al., 2012). This structure suggested that the 3'-5' polarity of the substrate came from the interactions of the KH domains with the RNA sugar-phosphate backbone. When bound to RNA the three KH domains collectively close upon the RNA and direct the 3' end towards the constricted aperture at the entrance of the central channel (Hardwick et al., 2012).

The catalytic site of PNPase is composed of structural elements of both PH1 and PH2 core domains and several mutations introduced into the PNPase core abolish or severely decrease all catalytic activities of the enzyme (Jarrige et al., 2002; Briani et al., 2007). However, other mutations in the core region were analysed that do not affect phosphorolytic or polymerase activities, but rather RNA-binding is severely impaired (Regonesi et al., 2006). *S. antibioticus* PNPase catalytic center has been identified using tungstate (a phosphate analogue), which is coordinated by T462 and S463 (Symmons et al., 2000). *E. coli* PNPase crystals obtained in the presence of Mn^{2+} (which can substitute for Mg^{2+} to support catalysis) showed that the metal is coordinated by the conserved residues D486, D492 and K494 (Nurmohamed et al., 2009). Indeed, substitution of D492 abolished both phosphorolysis and polymerization activities (Jarrige et al., 2002).

7.4 PNPase role in virulence

PNPase has been described to have a role in the establishment of virulence in several pathogens. In *Salmonella*, PNPase activity decreases the expression of genes from the pathogenicity islands SPI 1 (containing genes for invasion) and SPI 2 (containing genes for intracellular growth) (Clements et al., 2002). Similarly, in *Dichelobacter nodosus*, PNPase acts as a virulence repressor in benign strains by decreasing twitching motility (Palanisamy et al., 2009). On the contrary, in *Yersinia* PNPase modulates the type three secretion system (TTSS) by affecting the steady-

state levels of TTSS transcripts and controlling the secretion-rate (Rosenzweig et al., 2005; Rosenzweig et al., 2007). This is probably the reason why the *pnp* deletion results in a less virulent strain in a mouse model (Rosenzweig*, et al.*, 2007). Inactivation of the *C. jejuni* PNPase also results in a less virulent strain. *pnp* mutants showed distinct phenotypes such as limitations in swimming, substantial delay in the colonization of the chicken gut and a decreased ability to adhere and invade cells (Haddad et al., 2009). Defects in motility are suggested to be responsible for many of the attenuation of the virulent traits of *C. jejuni* in the mutant *pnp* strain. Furthermore, proteomic studies also showed that PNPase affects the synthesis of proteins involved in virulence, such as LuxS and PEB3 (Haddad et al., 2009; Haddad et al., 2012). Finally, in *Streptococcus pyogenes*, PNPase activity is rate-limiting for decay of *sagA* and *sda* which code for the important virulence factors streptolysin S and streptodornase (a DNase), respectively (Barnett et al., 2007).

8 Role of small RNAs and Hfq in RNA decay

Small noncoding RNAs (sRNAs) are molecules which are not translated into proteins. They are highly structured and very stable molecules that exert several regulatory functions in both prokaryotes and eukaryotes cells. Prokaryotic sRNAs can bind to proteins or to mRNA targets (antisense RNAs) with full (*cis*-encoded) or partial complementarity (*trans*-encoded) (Viegas & Arraiano, 2008). All *trans*-encoded sRNAs studied so far in *E. coli* bind the RNA chaperone Hfq, which has been shown to stabilize several sRNAs as well as promoting sRNA–mRNA duplex (Valentin-Hansen et al., 2004; Brennan & Link, 2007).

8.1 Small RNAs mode of action

The sRNA usually binds to the 5' end of the target mRNA, near the Shine Dalgarno sequence, blocking ribosome binding and affecting mRNA translation and/or stability (Urban & Vogel, 2007; Viegas & Arraiano, 2008) (Figure 2). However, some sRNAs can regulate mRNA levels by binding to the 5'end mRNA coding region (Argaman & Altuvia, 2000; Bouvier et al., 2008; Papenfort et al., 2010; Rice & Vanderpool, 2011). More recently it was shown that the sRNA RyhB blocking translation cannot fully explain sRNA-induced mRNA degradation. In fact, RyhB binding to the ribosome-binding site (RBS) of *sod*B promotes mRNA cleavage at a distal site more than 350 nt downstream from the RBS. The degradation of some sRNAs was shown to be coupled with the endoribonucleolytic inactivation of their target mRNAs (Massé et al., 2003). However, it was also demonstrated that 3'-5' exoribonucleolytic degradation can be important in the regulation of small RNAs (Viegas et al., 2007; Andrade & Arraiano, 2008).

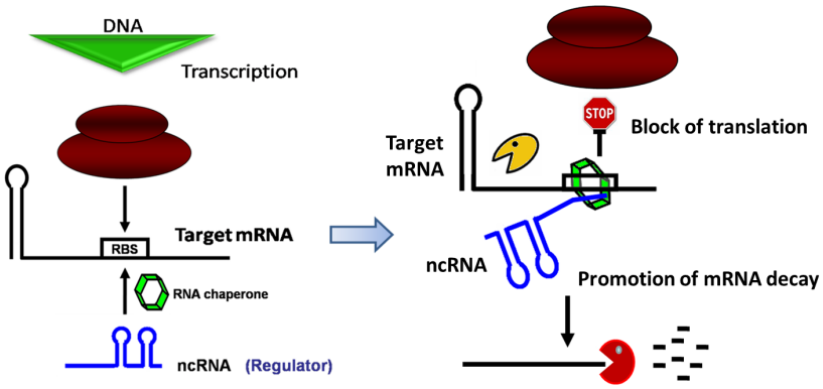

Figure 2 – Model for non-coding RNA mediated decay. Most of the ncRNA binds to the target mRNA with the help of Hfq. The binding of ncRNA to the mRNA blocks translation and promotes the target mRNA degradation.

The interaction between sRNAs and the target mRNA can include several regions of the sRNA. For example, OxyS RNA forms "kissing" complexes with its mRNA target *fhlA* at two different sites. This interaction occurs between two stem loops of OxyS RNA and two stem loops of *fhlA* mRNA (Argaman & Altuvia, 2000). The 5' end stem loop of OxyS RNA binds to a short sequence (9 nucleotides) of the *fhlA* mRNA coding region, while a 3' end stem loop binds to a 7 nucleotide sequence in the RBS site (Altuvia et al., 1998; Argaman & Altuvia, 2000). The binding of OxyS RNA to the coding region of *fhlA* mRNA facilitates the interaction of OxyS RNA to the RBS site of *fhlA* mRNA and a mutation in either one of the stem loops decreases the stability of the complex (Argaman & Altuvia, 2000). Nevertheless, generally is the 5' end of the sRNA that is involved in the interaction with the target mRNA.

In the last years it has become clear that a single sRNA can regulate several mRNA targets in a similar approach as miRNAs in eukaryotes. The major difference is in the length of the seed sequence (nucleotides involved in the base pairing). While miRNAs seed sequence is always the nucleotides 2-8 (Carthew & Sontheimer, 2009) the sRNAs in prokaryotes may have shorter or longer seed sequences. Also, it has been demonstrated that different seed sequences from the same sRNA can affect differently the mRNA targets (Guillier & Gottesman, 2008; Balbontín et al., 2010; Rice & Vanderpool, 2011). In the case of OmrA and OmrB RNAs, two redundant sRNAs from *E. coli*, their conserved 5' end region can regulate at least five different mRNA targets. However, the nucleotides and the length of the sequence involved in the regulation of the different targets may differ (Guillier & Gottesman, 2008). This is also true for SgrS sRNA and its mRNA targets (Rice & Vanderpool, 2011). In the case of SgrS RNA, it was also demonstrated that the secondary structure of the sRNA as well as the secondary structure of the mRNA targets are important for their interaction (Rice & Vanderpool, 2011). Another example as 5' end can be important in the regulation

of multiple mRNA targets is sRNA RybB. A seed sequence of the first 7 nucleotides in the 5' end of RybB RNA appears to be crucial for the interaction with *ompC*, *ompD* and *chiP* mRNA targets in *Salmonella* (Balbontín et al., 2010). The 5' end of RybB RNA was also shown to be a target recognition domain, meaning that the RybB 5' end by itself was able to target several *omp* mRNAs to degradation in *Salmonella* (Papenfort et al., 2010).

MicA RNA is a 78 nucleotide sRNA that controls the expression several mRNA targets (Gogol et al., 2011). The most well studied MicA RNA targets are ompA, an outer membrane protein, and lamB, a maltoporin (Rasmussen et al., 2005; Udekwu et al., 2005; Bossi & Figueroa-Bossi, 2007; Andrade & Arraiano, 2008). MicA binds to the 5'-UTR of the target *omp*A mRNA in an Hfq dependent manner (Udekwu et al., 2005). This binding blocks the ribosome entry (translational repression) promoting the *omp*A mRNA degradation. MicA RNA regulation of *ompA* mRNA is growth phase and growth rate dependent occurring during stationary phase (Rasmussen et al., 2005). MicA RNA binding sequence to *ompA* mRNA is different from the binding sequence to *lamB* mRNA. Although, the 5' end linear region of MicA RNA is involved in binding to both targets (Bossi & Figueroa-Bossi, 2007).

8.2 Hfq roles in the cell

Hfq is a RNA chaperone protein that enhances RNA annealing (Moll et al., 2003b; Rajkowitsch & Schroeder, 2007). It was originally discovered as a host factor required for replication of phage Qβ RNA (Franze de Fernandez et al., 1972). Hfq is a small protein of 102 a.a. encoded by the *hfq* gene (Kajitani et al., 1994). Hfq regulates its own expression at the translational level by binding to two different sites in the 5'-UTR region of *hfq* mRNA (Tsui et al., 1997; Vecerek et al.,

2005). Hfq is an ATP-binding protein and in the presence of ATP there is a significant destabilization of the Hfq-RNA complex (Arluison et al., 2007). Hfq is also involved in the metabolism of poly(A) tails (Hajnsdorf & Régnier, 2000; Le Derout et al., 2003; Folichon et al., 2005a). It was demonstrated that Hfq stimulates PAP I activity upon binding to the mRNA (Hajnsdorf & Régnier, 2000; Folichon et al., 2005a). Also, PAP I in the presence of Hfq becomes a processive enzyme extending RNAs harbouring oligo(A) tails (Hajnsdorf & Régnier, 2000). PNPase can also synthesize poly(A) tails, however, Hfq has a inhibitory effect on PNPase polymerization activity (Folichon et al., 2005a). Hfq affects not only PAP I activity but also the length and the frequency of the poly(A) tails (Le Derout et al., 2003). Hfq also appears to have a significant role in tRNA metabolism (Scheibe et al., 2007; Lee & Feig, 2008). Hfq stimulates the CCA-adding enzyme (catalyses the synthesis of the 3'-terminal sequence CCA to all tRNAs) activity after binding tRNAs (Scheibe et al., 2007).

Hfq mediates RNA-RNA interaction and is essential for the interaction of most of the known trans-acting sRNA with their mRNA targets (Moller et al., 2002). Hfq was found to accelerate the duplex formation between the sRNA SgrS and the mRNA *pts*G (Kawamoto et al., 2006). In some cases Hfq binds to the sRNA or to the mRNA destabilizing their structure and thus allowing binding between both RNAs, for example when Hfq binds *sod*B mRNA its structure is destabilized and allows the sRNA RyhB binding, and then triggers the degradation of both RNAs (Geissmann & Touati, 2004). On the other hand, there are also some examples where Hfq does not affect the RNA secondary structure (Brescia et al., 2003). Hfq binding to DsrA sRNA does not affect this RNA secondary structure although it might affect its tertiary conformation (Brescia et al., 2003). In some cases Hfq stabilizes a sRNA. RyhB RNA is very unstable in the absence of Hfq (Massé et al., 2003). This might be due to an identical binding site of Hfq and RNase E. In the presence of Hfq RyhB is protected against cleavage by the RNase E

(Massé et al., 2003; Moll et al., 2003a). This also happens with the sRNA DsrA (Moll et al., 2003a) and the mRNA *rps*O (Folichon et al., 2003). More recently, it was suggested that Hfq might also affect mRNAs at the transcriptional level independently of mRNA degradation, although the mechanism is still not clear (Le Derout et al., 2010).

8.3 Hfq structure and function

Hfq is a member of the Sm/Lsm superfamily and shares the OB fold (N-terminal α-helix followed by five stranded β-sheet) characteristic of this family of proteins (Schumacher et al., 2002). Hfq forms a symmetric hexameric ring with a doughnut like shape (Schumacher et al., 2002). Although Hfq is a Sm protein, it has two structural differences from the other members of this family. One is a shorter turn between its Sm1 and its C-terminal region ("Sm2" motif); the other is the hexamer formation instead of a heptamer, like the other Sm proteins (Schumacher et al., 2002). The pore of the Hfq hexamer has residues from the Sm1 and the "Sm2" motifs that form a six nucleotide binding pocket. RNA binds around this pocket in a circular and unwound manner (Schumacher et al., 2002). The Hfq binding to poly(A) tails is very different from the Hfq binding to A/U-rich regions. Poly(A) tails bind to Hfq in its distal face of the hexameric ring instead of the proximal face were RNA binds, also Hfq has the capacity to bind 18 nucleotides from the poly(A) tail (Link et al., 2009). The proximal face of Hfq was also identified as a tRNA binding site (Lee & Feig, 2008). So, it is possible to identify different Hfq interaction surfaces for different substrates (Mikulecky et al., 2004).

The N-terminal region of the Sm proteins is much conserved; on the other hand the C-terminal region is extremely variable. The biological role for the C-

terminal region of Hfq is still not clear. Although some studies with a C-terminally truncated variant of Hfq (Hfq_{65}) suggested that this region was involved in regulation and that this Hfq variant was defective in mRNA binding (Vecerek et al., 2008), other studies seems to demonstrate that the C-terminal region is not involved in riboregulation (Olsen et al., 2010). However, it was demonstrated that the C-terminal domain stabilizes the Hfq hexameric structure (Arluison et al., 2004).

8.4 Hfq complexes

Hfq can form complexes with other several other proteins. One of these proteins is the ribosomal protein S1 (Sukhodolets & Garges, 2003; Morita et al., 2005). Hfq can form a complex with the S1 and with the RNA polymerase, although by itself, Hfq has no affinity for the RNA polymerase (Sukhodolets & Garges, 2003). Hfq can also form complexes with both PNPase and Poly(A) polymerase (PAP I). These complexes can be Hfq-PNPase-PAP I, but also only Hfq-PNPase or Hfq-PAP I (Mohanty et al., 2004). Hfq directly associates with the C-terminal scaffold region of RNase E (Morita et al., 2005; Worrall et al., 2008; Ikeda et al., 2011). However, the overexpression of RhlB can inhibit Hfq binding to RNase E (Ikeda et al., 2011). It was also demonstrated that SgrS and RyhB RNAs can also associate with RNase E through Hfq, forming ribonucleoprotein complexes that act as specialized RNA decay machines (Morita et al., 2005).

9 Concluding Remarks

Maintenance of optimal levels of RNAs at any time and under any circumstance is an extremely difficult task to achieve and requires great coordination among all the factors involved in this control. It is also assumed that there is a cross-talk between transcription and degradation to maintain the balance that is best for the survival of microorganisms. There are several examples where this is obvious, and when a specific message is more transcribed, it is also more stabilized, and vice versa. Transcripts can have a different half-life under different growth conditions to rapidly carry out the necessary changes and adjust to adequate RNA levels. The same RNA can have a 'preferred' decay pathway, but there are examples where there is alternative degradation pathways for the same transcript, depending on which enzyme cleaves first. After cleavage, the RNA breakdown product(s) can have a distinct half-life depending on sequence and structure. Therefore, the structural characteristics of RNA stability and instability predetermine the 'fate' of RNA, but the environment and the consequent levels and nature of the degradative enzymes will also play a determinant role in its turnover. For instance, the mRNAs expressed in heterologous systems can have a very different half-life than if they are expressed in their own microorganism. The directionality of the decay process depends on the transcript analysed. Once we characterise the enzymes from one microorganism, we can design strategies to stabilize RNAs. Mutants have been instrumental in characterizing degradation pathways and in changing the turnover of specific transcripts, especially because a limited number of RNases intervene in the maturation and degradation of RNAs. There are fundamental principles that govern RNA decay in all organisms. Evolution has resulted in similar functions performed by different enzymes. For instance, in *E. coli*, RNase E is one of the major endoribonucleases, but this enzyme is absent in *B. subtilis*. In *B. subtilis*,

RNase J1 seems to take over the same function, and this enzyme is not present in *E. coli*. In yeast, 5'–3' decay is prominent, and Rrp44/ Dis3, an RNase II family enzyme, has dual endo and 3'–5' exo activities, being an example of an optimized 'RNA degradation machine'. Sometimes, RNases also combine into complexes to speed up the decay process or confer specificity to certain targets. It is fascinating to know that RNases themselves are strictly regulated proteins and have mechanisms to adapt them to the environment and to the levels of the other RNases. For instance, RNase R is highly increased under cold shock; the levels of PNPase and RNase II are inter-regulated and the level of RNase E is autoregulated. Recent studies demonstrate that, between prokaryotic and eukaryotic systems, the RNA degradation mechanisms have much more similarities than expected. The mechanism of RNA interference in eukaryotes has shown the power of RNA degradation mechanisms involving RNases. It is now obvious that the modulation of RNA levels and their respective proteins can be rapidly achieved. The field of RNA still holds many unanswered questions. The continuous study of these diverse enzymes, small RNAs and other factors involved in RNA degradation will surely guarantee a more detailed understanding of RNA metabolism.

10 References

Afonyushkin T, Vecerek B, Moll I, Blasi U, Kaberdin VR. 2005. Both RNase E and RNase III control the stability of sodB mRNA upon translational inhibition by the small regulatory RNA RyhB. *Nucleic Acids Res 33*:1678-1689.

Agrawal N, Dasaradhi PV, Mohmmed A, Malhotra P, Bhatnagar RK, Mukherjee SK. 2003. RNA interference: biology, mechanism, and applications. *Microbiol Mol Biol Rev 67*:657-685.

Altuvia S, Zhang A, Argaman L, Tiwari A, Storz G. 1998. The *Escherichia coli* OxyS regulatory RNA represses *fhl*A translation by blocking ribosome binding. *EMBO J 17*:6069-6075.

Amblar M, Arraiano CM. 2005. A single mutation in *Escherichia coli* ribonuclease II inactivates the enzyme without affecting RNA binding. *FEBS J 272*:363-374.

Amblar M, Barbas A, Fialho AM, Arraiano CM. 2006. Characterization of the Functional Domains of *Escherichia coli* RNase II. *Journal of molecular biology 360*:921-933.

Amblar M, Barbas A, Gomez-Puertas P, Arraiano CM. 2007. The role of the S1 domain in exoribonucleolytic activity: substrate specificity and multimerization. *RNA 13*:317-327.

Andrade JM, Arraiano CM. 2008. PNPase is a key player in the regulation of small RNAs that control the expression of outer membrane proteins. *RNA 14*:543-551.

Andrade JM, Cairrão F, Arraiano CM. 2006. RNase R affects gene expression in stationary phase: regulation of *ompA*. *Mol Microbiol 60*:219-228.

Andrade JM, Hajnsdorf E, Régnier P, Arraiano CM. 2009a. The poly(A)-dependent degradation pathway of *rps*O mRNA is primarily mediated by RNase R. *RNA 15*:316-326.

Andrade JM, Pobre V, Silva IJ, Domingues S, Arraiano CM. 2009b. The role of 3'-5' exoribonucleases in RNA degradation. *Prog Mol Biol Transl Sci 85*:187-229.

Apirion D, Lassar AB. 1978. A conditional lethal mutant of Escherichia coli which affects the processing of ribosomal RNA. *J Biol Chem 253*:1738-1742.

Argaman L, Altuvia S. 2000. *fhl*A repression by *OxyS* RNA: kissing complex formation at two sites results in a stable antisense-target RNA complex. *J Mol Biol 300*:1101-1112.

Arluison V, Folichon M, Marco S, Derreumaux P, Pellegrini O, Seguin J, Hajnsdorf E, Régnier P. 2004. The C-terminal domain of *Escherichia coli* Hfq increases the stability of the hexamer. *Eur J Biochem 271*:1258-1265.

Arluison V, Mutyam SK, Mura C, Marco S, Sukhodolets MV. 2007. Sm-like protein Hfq: location of the ATP-binding site and the effect of ATP on Hfq-- RNA complexes. *Protein Sci 16*:1830-1841.

Arraiano CM, Andrade JM, Domingues S, Guinote IB, Malecki M, Matos RG, Moreira RN, Pobre V, Reis FP, Saramago M, Silva IJ, Viegas SC. 2010. The critical role of RNA processing and degradation in the control of gene expression. *FEMS Microbiol Rev 34*:883-923.

Arraiano CM, Barbas A, Amblar M. 2008. Characterizing ribonucleases in vitro examples of synergies between biochemical and structural analysis. *Methods Enzymol 447*:131-160.

Arraiano CM, Maquat LE. 2003. Post-transcriptional control of gene expression: effectors of mRNA decay. *Mol Microbiol 49*:267-276.

Arraiano CM, Yancey SD, Kushner SR. 1988. Stabilization of discrete mRNA breakdown products in *ams pnp rnb* multiple mutants of *Escherichia coli* K-12. *J Bacteriol 170*:4625-4633.

Awano N, Inouye M, Phadtare S. 2008. RNase activity of polynucleotide phosphorylase is critical at low temperature in *Escherichia coli* and is complemented by RNase II. *Journal of bacteriology 190*:5924-5933.

Awano N, Rajagopal V, Arbing M, Patel S, Hunt J, Inouye M, Phadtare S. 2010. *Escherichia coli* RNase R has dual activities, helicase and RNase. *Journal of bacteriology 192*:1344-1352.

Babitzke P, Kushner SR. 1991. The Ams (altered mRNA stability) protein and ribonuclease E are encoded by the same structural gene of *Escherichia coli*. *Proc Natl Acad Sci U S A 88*:1-5.

Baker KE, Mackie GA. 2003. Ectopic RNase E sites promote bypass of 5'-end-dependent mRNA decay in *Escherichia coli*. *Mol Microbiol 47*:75-88.

Balbontín R, Fiorini F, Figueroa-Bossi N, Casadesus J, Bossi L. 2010. Recognition of heptameric seed sequence underlies multi-target regulation by RybB small RNA in *Salmonella enterica*. *Mol Microbiol 78*:380-394.

Barbas A, Matos RG, Amblar M, Lopez-Vinas E, Gomez-Puertas P, Arraiano CM. 2008. New Insights into the Mechanism of RNA Degradation by Ribonuclease II: Identification of the residue responsible for setting the RNase II end-product. *The Journal of biological chemistry 283*:13070-13076.

Barbas A, Matos RG, Amblar M, Lopez-Vinas E, Gomez-Puertas P, Arraiano CM. 2009. Determination of key residues for catalysis and RNA cleavage specificity: one mutation turns RNase II into a "SUPER-ENZYME". *J Biol Chem 284*:20486-20498.

Bardwell JC, Régnier P, Chen SM, Nakamura Y, Grunberg-Manago M, Court DL. 1989. Autorregulation of RNase III operon by mRNA processing. *EMBO J 8*:3401-3407.

Barnett TC, Bugrysheva JV, Scott JR. 2007. Role of mRNA stability in growth phase regulation of gene expression in the group A *streptococcus*. *Journal of bacteriology 189*:1866-1873.

Beran RK, Simons RW. 2001. Cold-temperature induction of *Escherichia coli* polynucleotide phosphorylase occurs by reversal of its autoregulation. *Mol Microbiol 39*:112-125.

Bermúdez-Cruz RM, Fernandez-Ramírez F, Kameyama-Kawabe L, Montañez C. 2005. Conserved domains in polynucleotide phosphorylase among eubacteria. *Biochimie 87*:737-745.

Blaszczyk J, Gan J, Tropea JE, Court DL, Waugh DS, Ji X. 2004. Noncatalytic assembly of ribonuclease III with double-stranded RNA. *Structure 12*:457-466.

Blaszczyk J, Tropea JE, Bubunenko M, Routzahn KM, Waugh DS, Court DL, Ji X. 2001. Crystallographic and modeling studies of RNase III suggest a mechanism for double-stranded RNA cleavage. *Structure 9*:1225-1236.

Bossi L, Figueroa-Bossi N. 2007. A small RNA downregulates LamB maltoporin in *Salmonella*. *Mol Microbiol 65*:799-810.

Bouvier M, Sharma CM, Mika F, Nierhaus KH, Vogel J. 2008. Small RNA binding to 5' mRNA coding region inhibits translational initiation. *Mol Cell 32*:827-837.

Bralley P, Gust B, Chang S, Chater KF, Jones GH. 2006. RNA 3'-tail synthesis in Streptomyces: *in vitro* and *in vivo* activities of RNase PH, the SCO3896 gene product and polynucleotide phosphorylase. *Microbiology 152*:627-636.

Bralley P, Jones GH. 2003. Overexpression of the polynucleotide phosphorylase gene (*pnp*) of *Streptomyces antibioticus* affects mRNA stability and poly(A) tail length but not ppGpp levels. *Microbiology 149*:2173-2182.

Brennan RG, Link TM. 2007. Hfq structure, function and ligand binding. *Curr Opin Microbiol 10*:125-133.

Brescia CC, Mikulecky PJ, Feig AL, Sledjeski DD. 2003. Identification of the Hfq-binding site on *DsrA* RNA: Hfq binds without altering *DsrA* secondary structure. *RNA 9*:33-43.

Briani F, Curti S, Rossi F, Carzaniga T, Mauri P, Deho G. 2008. Polynucleotide phosphorylase hinders mRNA degradation upon ribosomal protein S1 overexpression in *Escherichia coli*. *RNA 14*:2417-2429.

Briani F, Del Favero M, Capizzuto R, Consonni C, Zangrossi S, Greco C, De Gioia L, Tortora P, Dehò G. 2007. Genetic analysis of polynucleotide phosphorylase structure and functions. *Biochimie 89*:145-157.

Cairrão F, Arraiano CM. 2006. The role of endoribonucleases in the regulation of RNase R. *Biochemical and biophysical research communications 343*:731-737.

Cairrão F, Chora A, Zilhão R, Carpousis AJ, Arraiano CM. 2001. RNase II levels change according to the growth conditions: characterization of *gmr*, a new *Escherichia coli* gene involved in the modulation of RNase II. *Mol Microbiol 39*:1550-1561.

Cairrão F, Cruz A, Mori H, Arraiano CM. 2003. Cold shock induction of RNase R and its role in the maturation of the quality control mediator SsrA/tmRNA. *Mol Microbiol 50*:1349-1360.

Calin-Jageman I, Nicholson AW. 2003. RNA structure-dependent uncoupling of substrate recognition and cleavage by *Escherichia coli* ribonuclease III. *Nucleic Acids Res 31*:2381-2392.

Callaghan AJ, Aurikko JP, Ilag LL, Gunter Grossmann J, Chandran V, Kühnel K, Poljak L, Carpousis AJ, Robinson CV, Symmons MF, Luisi BF. 2004. Studies of the RNA degradosome-organizing domain of the *Escherichia coli* ribonuclease RNase E. *J Mol Biol 340*:965-979.

Callaghan AJ, Marcaida MJ, Stead JA, McDowall KJ, Scott WG, Luisi BF. 2005. Structure of *Escherichia coli* RNase E catalytic domain and implications for RNA turnover. *Nature 437*:1187-1191.

Cannistraro VJ, Kennell D. 1994. The processive reaction mechanism of ribonuclease II. *Journal of molecular biology 243*:930-943.

Cannistraro VJ, Kennell D. 1999. The reaction mechanism of ribonuclease II and its interaction with nucleic acid secondary structures. *Biochem et Biophys Acta 1433*:170-187.

Cardenas PP, Carrasco B, Sanchez H, Deikus G, Bechhofer DH, Alonso JC. 2009. *Bacillus subtilis* polynucleotide phosphorylase 3'-to-5' DNase activity is involved in DNA repair. *Nucleic Acids Res 37*:4157-4169.

Carpousis AJ, Luisi BF, McDowall KJ. 2009. Endonucleolytic initiation of mRNA decay in *Escherichia coli*. *Prog Mol Biol Transl Sci 85*:91-135.

Carpousis AJ, Vanzo NF, Raynal LC. 1999. mRNA degradation. A tale of poly(A) and multiprotein machines. *Trends Genet 15*:24-28.

Carthew RW, Sontheimer EJ. 2009. Origins and Mechanisms of miRNAs and siRNAs. *Cell 136*:642-655.

Carzaniga T, Briani F, Zangrossi S, Merlino G, Marchi P, Dehò G. 2009. Autogenous regulation of *Escherichia coli* polynucleotide phosphorylase expression revisited. *Journal of bacteriology 191*:1738-1748.

Celesnik H, Deana A, Belasco JG. 2007. Initiation of RNA decay in *Escherichia coli* by 5' pyrophosphate removal. *Mol Cell 27*:79-90.

Charpentier X, Faucher SP, Kalachikov S, Shuman HA. 2008. Loss of RNase R induces competence development in *Legionella pneumophila*. *Journal of bacteriology 190*:8126-8136.

Chen C, Deutscher MP. 2010. RNase R is a highly unstable protein regulated by growth phase and stress. *RNA 16*:667-672.

Cheng ZF, Deutscher MP. 2002. Purification and characterization of the *Escherichia coli* exoribonuclease RNase R. Comparison with RNase II. *J Biol Chem 277*:21624-21629.

Cheng ZF, Deutscher MP. 2003. Quality control of ribosomal RNA mediated by polynucleotide phosphorylase and RNase R. *Proc Natl Acad Sci USA 100*:6388-6393.

Cheng ZF, Deutscher MP. 2005. An important role for RNase R in mRNA decay. *Mol Cell 17*:313-318.

Cheng ZF, Zuo Y, Li Z, Rudd KE, Deutscher MP. 1998. The *vacB* gene required for virulence in *Shigella flexneri* and *Escherichia coli* encodes the exoribonuclease RNase R. *J Biol Chem 273*:14077-14080.

Clements MO, Eriksson S, Thompson A, Lucchini S, Hinton JC, Normark S, Rhen M. 2002. Polynucleotide phosphorylase is a global regulator of virulence and persistency in *Salmonella enterica*. *Proc Natl Acad Sci USA 99*:8784-8789.

Coburn GA, Mackie GA. 1996. Overexpression, purification, and properties of *Escherichia coli* ribonuclease II. *J Biol Chem 271*:1048-1053.

Condon C, Putzer H. 2002. The phylogenetic distribution of bacterial ribonucleases. *Nucleic acids research 30*:5339-5346.

Court DL. 1993. RNA processing and degradation by RNase III. In: G. BJGaB, ed. *Control of Messenger RNA Stability*: Academic Press. pp 71-116.

Dasgupta S, Fernandez L, Kameyama L, Inada T, Nakamura Y, Pappas A, Court DL. 1998. Genetic uncoupling of the dsRNA-binding and RNA cleavage activities of the *Escherichia coli* endoribonuclease RNase III - the effect of dsRNA binding on gene expression. *Mol Microbiol 28*:629-640.

Deana A, Celesnik H, Belasco JG. 2008. The bacterial enzyme RppH triggers messenger RNA degradation by 5' pyrophosphate removal. *Nature 451*:355-358.

Del Favero M, Mazzantini E, Briani F, Zangrossi S, Tortora P, Dehò G. 2008. Regulation of *Escherichia coli* polynucleotide phosphorylase by ATP. *J Biol Chem 283*:27355-27359.

Deutscher MP, Reuven NB. 1991. Enzymatic basis for hydrolytic versus phosphorolytic mRNA degradation in *Escherichia coli* and *Bacillus subtilis*. *Proc Natl Acad Sci USA 88*:3277-3280.

Diwa A, Bricker AL, Jain C, Belasco JG. 2000. An evolutionarily conserved RNA stem-loop functions as a sensor that directs feedback regulation of RNase E gene expression. *Genes Dev 14*:1249-1260.

Domingues S, Matos RG, Reis FP, Fialho AM, Barbas A, Arraiano CM. 2009. Biochemical characterization of the RNase II family of exoribonucleases from the human pathogens *Salmonella typhimurium* and *Streptococcus pneumoniae*. *Biochemistry 48*:11848-11857.

Donovan WP, Kushner SR. 1986. Polynucleotide phosphorylase and ribonuclease II are required for cell viability and mRNA turnover in *Escherichia coli* K-12. *Proc Natl Acad Sci USA 83*:120-124.

Dreyfus M, Régnier P. 2002. The poly(A) tail of mRNAs: bodyguard in eukaryotes, scavenger in bacteria. *Cell 111*:611-613.

Dziembowski A, Lorentzen E, Conti E, Seraphin B. 2007. A single subunit, Dis3, is essentially responsible for yeast exosome core activity. *Nat Struct Mol Biol 14*:15-22.

Erova TE, Kosykh VG, Fadl AA, Sha J, Horneman AJ, Chopra AK. 2008. Cold shock exoribonuclease R (*VacB*) is involved in *Aeromonas hydrophila* pathogenesis. *J Bacteriol 190*:3467-3474.

Evguenieva-Hackenberg E, Klug G. 2000. RNase III processing of intervening sequences found in helix 9 of 23S rRNA in the alpha subclass of Proteobacteria. *Journal of bacteriology 182*:4719-4729.

Falaleeva MV, Chetverina HV, Ugarov VI, Uzlova EA, Chetverin AB. 2008. Factors influencing RNA degradation by Thermus thermophilus polynucleotide phosphorylase. *Febs J 275*:2214-2226.

Folichon M, Allemand F, Régnier P, Hajnsdorf E. 2005a. Stimulation of poly(A) synthesis by *Escherichia coli* poly(A)polymerase I is correlated with Hfq binding to poly(A) tails. *Febs J 272*:454-463.

Folichon M, Arluison V, Pellegrini O, Huntzinger E, Régnier P, Hajnsdorf E. 2003. The poly(A) binding protein Hfq protects RNA from RNase E and exoribonucleolytic degradation. *Nucleic acids research 31*:7302-7310.

Folichon M, Marujo PE, Arluison V, Le Derout J, Pellegrini O, Hajnsdorf E, Régnier P. 2005b. Fate of mRNA extremities generated by intrinsic termination: detailed analysis of reactions catalyzed by ribonuclease II and poly(A) polymerase. *Biochimie 87*:819-826.

Franze de Fernandez MT, Hayward WS, August JT. 1972. Bacterial proteins required for replication of phage Q ribonucleic acid. Pruification and properties of host factor I, a ribonucleic acid-binding protein. *J Biol Chem 247*:824-831.

Frazão C, McVey CE, Amblar M, Barbas A, Vonrhein C, Arraiano CM, Carrondo MA. 2006. Unravelling the dynamics of RNA degradation by ribonuclease II and its RNA-bound complex. *Nature 443*:110-114.

Freire P, Amaral JD, Santos JM, Arraiano CM. 2006. Adaptation to carbon starvation: RNase III ensures normal expression levels of bolA1p mRNA and sigma(S). *Biochimie 88*:341-346.

Gan J, Tropea JE, Austin BP, Court DL, Waugh DS, Ji X. 2006. Structural insight into the mechanism of double-stranded RNA processing by ribonuclease III. *Cell 124*:355-366.

Gao J, Lee K, Zhao M, Qiu J, Zhan X, Saxena A, Moore CJ, Cohen SN, Georgiou G. 2006. Differential modulation of *E. coli* mRNA abundance by inhibitory

proteins that alter the composition of the degradosome. *Mol Microbiol 61*:394-406.

García-Mena J, Das A, Sánchez-Trujillo A, Portier C, Montañez C. 1999. A novel mutation in the KH domain of polynucleotide phosphorylase affects autoregulation and mRNA decay in *Escherichia coli*. *Mol Microbiol 33*:235-248.

Garrey SM, Mackie GA. 2011. Roles of the 5'-phosphate sensor domain in RNase E. *Mol Microbiol 80*:1613-1624.

Gatewood ML, Jones GH. 2010. (p)ppGpp inhibits polynucleotide phosphorylase from streptomyces but not from *Escherichia coli* and increases the stability of bulk mRNA in *Streptomyces coelicolor*. *Journal of bacteriology 192*:4275-4280.

Geissmann TA, Touati D. 2004. Hfq, a new chaperoning role: binding to messenger RNA determines access for small RNA regulator. *EMBO J 23*:396-405.

Ghora BK, Apirion D. 1978. Structural analysis and *in vitro* processing to p5 rRNA of a 9S RNA molecule isolated from an rne mutant of *E. coli*. *Cell 15*:1055-1066.

Godefroy T. 1970. Kinetics of polymerization and phosphorolysis reactions of *Escherichia coli* polynucleotide phosphorylase. Evidence for multiple binding of polynucleotide in phosphorolysis. *Eur J Biochem 14*:222-231.

Gogol EB, Rhodius VA, Papenfort K, Vogel J, Gross CA. 2011. Small RNAs endow a transcriptional activator with essential repressor functions for single-tier control of a global stress regulon. *Proc Natl Acad Sci U S A 108*:12875-12880.

Grossman D, van Hoof A. 2006. RNase II structure completes group portrait of 3' exoribonucleases. *Nat Struct Mol Biol 13*:760-761.

Grunberg-Manago M. 1999. Messenger RNA stability and its role in control of gene expression in bacteria and phages. *Annu Rev Genet 33*:193-227.

Guillier M, Gottesman S. 2008. The 5' end of two redundant sRNAs is involved in the regulation of multiple targets, including their own regulator. *Nucleic Acids Res 36*:6781-6794.

Guillier M, Gottesman S, Storz G. 2006. Modulating the outer membrane with small RNAs. *Genes Dev 20*:2338-2348.

Haddad N, Burns CM, Bolla JM, Prevost H, Federighi M, Drider D, Cappelier JM. 2009. Long-term survival of *Campylobacter jejuni* at low temperatures is dependent on polynucleotide phosphorylase activity. *Applied and Environmental Microbiology 75*:7310-7318.

Haddad N, Tresse O, Rivoal K, Chevret D, Nonglaton Q, Burns CM, Prévost H, Cappelier JM. 2012. Polynucleotide phosphorylase has an impact on cell biology of *Campylobacter jejuni*. *Front Cell Inf Microbio*:doi: 10.3389/fcimb.2012.00030

Hahn J, Luttinger A, Dubnau D. 1996. Regulatory inputs for the synthesis of ComK, the competence transcription factor of *Bacillus subtilis*. *Mol Microbiol 21*:763-775.

Hajnsdorf E, Régnier P. 2000. Host factor Hfq of *Escherichia coli* stimulates elongation of poly(A) tails by poly(A) polymerase I. *Proc Natl Acad Sci USA 97*:1501-1505.

Hajnsdorf E, Steier O, Coscoy L, Teysset L, Régnier P. 1994. Roles of RNase E, RNase II and PNPase in the degradation of the *rpsO* transcripts of *Escherichia coli*: stabilizing function of RNase II and evidence for efficient degradation in an *ams pnp rnb* mutant. *EMBO J 13*:3368-3377.

Hankins JS, Zappavigna C, Prud'homme-Genereux A, Mackie GA. 2007. Role of RNA structure and susceptibility to RNase E in regulation of a cold shock mRNA, *cspA* mRNA. *Journal of bacteriology 189*:4353-4358.

Hardwick SW, Gubbey T, Hug I, Jenal U, Luisi BF. 2012. Crystal structure of *Caulobacter crescentus* polynucleotide phosphorylase reveals a mechanism of RNA substrate channelling and RNA degradosome assembly. *Open biology 2*:120028.

Huntzinger E, Boisset S, Saveanu C, Benito Y, Geissmann T, Namane A, Lina G, Etienne J, Ehresmann B, Ehresmann C, Jacquier A, Vandenesch F, Romby P. 2005. *Staphylococcus aureus* RNAIII and the endoribonuclease III coordinately regulate *spa* gene expression. *EMBO J 24*:824-835.

Hutchison CA, Peterson SN, Gill SR, Cline RT, White O, Fraser CM, Smith HO, Venter JC. 1999. Global transposon mutagenesis and a minimal Mycoplasma genome. *Science 286*:2165-2169.

Ikeda Y, Yagi M, Morita T, Aiba H. 2011. Hfq binding at RhlB-recognition region of RNase E is crucial for the rapid degradation of target mRNAs mediated by sRNAs in *Escherichia coli*. *Mol Microbiol 79*:419-432.

Jagannath A, Wood M. 2007. RNA interference based gene therapy for neurological disease. *Brief Funct Genomic Proteomic 6*:40-49.

Jager S, Evguenieva-Hackenberg E, Klug G. 2004. Temperature-dependent processing of the *cspA* mRNA in *Rhodobacter capsulatus*. *Microbiology 150*:687-695.

Jager S, Fuhrmann O, Heck C, Hebermehl M, Schiltz E, Rauhut R, Klug G. 2001. An mRNA degrading complex in *Rhodobacter capsulatus*. *Nucleic Acids Res 29*:4581-4588.

Jain C, Belasco JG. 1995. Autoregulation of RNase E synthesis in *Escherichia coli*. *Nucleic acids symposium series*:85-88.

Jarrige A, Brechemier-Baey D, Mathy N, Duche O, Portier C. 2002. Mutational analysis of polynucleotide phosphorylase from *Escherichia coli*. *Journal of molecular biology 321*:397-409.

Jarrige AC, Mathy N, Portier C. 2001. PNPase autocontrols its expression by degrading a double-stranded structure in the *pnp* mRNA leader. *EMBO J 20*:6845-6855.

Jaskiewicz L, Filipowicz W. 2008. Role of Dicer in posttranscriptional RNA silencing. *Current topics in microbiology and immunology 320*:77-97.

Jiang X, Belasco JG. 2004. Catalytic activation of multimeric RNase E and RNase G by 5'-monophosphorylated RNA. *Proc Natl Acad Sci U S A 101*:9211-9216.

Jiang X, Diwa A, Belasco JG. 2000. Regions of RNase E important for 5'-end-dependent RNA cleavage and autoregulated synthesis. *Journal of bacteriology 182*:2468-2475.

Jinek M, Doudna JA. 2009. A three-dimensional view of the molecular machinery of RNA interference. *Nature 457*:405-412.

Jourdan SS, Kime L, McDowall KJ. 2010. The sequence of sites recognised by a member of the RNase E/G family can control the maximal rate of cleavage, while a 5'-monophosphorylated end appears to function cooperatively in mediating RNA binding. *Biochemical and biophysical research communications 391*:879-883.

Kaberdin VR, Blasi U. 2006. Translation initiation and the fate of bacterial mRNAs. *FEMS Microbiol Rev 30*:967-979.

Kaberdin VR, Miczak A, Jakobsen JS, Lin-Chao S, McDowall KJ, von Gabain A. 1998. The endoribonucleolytic N-terminal half of *Escherichia coli* RNase E is evolutionarily conserved in *Synechocystis sp*. and other bacteria but not the C-terminal half, which is sufficient for degradosome assembly. *Proc Natl Acad Sci U S A 95*:11637-11642.

Kajitani M, Kato A, Wada A, Inokuchi Y, Ishihama A. 1994. Regulation of the *Escherichia coli* hfq gene encoding the host factor for phage Q beta. *Journal of bacteriology 176*:531-534.

Kawamoto H, Koide Y, Morita T, Aiba H. 2006. Base-pairing requirement for RNA silencing by a bacterial small RNA and acceleration of duplex formation by Hfq. *Mol Microbiol 61*:1013-1022.

Khemici V, Poljak L, Luisi BF, Carpousis AJ. 2008. The RNase E of *Escherichia coli* is a membrane-binding protein. *Mol Microbiol 70*:799-813.

Kime L, Jourdan SS, Stead JA, Hidalgo-Sastre A, McDowall KJ. 2010. Rapid cleavage of RNA by RNase E in the absence of 5' monophosphate stimulation. *Mol Microbiol 76*:590-604.

Ko JH, Han K, Kim Y, Sim S, Kim KS, Lee SJ, Cho B, Lee K, Lee Y. 2008. Dual function of RNase E for control of M1 RNA biosynthesis in *Escherichia coli*. *Biochemistry 47*:762-770.

Koslover DJ, Callaghan AJ, Marcaida MJ, Garman EF, Martick M, Scott WG, Luisi BF. 2008. The crystal structure of the *Escherichia coli* RNase E apoprotein and a mechanism for RNA degradation. *Structure 16*:1238-1244.

LaCava J, Houseley J, Saveanu C, Petfalski E, Thompson E, Jacquier A, Tollervey D. 2005. RNA degradation by the exosome is promoted by a nuclear polyadenylation complex. *Cell 121*:713-724.

Lalonde MS, Zuo Y, Zhang J, Gong X, Wu S, Malhotra A, Li Z. 2007. Exoribonuclease R in *Mycoplasma genitalium* can carry out both RNA processing and degradative functions and is sensitive to RNA ribose methylation. *RNA 13*:1957-1968.

Lamontagne B, Larose S, Boulanger J, Elela SA. 2001. The RNase III family: a conserved structure and expanding functions in eukaryotic dsRNA metabolism. *Curr Issues Mol Biol 3*:71-78.

Lasa I, Toledo-Arana A, Dobin A, Villanueva M, de los Mozos IR, Vergara-Irigaray M, Segura V, Fagegaltier D, Penades JR, Valle J, Solano C, Gingeras TR. 2011. Genome-wide antisense transcription drives mRNA processing in bacteria. *Proc Natl Acad Sci U S A 108*:20172-20177.

Le Derout J, Boni IV, Régnier P, Hajnsdorf E. 2010. Hfq affects mRNA levels independently of degradation. *BMC Mol Biol 11*:17.

Le Derout J, Folichon M, Briani F, Dehò G, Régnier P, Hajnsdorf E. 2003. Hfq affects the length and the frequency of short oligo(A) tails at the 3' end of *Escherichia coli rps*O mRNAs. *Nucleic Acids Res 31*:4017-4023.

Lee K, Bernstein JA, Cohen SN. 2002. RNase G complementation of rne null mutation identifies functional interrelationships with RNase E in *Escherichia coli*. *Mol Microbiol 43*:1445-1456.

Lee K, Cohen SN. 2003. A *Streptomyces coelicolor* functional orthologue of *Escherichia coli* RNase E shows shuffling of catalytic and PNPase-binding domains. *Mol Microbiol 48*:349-360.

Lee K, Zhan X, Gao J, Qiu J, Feng Y, Meganathan R, Cohen SN, Georgiou G. 2003. RraA. a protein inhibitor of RNase E activity that globally modulates RNA abundance in *E. coli*. *Cell 114*:623-634.

Lee T, Feig AL. 2008. The RNA binding protein Hfq interacts specifically with tRNAs. *RNA 14*:514-523.

Li H, Nicholson AW. 1996. Defining the enzyme binding domain of a ribonuclease III processing signal. Ethylation interference and hydroxyl radical footprinting using catalytically inactive RNase III mutants. *EMBO J 15*:1421-1433.

Li Z, Pandit S, Deutscher MP. 1999. RNase G (CafA protein) and RNase E are both required for the 5' maturation of 16S ribosomal RNA. *EMBO J 18*:2878-2885.

Li Z, Reimers S, Pandit S, Deutscher MP. 2002. RNA quality control: degradation of defective transfer RNA. *EMBO J 21*:1132-1138.

Liang W, Deutscher MP. 2010. A novel mechanism for ribonuclease regulation: transfer-messenger RNA (tmRNA) and its associated protein SmpB regulate the stability of RNase R. *J Biol Chem 285*:29054-29058.

Liang W, Deutscher MP. 2012. Post-translational modification of RNase R is regulated by stress-dependent reduction in the acetylating enzyme Pka (YfiQ). *RNA 18*:37-41.

Lin-Chao S, Wei CL, Lin YT. 1999. RNase E is required for the maturation of *ssr*A RNA and normal *ssr*A RNA peptide-tagging activity. *Proc Natl Acad Sci U S A 96*:12406-12411.

Lin PH, Lin-Chao S. 2005. RhlB helicase rather than enolase is the beta-subunit of the *Escherichia coli* polynucleotide phosphorylase (PNPase)-exoribonucleolytic complex. *Proc Natl Acad Sci USA 102*:16590-16595.

Link TM, Valentin-Hansen P, Brennan RG. 2009. Structure of *Escherichia coli* Hfq bound to polyriboadenylate RNA. *Proc Natl Acad Sci U S A 106*:19292-19297.

Lioliou E, Sharma CM, Caldelari I, Helfer AC, Fechter P, Vandenesch F, Vogel J, Romby P. 2012. Global regulatory functions of the *Staphylococcus aureus* endoribonuclease III in gene expression. *PLoS genetics 8*:e1002782.

Liou GG, Chang HY, Lin CS, Lin-Chao S. 2002. DEAD box RhlB RNA helicase physically associates with exoribonuclease PNPase to degrade double-stranded RNA independent of the degradosome-assembling region of RNase E. *J Biol Chem 277*:41157-41162.

Liou GG, Jane WN, Cohen SN, Lin NS, Lin-Chao S. 2001. RNA degradosomes exist *in vivo* in *Escherichia coli* as multicomponent complexes associated with the cytoplasmic membrane via the N-terminal region of ribonuclease E. *Proc Natl Acad Sci U S A 98*:63-68.

Littauer YZ, Soreq H. 1982. *Polynucleotide Phosphorylase*. New York: Academic Press.

Liu Q, Greimann JC, Lima CD. 2006. Reconstitution, activities, and structure of the eukaryotic RNA exosome. *Cell 127*:1223-1237.

Lopez PJ, Marchand I, Joyce SA, Dreyfus M. 1999. The C-terminal half of RNase E, which organizes the *Escherichia coli* degradosome, participates in mRNA degradation but not rRNA processing *in vivo*. *Mol Microbiol 33*:188-199.

Lundberg U, Altman S. 1995. Processing of the precursor to the catalytic RNA subunit of RNase P from *Escherichia coli*. *RNA 1*:327-334.

Luttinger A, Hahn J, Dubnau D. 1996. Polynucleotide phosphorylase is necessary for competence development in *Bacillus subtilis*. *Mol Microbiol 19*:343-356.

Ma Y, Chan CY, He ML. 2007. RNA interference and antiviral therapy. *World J Gastroenterol 13*:5169-5179.

Mackie GA. 1992. Secondary structure of the mRNA for ribosomal protein S20. Implications for cleavage by ribonuclease E. *J Biol Chem 267*:1054-1061.

Mackie GA. 1998. Ribonuclease E is a 5'-end-dependent endonuclease. *Nature 395*:720-723.

Mackie GA. 2000. Stabilization of circular *rpsT* mRNA demonstrates the 5'-end dependence of RNase E action *in vivo*. *J Biol Chem 275*:25069-25072.

MacRae IJ, Doudna JA. 2007. Ribonuclease revisited: structural insights into ribonuclease III family enzymes. *Current opinion in structural biology 17*:138-145.

Malecki M, Viegas S, Carneiro T, Golik P, Dressaire C, Ferreira MG, Arraiano CM. 2012. Characterization of the Exoribonuclease Dis3L2 Discloses a Novel Degradation Pathway in Eukaryotes. *Submited*.

Marcaida MJ, DePristo MA, Chandran V, Carpousis AJ, Luisi BF. 2006. The RNA degradosome: life in the fast lane of adaptive molecular evolution. *Trends in biochemical sciences 31*:359-365.

Marujo PE, Hajnsdorf E, Le Derout J, Andrade R, Arraiano CM, Régnier P. 2000. RNase II removes the oligo(A) tails that destabilize the *rpsO* mRNA of *Escherichia coli*. *RNA 6*:1185-1193.

Massé E, Escorcia FE, Gottesman S. 2003. Coupled degradation of a small regulatory RNA and its mRNA targets in *Escherichia coli*. *Genes Dev 17*:2374-2383.

Mathy N, Jarrige AC, Robert-Le Meur M, Portier C. 2001. Increased expression of *Escherichia coli* polynucleotide phosphorylase at low temperatures is linked to a decrease in the efficiency of autocontrol. *J Bacteriol 183*:3848-3854.

Matos RG, Barbas A, Arraiano CM. 2009. RNase R mutants elucidate the catalysis of structured RNA: RNA-binding domains select the RNAs targeted for degradation. *Biochem J 423*:291-301.

Matsunaga J, Simons EL, Simons RW. 1996. RNase III autoregulation: structure and function of rncO, the posttranscriptional "operator". *RNA 2*:1228-1240.

Matus-Ortega ME, Regonesi ME, Pina-Escobedo A, Tortora P, Dehò G, García-Mena J. 2007. The KH and S1 domains of *Escherichia coli* polynucleotide phosphorylase are necessary for autoregulation and growth at low temperature. *Biochim Biophys Acta 1769*:194-203.

McDowall KJ, Hernandez RG, Lin-Chao S, Cohen SN. 1993. The *ams*-1 and *rne*-3071 temperature-sensitive mutations in the ams gene are in close proximity to each other and cause substitutions within a domain that resembles a product of the *Escherichia coli mre* locus. *J Bacteriol 175*:4245-4249.

McDowall KJ, Kaberdin VR, Wu SW, Cohen SN, Lin-Chao S. 1995. Site-specific RNase E cleavage of oligonucleotides and inhibition by stem-loops. *Nature 374*:287-290.

Melefors O, von Gabain A. 1991. Genetic studies of cleavage-initiated mRNA decay and processing of ribosomal 9S RNA show that the *Escherichia coli* ams and rne loci are the same. *Mol Microbiol 5*:857-864.

Meng W, Nicholson AW. 2008. Heterodimer-based analysis of subunit and domain contributions to double-stranded RNA processing by *Escherichia coli* RNase III *in vitro*. *Biochem J 410*:39-48.

Mian IS. 1997. Comparative sequence analysis of ribonucleases HII, III, II PH and D. *Nucleic acids research 25*:3187-3195.

Mikulecky PJ, Kaw MK, Brescia CC, Takach JC, Sledjeski DD, Feig AL. 2004. *Escherichia coli* Hfq has distinct interaction surfaces for *Dsr*A, *rpo*S and poly(A) RNAs. *Nat Struct Mol Biol 11*:1206-1214.

Misra TK, Apirion D. 1979. RNase E, an RNA processing enzyme from *Escherichia coli*. *J Biol Chem 254*:11154-11159.

Mitchell P, Petfalski E, Shevchenko A, Mann M, Tollervey D. 1997. The exosome: a conserved eukaryotic RNA processing complex containing multiple 3' - 5' exoribonucleases. *Cell 91*:457-466.

Mohanty BK, Kushner SR. 2000. Polynucleotide phosphorylase, RNase II and RNase E play different roles in the *in vivo* modulation of polyadenylation in *Escherichia coli*. *Mol Microbiol 36*:982-994.

Mohanty BK, Kushner SR. 2000 Polynucleotide phosphorylase functions both as a 3' right-arrow 5' exonuclease and a poly(A) polymerase in *Escherichia coli*. *Proc Natl Acad Sci USA 97*:11966-11971.

Mohanty BK, Kushner SR. 2002. Polyadenylation of *Escherichia coli* transcripts plays an integral role in regulating intracellular levels of polynucleotide phosphorylase and RNase E. *Mol Microbiol 45*:1315-1324.

Mohanty BK, Kushner SR. 2003. Genomic analysis in *Escherichia coli* demonstrates differential roles for polynucleotide phosphorylase and RNase II in mRNA abundance and decay. *Mol Microbiol 50*:645-658.

Mohanty BK, Kushner SR. 2006. The majority of *Escherichia coli* mRNAs undergo post-transcriptional modification in exponentially growing cells. *Nucleic acids research 34*:5695-5704.

Mohanty BK, Kushner SR. 2009. Processing of the *Escherichia coli leuX* tRNA transcript, encoding tRNALeu5, requires either the 3'->5' exoribonuclease polynucleotide phosphorylase or RNase P to remove the Rho-independent transcription terminator. *Nucleic Acids Res*.

Mohanty BK, Maples VF, Kushner SR. 2004. The Sm-like protein Hfq regulates polyadenylation dependent mRNA decay in *Escherichia coli*. *Mol Microbiol 54*:905-920.

Moll I, Afonyushkin T, Vytvytska O, Kaberdin VR, Blasi U. 2003a. Coincident Hfq binding and RNase E cleavage sites on mRNA and small regulatory RNAs. *RNA 9*:1308-1314.

Moll I, Leitsch D, Steinhauser T, Blasi U. 2003b. RNA chaperone activity of the Sm-like Hfq protein. *EMBO Rep 4*:284-289.

Moller T, Franch T, Hojrup P, Keene DR, Bachinger HP, Brennan RG, Valentin-Hansen P. 2002. Hfq: a bacterial Sm-like protein that mediates RNA-RNA interaction. *Mol Cell 9*:23-30.

Morita T, Maki K, Aiba H. 2005. RNase E-based ribonucleoprotein complexes: mechanical basis of mRNA destabilization mediated by bacterial noncoding RNAs. *Genes Dev 19*:2176-2186.

Mudd EA, Higgins CF. 1993. *Escherichia coli* endoribonuclease RNase E: autoregulation of expression and site-specific cleavage of mRNA. *Mol Microbiol 9*:557-568.

Mudd EA, Krisch HM, Higgins CF. 1990. RNase E, an endoribonuclease, has a general role in the chemical decay of *Escherichia coli* mRNA: evidence that rne and ams are the same genetic locus. *Mol Microbiol 4*:2127-2135.

Murashko ON, Kaberdin VR, Lin-Chao S. 2012. Membrane binding of *Escherichia coli* RNase E catalytic domain stabilizes protein structure and increases RNA substrate affinity. *Proc Natl Acad Sci U S A 109*:7019-7024.

Nicholson AW. 1999. Function, mechanism and regulation of bacterial ribonucleases. *FEMS Microbiol Rev 23*:371-390.

Nurmohamed S, Vaidialingam B, Callaghan AJ, Luisi BF. 2009. Crystal structure of *Escherichia coli* polynucleotide phosphorylase core bound to RNase E, RNA and manganese: implications for catalytic mechanism and RNA degradosome assembly. *J Mol Biol 389*:17-33.

Nurmohamed S, Vincent HA, Titman CM, Chandran V, Pears MR, Du D, Griffin JL, Callaghan AJ, Luisi BF. 2011. Polynucleotide Phosphorylase Activity May Be Modulated by Metabolites in *Escherichia coli*. *J Biol Chem 286*:14315-14323.

Okada Y, Wachi M, Hirata A, Suzuki K, Nagai K, Matsuhashi M. 1994. Cytoplasmic axial filaments in *Escherichia coli* cells: possible function in the mechanism of chromosome segregation and cell division. *Journal of bacteriology 176*:917-922.

Olsen AS, Moller-Jensen J, Brennan RG, Valentin-Hansen P. 2010. C-Terminally Truncated Derivatives of *Escherichia coli* Hfq Are Proficient in Riboregulation. *J Mol Biol*.

Ono M, Kuwano M. 1979. A conditional lethal mutation in an *Escherichia coli* strain with a longer chemical lifetime of messenger RNA. *J Mol Biol 129*:343-357.

Ono M, Kuwano M. 1980. Chromosomal location of a gene for chemical longevity of messenger ribonculeic acid in a temperature-sensitive mutant of *Escherichia coli*. *Journal of bacteriology 142*:325-326.

Oppenheim AB, Kornitzer D, Altuvia S, Court DL. 1993. Posttranscriptional control of the lysogenic pathway in bacteriophage *lambda*. *Prog Nucleic Acid Res Mol Biol 46*:37-49.

Oussenko IA, Abe T, Ujiie H, Muto A, Bechhofer DH. 2005. Participation of 3'-to-5' exoribonucleases in the turnover of *Bacillus subtilis* mRNA. *J Bacteriol 187*:2758-2767.

Ow MC, Kushner SR. 2002. Initiation of tRNA maturation by RNase E is essential for cell viability in *E. coli*. *Genes Dev 16*:1102-1115.

Ow MC, Liu Q, Kushner SR. 2000. Analysis of mRNA decay and rRNA processing in *Escherichia coli* in the absence of RNase E-based degradosome assembly. *Mol Microbiol 38*:854-866.

Ow MC, Liu Q, Mohanty BK, Andrew ME, Maples VF, Kushner SR. 2002. RNase E levels in *Escherichia coli* are controlled by a complex regulatory system that involves transcription of the rne gene from three promoters. *Mol Microbiol 43*:159-171.

Palanisamy SK, Fletcher C, Tanjung L, Katz ME, Cheetham BF. 2009. Deletion of the C-terminus of polynucleotide phosphorylase increases twitching motility, a virulence characteristic of the anaerobic bacterial pathogen *Dichelobacter nodosus*. *FEMS Microbiol Lett 302*:39-45.

Papenfort K, Bouvier M, Mika F, Sharma CM, Vogel J. 2010. Evidence for an autonomous 5' target recognition domain in an Hfq-associated small RNA. *Proc Natl Acad Sci U S A 107*:20435-20440.

Parker R, Song H. 2004. The enzymes and control of eukaryotic mRNA turnover. *Nat Struct Mol Biol 11*:121-127.

Pepe CM, Maslesa-Galic S, Simons RW. 1994. Decay of the IS10 antisense RNA by 3' exoribonucleases: evidence that RNase II stabilizes RNA-OUT against PNPase attack. *Mol Microbiol 13*:1133-1142.

Pertzev AV, Nicholson AW. 2006. Characterization of RNA sequence determinants and antideterminants of processing reactivity for a minimal substrate of *Escherichia coli* ribonuclease III. *Nucleic Acids Res 34*:3708-3721.

Piazza F, Zappone M, Sana M, Briani F, Dehò G. 1996. Polynucleotide phosphorylase of *Escherichia coli* is required for the establishment of bacteriophage P4 immunity. *J Bacteriol 178*:5513-5521.

Portier C, Dondon L, Grunberg-Manago M, Régnier P. 1987. The first step in the functional inactivation of the *Escherichia coli* polynucleotide phosphorylase messenger is a ribonuclease III processing at the 5' end. *EMBO J 6*:2165-2170.

Portier C, Régnier P. 1984. Expression of the *rps*O and *pnp* genes: structural analysis of a DNA fragment carrying their control regions. *Nucleic Acids Res 12*:6091-6102.

Pruijn GJ. 2005. Doughnuts dealing with RNA. *Nat Struct Mol Biol 12*:562-564.

Purusharth RI, Klein F, Sulthana S, Jager S, Jagannadham MV, Evguenieva-Hackenberg E, Ray MK, Klug G. 2005. Exoribonuclease R interacts with endoribonuclease E and an RNA helicase in the psychrotrophic bacterium *Pseudomonas syringae Lz4W*. *J Biol Chem 280*:14572-14578.

Purusharth RI, Madhuri B, Ray MK. 2007. Exoribonuclease R in *Pseudomonas syringae* is essential for growth at low temperature and plays a novel role in the 3' end processing of 16 and 5 S ribosomal RNA. *J Biol Chem 282*:16267-16277.

Py B, Higgins CF, Krisch HM, Carpousis AJ. 1996. A DEAD-box RNA helicase in the *Escherichia coli* RNA degradosome. *Nature 381*:169-172.

Rajkowitsch L, Schroeder R. 2007. Dissecting RNA chaperone activity. *RNA 13*:2053-2060.

Rasmussen AA, Eriksen M, Gilany K, Udesen C, Franch T, Petersen C, Valentin-Hansen P. 2005. Regulation of *omp*A mRNA stability: the role of a small regulatory RNA in growth phase-dependent control. *Mol Microbiol 58*:1421-1429.

Redko Y, Bechhofer DH, Condon C. 2008. Mini-III, an unusual member of the RNase III family of enzymes, catalyses 23S ribosomal RNA maturation in *B. subtilis*. *Mol Microbiol 68*:1096-1106.

Régnier P, Arraiano CM. 2000. Degradation of mRNA in bacteria: emergence of ubiquitous features. *Bioessays 22*:235-244.

Régnier P, Grunberg-Manago M. 1990. RNase III cleavages in non-coding leaders of *Escherichia coli* transcripts control mRNA stability and genetic expression. *Biochimie 72*:825-834.

Régnier P, Portier C. 1986. Initiation, attenuation and RNase III processing of transcripts from the *Escherichia coli* operon encoding ribosomal protein S15 and polynucleotide phosphorylase. *Journal of molecular biology 187*:23-32.

Regonesi ME, Del Favero M, Basilico F, Briani F, Benazzi L, Tortora P, Mauri P, Dehò G. 2006. Analysis of the *Escherichia coli* RNA degradosome composition by a proteomic approach. *Biochimie 88*:151-161.

Reva ON, Weinel C, Weinel M, Bohm K, Stjepandic D, Hoheisel JD, Tummler B. 2006. Functional genomics of stress response in *Pseudomonas putida* KT2440. *Journal of bacteriology 188*:4079-4092.

Rice JB, Vanderpool CK. 2011. The small RNA *SgrS* controls sugar-phosphate accumulation by regulating multiple PTS genes. *Nucleic Acids Res 39*:3806-3819.

Robert-Le Meur M, Portier C. 1992. *E.coli* polynucleotide phosphorylase expression is autoregulated through an RNase III-dependent mechanism. *EMBO J 11*:2633-2641.

Robert-Le Meur M, Portier C. 1994. Polynucleotide phosphorylase of *Escherichia coli* induces the degradation of its RNase III processed messenger by preventing its translation. *Nucleic acids research 22*:397-403.

Robertson HD, Webster RE, Zinder ND. 1968. Purification and properties of ribonuclease III from *Escherichia coli*. *J Biol Chem 243*:82-91.

Rosenzweig JA, Chromy B, Echeverry A, Yang J, Adkins B, Plano GV, McCutchen-Maloney S, Schesser K. 2007. Polynucleotide phosphorylase independently controls virulence factor expression levels and export in *Yersinia spp*. *FEMS Microbiol Lett 270*:255-264.

Rosenzweig JA, Weltman G, Plano GV, Schesser K. 2005. Modulation of yersinia type three secretion system by the S1 domain of polynucleotide phosphorylase. *J Biol Chem 280*:156-163.

Rott R, Zipor G, Portnoy V, Liveanu V, Schuster G. 2003. RNA polyadenylation and degradation in cyanobacteria are similar to the chloroplast but different from *Escherichia coli*. *J Biol Chem 278*:15771-15777.

Santos JM, Drider D, Marujo PE, Lopez P, Arraiano CM. 1997. Determinant role of *E. coli* RNase III in the decay of both specific and heterologous mRNAs. *FEMS Microbiol Lett 157*:31-38.

Schaeffer D, Tsanova B, Barbas A, Reis FP, Dastidar EG, Sanchez-Rotunno M, Arraiano CM, van Hoof A. 2009. The exosome contains domains with specific endoribonuclease, exoribonuclease and cytoplasmic mRNA decay activities. *Nat Struct Mol Biol 16*:56-62.

Scheibe M, Bonin S, Hajnsdorf E, Betat H, Morl M. 2007. Hfq stimulates the activity of the CCA-adding enzyme. *BMC Mol Biol 8*:92.

Schumacher MA, Pearson RF, Moller T, Valentin-Hansen P, Brennan RG. 2002. Structures of the pleiotropic translational regulator Hfq and an Hfq-RNA complex: a bacterial Sm-like protein. *EMBO J 21*:3546-3556.

Shi Z, Yang WZ, Lin-Chao S, Chak KF, Yuan HS. 2008. Crystal structure of *Escherichia coli* PNPase: central channel residues are involved in processive RNA degradation. *RNA 14*:2361-2371.

Silva IJ, Saramago M, Dressaire C, Domingues S, Viegas SC, Arraiano CM. 2011. Importance and key events of prokaryotic RNA decay: the ultimate fate of an RNA molecule. *Wiley interdisciplinary reviews RNA 2*:818-836.

Sim SH, Yeom JH, Shin C, Song WS, Shin E, Kim HM, Cha CJ, Han SH, Ha NC, Kim SW, Hahn Y, Bae J, Lee K. 2010. *Escherichia coli* ribonuclease III activity is downregulated by osmotic stress: consequences for the degradation of *bdm* mRNA in biofilm formation. *Mol Microbiol 75*:413-425.

Slomovic S, Portnoy V, Yehudai-Resheff S, Bronshtein E, Schuster G. 2008. Polynucleotide phosphorylase and the archaeal exosome as poly(A)-polymerases. *Biochim Biophys Acta 1779*:247-255.

Sohlberg B, Huang J, Cohen SN. 2003. The *Streptomyces coelicolor* polynucleotide phosphorylase homologue, and not the putative poly(A) polymerase, can polyadenylate RNA. *J Bacteriol 185*:7273-7278.

Sousa S, Marchand I, Dreyfus M. 2001. Autoregulation allows *Escherichia coli* RNase E to adjust continuously its synthesis to that of its substrates. *Mol Microbiol 42*:867-878.

Spickler C, Mackie A. 2000. Action of RNases II and Polynucleotide Phosphorylase against RNAs containing stem-loops of defined structure. *J Bacteriol 182*:2422-2427.

Stickney LM, Hankins JS, Miao X, Mackie GA. 2005. Function of the conserved S1 and KH domains in polynucleotide phosphorylase. *J Bacteriol 187*:7214-7221.

Sukhodolets MV, Garges S. 2003. Interaction of *Escherichia coli* RNA polymerase with the ribosomal protein S1 and the Sm-like ATPase Hfq. *Biochemistry 42*:8022-8034.

Sulewski M, Marchese-Ragona SP, Johnson KA, Benkovic SJ. 1989. Mechanism of polynucleotide phosphorylase. *Biochemistry 28*:5855-5864.

Symmons MF, Jones GH, Luisi BF. 2000. A duplicated fold is the structural basis for polynucleotide phosphorylase catalytic activity, processivity, and regulation. *Structure 8*:1215-1226.

Taghbalout A, Rothfield L. 2008. RNaseE and RNA helicase B play central roles in the cytoskeletal organization of the RNA degradosome. *J Biol Chem 283*:13850-13855.

Taraseviciene L, Miczak A, Apirion D. 1991. The gene specifying RNase E (*rne*) and a gene affecting mRNA stability (*ams*) are the same gene. *Mol Microbiol 5*:851-855.

Tobe T, Sasakawa C, Okada N, Honma Y, Yoshikawa M. 1992. *vacB*, a novel chromosomal gene required for expression of virulence genes on the large plasmid of *Shigella flexneri*. *J Bacteriol 174*:6359-6367.

Tock MR, Walsh AP, Carroll G, McDowall KJ. 2000. The CafA protein required for the 5'-maturation of 16 S rRNA is a 5'-end-dependent ribonuclease that has context-dependent broad sequence specificity. *J Biol Chem 275*:8726-8732.

Tomecki R, Kristiansen MS, Lykke-Andersen S, Chlebowski A, Larsen KM, Szczesny RJ, Drazkowska K, Pastula A, Andersen JS, Stepien PP, Dziembowski A, Jensen TH. 2010. The human core exosome interacts with differentially localized processive RNases: hDIS3 and hDIS3L. *EMBO J 29*:2342-2357.

Tsui HC, Feng G, Winkler ME. 1997. Negative regulation of *mutS* and *mutH* repair gene expression by the Hfq and RpoS global regulators of *Escherichia coli* K-12. *Journal of bacteriology 179*:7476-7487.

Tuckerman JR, Gonzalez G, Gilles-Gonzalez MA. 2011. Cyclic di-GMP activation of polynucleotide phosphorylase signal-dependent RNA processing. *J Mol Biol 407*:633-639.

Udekwu KI, Darfeuille F, Vogel J, Reimegard J, Holmqvist E, Wagner EG. 2005. Hfq-dependent regulation of *Omp*A synthesis is mediated by an antisense RNA. *Genes Dev 19*:2355-2366.

Umitsuki G, Wachi M, Takada A, Hikichi T, Nagai K. 2001. Involvement of RNase G in *in vivo* mRNA metabolism in *Escherichia coli*. *Genes to cells : devoted to molecular & cellular mechanisms 6*:403-410.

Urban JH, Vogel J. 2007. Translational control and target recognition by *Escherichia coli* small RNAs *in vivo*. *Nucleic Acids Res 35*:1018-1037.

Valentin-Hansen P, Eriksen M, Udesen C. 2004. The bacterial Sm-like protein Hfq: a key player in RNA transactions. *Mol Microbiol 51*:1525-1533.

Vanácová S, Wolf J, Martin G, Blank D, Dettwiler S, Friedlein A, Langen H, Keith G, Keller W. 2005. A new yeast poly(A) polymerase complex involved in RNA quality control. *PLoS Biol 3*:e189.

Vanzo NF, Li YS, Py B, Blum E, Higgins CF, Raynal LC, Krisch HM, Carpousis AJ. 1998. Ribonuclease E organizes the protein interactions in the *Escherichia coli* RNA degradosome. *Genes Dev 12*:2770-2781.

Vecerek B, Moll I, Blasi U. 2005. Translational autocontrol of the *Escherichia coli* hfq RNA chaperone gene. *RNA 11*:976-984.

Vecerek B, Rajkowitsch L, Sonnleitner E, Schroeder R, Blasi U. 2008. The C-terminal domain of *Escherichia coli* Hfq is required for regulation. *Nucleic Acids Res 36*:133-143.

Viegas SC, Arraiano CM. 2008. Regulating the regulators: How ribonucleases dictate the rules in the control of small non-coding RNAs. *RNA Biol 5*:230-243.

Viegas SC, Pfeiffer V, Sittka A, Silva IJ, Vögel J, Arraiano CM. 2007. Characterization of the role of ribonucleases in *Salmonella* small RNA decay. *Nucleic Acids Res 35*:7651-7664.

Vincent HA, Deutscher MP. 2006. Substrate recognition and catalysis by the exoribonuclease RNase R. *J Biol Chem 281*:29769-29775.

Vincent HA, Deutscher MP. 2009. The roles of individual domains of RNase R in substrate binding and exoribonuclease activity. The nuclease domain is sufficient for digestion of structured RNA. *J Biol Chem 284*:486-494.

Vogel J, Argaman L, Wagner EG, Altuvia S. 2004. The small RNA *IstR* inhibits synthesis of an SOS-induced toxic peptide. *Curr Biol 14*:2271-2276.

Wachi M, Umitsuki G, Shimizu M, Takada A, Nagai K. 1999. *Escherichia coli cafA* gene encodes a novel RNase, designated as RNase G, involved in processing of the 5' end of 16S rRNA. *Biochemical and biophysical research communications 259*:483-488.

Wagner EG, Altuvia S, Romby P. 2002. Antisense RNAs in bacteria and their genetic elements. *Advances in genetics 46*:361-398.

Wang W, Bechhofer DH. 1996. Properties of a *Bacillus subtilis* polynucleotide phosphorylase deletion strain. *Journal of bacteriology 178*:2375-2382.

Worrall JA, Gorna M, Crump NT, Phillips LG, Tuck AC, Price AJ, Bavro VN, Luisi BF. 2008. Reconstitution and analysis of the multienzyme *Escherichia coli* RNA degradosome. *J Mol Biol 382*:870-883.

Wu J, Jiang Z, Liu M, Gong X, Wu S, Burns CM, Li Z. 2009. Polynucleotide phosphorylase protects *Escherichia coli* against oxidative stress. *Biochemistry 48*:2012-2020.

Wyers F, Rougemaille M, Badis G, Rousselle JC, Dufour ME, Boulay J, Regnault B, Devaux F, Namane A, Seraphin B, Libri D, Jacquier A. 2005. Cryptic pol II transcripts are degraded by a nuclear quality control pathway involving a new poly(A) polymerase. *Cell 121*:725-737.

Xu F, Cohen SN. 1995. RNA degradation in *Escherichia coli* regulated by 3' adenylation and 5' phosphorylation. *Nature 374*:180-183.

Yehudai-Resheff S, Hirsh M, Schuster G. 2001. Polynucleotide phosphorylase functions as both an exonuclease and a poly(A) polymerase in spinach chloroplasts. *Mol Cell Biol 21*:5408-5416.

Zangrossi S, Briani F, Ghisotti D, Regonesi ME, Tortora P, Dehò G. 2000. Transcriptional and post-transcriptional control of polynucleotide phosphorylase during cold acclimation in *Escherichia coli*. *Mol Microbiol 36*:1470-1480.

Zhang K, Nicholson AW. 1997. Regulation of ribonuclease III processing by double-helical sequence antideterminants. *Proc Natl Acad Sci U S A 94*:13437-13441.

Zilhão R, Caillet J, Régnier P, Arraiano CM. 1995a. Precise physical mapping of the *Escherichia coli rnb* gene, encoding ribonuclease II. *Mol Gen Genet 248*:242-246.

Zilhão R, Cairrão F, Régnier P, Arraiano CM. 1996. PNPase modulates RNase II expression in *Escherichia coli*: implications for mRNA decay and cell metabolism. *Mol Microbiol 20*:1033-1042.

Zilhão R, Camelo L, Arraiano CM. 1993. DNA sequencing and expression of the gene *rnb* encoding *Escherichia coli* ribonuclease II. *Mol Microbiol 8*:43-51.

Zilhão R, Régnier P, Arraiano CM. 1995b. The role of endonucleases in the expression of ribonuclease II in *Escherichia coli*. *FEMS Microbiol Lett 130*:237-244.

Zuo Y, Deutscher MP. 2001. Exoribonuclease superfamilies: structural analysis and phylogenetic distribution. *Nucleic Acids Res 29*:1017-1026.

Zuo Y, Vincent HA, Zhang J, Wang Y, Deutscher MP, Malhotra A. 2006. Structural basis for processivity and single-strand specificity of RNase II. *Mol Cell 24*:149-156.

Chapter 2

The crucial role of PNPase in the degradation of small RNAs that are not associated with Hfq

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For this work I helped carried out the experiments, the analysis of the data and writing of the manuscript.

Table of Contents

Abstract

The transient existence of small RNAs free of binding to the RNA chaperone Hfq is part of the normal dynamic lifecycle of a sRNA. Small RNAs are extremely labile when are not associated with Hfq, but the mechanism by which Hfq stabilises sRNAs has been elusive. In this work, we have found that polynucleotide phosphorylase (PNPase) is the major factor involved in the rapid degradation of small RNAs, especially those that are free of binding to Hfq. The levels of MicA, GlmY, RyhB and SgrS RNAs are drastically increased upon PNPase inactivation in Hfq^{-} cells. In the absence of Hfq, all sRNAs are slightly shorter than their full-length species as result of 3'-end trimming. We show that the turnover of Hfq-free small RNAs is growth phase regulated and that PNPase activity is particularly important in stationary-phase. Indeed, PNPase makes a greater contribution than RNase E, which is commonly believed to be the main enzyme in the decay of small RNAs. Lack of poly(A) polymerase I (PAP I) is also found to affect the rapid degradation of Hfq-free small RNAs although to a lesser extent. Our data also suggests that when the sRNA is not associated with Hfq, the degradation occurs mainly in a target-independent pathway in which RNase III has a reduced impact. This work demonstrated that small RNAs free of Hfq binding are preferably degraded by PNPase. Overall, our data highlights the impact of 3'-exonucleolytic RNA decay pathways and re-evaluates the degradation mechanisms of Hfq-free small RNAs.

Introduction

The bacterial Hfq is a member of the Sm/Lsm superfamily of proteins involved in RNA metabolism (Wilusz & Wilusz, 2005). It is a global regulator of cell physiology with particular impact on stress responses and affects the virulence traits of many pathogens (Tsui et al., 1994; Chao & Vogel, 2010). Hfq plays a relevant role as a mediator of small noncoding RNA–mRNA interactions (Valentin-Hansen et al., 2004; Waters & Storz, 2009). Base pairing of small RNAs with their target mRNAs can alter mRNA translation and/or stability. The majority of small RNAs act as inhibitors of translation usually triggering mRNA decay, although some other sRNAs act as positive regulators (Massé et al., 2003; Vecerek et al., 2007; Soper et al., 2010).

Hfq forms a stable hexamer with a ring-shaped structure displaying two distinct RNA-binding surfaces (Brennan & Link, 2007). Biochemical and structural data support that the Hfq hexamer can bind simultaneously the sRNA on its proximal face and mRNA on its distal face increasing the probability of RNA-RNA interactions in order to form a heteroduplex (Schumacher et al., 2002; Lease & Woodson, 2004; Mikulecky et al., 2004; Link et al., 2009). In agreement, it has been suggested that the Hfq hexamer forms a ternary complex with oligo A_{18} and the small RNA $DsrA_{DII}$ with a stoichiometry of 1:1:1 (Updegrove et al., 2011). Hfq can also work as a RNA chaperone and induce structural rearrangement of the RNA molecules to enable the contact between the two partner RNAs (Moll et al., 2003b; Geissmann & Touati, 2004; Afonyushkin et al., 2005; Arluison et al., 2007).

Bacterial small RNAs that act as repressors bind at or near the ribosome binding site (RBS) of the target mRNA blocking its translation (Morita et al., 2006; Bouvier et al., 2008). Most of the time, this promotes cleavages in the mRNA not only on the vicinity of the duplex (as happens with *ompA* mRNA/MicA) (Udekwu et al., 2005), but also downstream into the coding region (as reported for *sodB*

mRNA/RyhB) (Prévost et al., 2011). Ribonuclease (RNase) III is an important endoribonuclease in the degradation of sRNA coupled to their target mRNAs (Afonyushkin et al., 2005; Deltcheva et al., 2011). However other sRNAs mediate the destabilisation of the target mRNA in an RNase E-dependent manner (Massé et al., 2003; Afonyushkin et al., 2005; Morita et al., 2005; Udekwu et al., 2005). RNase E is a single-stranded RNA endoribonuclease involved in mRNA decay in *Escherichia coli* (Arraiano et al., 2010). Hfq can associate with RNase E and sRNA in ribonucleoprotein complexes that are thought to make the degradation of target mRNAs more efficient (Aiba, 2007).

Hfq is also found to interact with other proteins involved in mRNA decay. One of these proteins is the poly(A) polymerase I (PAP I), responsible for the majority of polyadenylation in *E. coli* cells (Régnier & Hajnsdorf, 2009). Hfq is suggested to regulate polyadenylation by stimulating PAP I activity on mRNA (Hajnsdorf & Régnier, 2000; Folichon et al., 2005). In the absence of Hfq, the poly(A) levels are reduced and the poly(A) tails are suggested to become smaller (Le Derout et al., 2003; Mohanty et al., 2004). Hfq was also shown to interact with the polynucleotide phosphorylase (PNPase) (Mohanty et al., 2004), a major 3'-5' exoribonuclease involved in RNA degradation (Andrade et al., 2009b). PNPase responds to environmental stimuli and its activity is modulated by metabolites, such as ATP, citrate and cyclic di-GMP (Del Favero et al., 2008; Nurmohamed et al., 2011; Tuckerman et al., 2011). We have previously shown that PNPase is a key factor in the turnover of small RNAs controlling the expression of outer membrane proteins in the stationary-phase of growth (Andrade & Arraiano, 2008). It was recently suggested that PNPase can also have a protector role for some sRNAs in exponentially growing cells (De Lay & Gottesman, 2011). However, the details of the interplay between PNPase and Hfq in the function of sRNA are still not clear.

The interaction of Hfq with small RNAs is dynamic. Small RNAs compete for access to Hfq and it was shown that the expression of unrelated sRNAs can dissociate Hfq-sRNA complexes already formed (Fender et al., 2010; Hussein & Lim, 2011). The transient existence of small RNAs free from Hfq binding is thus part of the normal dynamic lifecycle of a sRNA. In addition, variations in the Hfq expression levels or in the availability of the free pool of Hfq can result in the reduction of Hfq-sRNA complexes. A small RNA that is not associated with Hfq is rapidly degraded, although the mechanism by which Hfq stabilises small RNAs is not yet fully understood. RNase E was considered to be the responsible for the rapid degradation of the small RNAs and was shown to compete with Hfq for access to the same RNA sequences (Massé et al., 2003; Moll et al., 2003a). However, the activity of RNase E may not be as generalised; for example, RNase E was not found to be important for the *in vivo* degradation of OxyS upon Hfq inactivation (Basineni et al., 2009).

In this work, we have characterised the degradation of small RNAs that are unassociated with Hfq. We have constructed multiple *hfq* mutants defective in RNases or in the poly(A) polymerase and studied the impact of these factors in the expression of several small RNAs. We have shown that small RNAs in their Hfq-free state are rapidly degraded by PNPase, particularly in the stationary-phase of growth. Moreover, PNPase was found to be more relevant than RNase E or RNase III in the degradation of small RNAs when these were not associated to Hfq. PNPase-mediated degradation of small RNAs is also found to be an active regulatory pathway in the cells expressing Hfq. Together, results show that PNPase has a predominant role in the degradation of Hfq-free small RNAs.

Results

Inactivation of PNPase markedly increases the levels of small RNAs not associated with Hfq

It is commonly believed that the RNA chaperone Hfq protects regulatory RNAs from RNase E endonucleolytic cleavages (Massé et al., 2003; Moll et al., 2003a). Nevertheless, we have previously showed that 3'-5' exonucleolytic activity can be determinant for the degradation of small RNAs even in the presence of Hfq (Andrade & Arraiano, 2008). In this work, we wanted to characterise the role of 3'-5' exoribonucleases in the degradation of small RNAs while are not associated with Hfq. Therefore, we have performed most of our studies in strains lacking Hfq.

In this work, we have analysed four well characterised *E. coli* sRNAs; MicA, SgrS, RyhB and GlmY. Most of these small RNAs are induced under specific conditions of stress and in stationary-phase. Therefore, we decided to focus our work in this growth phase. Total RNA was extracted from stationary-phase cultures and the steady-state levels of these small RNAs were analysed by Northern blotting. Inactivation of Hfq resulted in the high reduction of all the small RNAs analysed, when compared to wild-type strain (Figure 1, central panel). Only GlmY levels seemed not to be so strongly affected by the absence of Hfq in the conditions analysed. Subsequently, a set of multiple mutants lacking both Hfq and one of the main 3'-5' exoribonucleases (PNPase, RNase II or RNase R) was constructed. RNase II and RNase R had a reduced impact in this regulation; RNase II mutant was only shown to change GlmY levels and the absence of RNase R did not affect the levels of any of these small RNAs. Upon inactivation of PNPase in cells lacking Hfq, it was possible to detect a very strong signal for all the small RNAs analysed. These results indicate that PNPase is a major factor controlling the levels of small RNA that are not associated with Hfq.

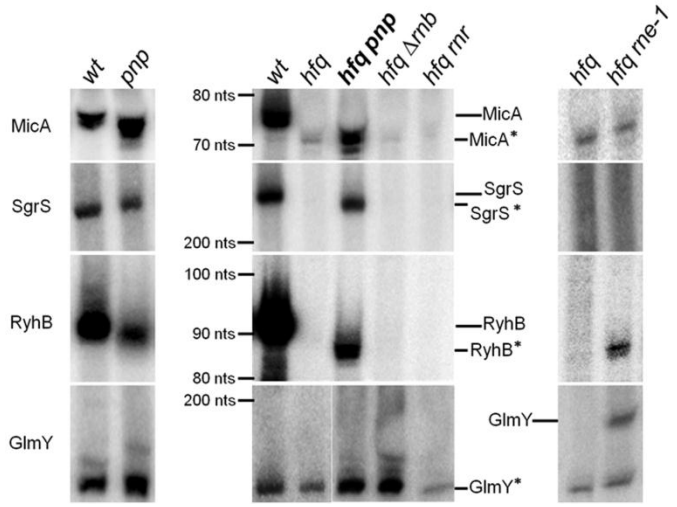


Figure 1. PNPase strongly affects the levels of several small RNAs that are not bound to Hfq.
Small RNA expression was analysed by Northern blot. *(Left panel)* The levels of MicA, SgrS, RyhB and GlmY were analysed in the wild-type (*hfq*$^{+}$ *pnp*$^{+}$) and a PNPase mutant (*hfq*$^{+}$ *pnp*$^{-}$). Total RNA was extracted from stationary-phase cultures grown at 37^{o}C as mentioned in *Material and Methods*. *(Central panel)* Hfq mutants lacking one of the 3'-5' exoribonucleases PNPase (*pnp*), RNase II (*rnb*) and RNase R (*rnr*) were compared to wild-type (*wt*) and *hfq* single mutant. (*Right panel)* To study the impact of the essential RNase E (*rne*), the double *hfq rne*-1 mutant was grown at 30^{o}C until it reached stationary-phase and then shifted to the non-permissive temperature of 44^{o}C for inactivation of the thermosensitive RNase E. Samples were withdrawn after 5 min incubation. For comparison, the single *hfq* mutant was treated in the same conditions. Specific [32P]-labelled probes were used to detect the small RNAs. Full-length small RNAs are clearly detected on wild-type (except for GlmY), showing the expected sizes: MicA (74 nts), RyhB (90 nts), GlmY (180 nts) and SgrS (227 nts), as estimated from markers run along the gels. Small RNAs detected on *hfq* mutants (namely in the *hfq pnp*) are slightly shorter than the corresponding full-length sRNAs; these shorter small RNA are designated by an *asterisk* (∗). The positions of both the full-length and the shorter small RNAs are indicated. 5S RNA or tmRNA were used as loading controls.

In Hfq^{-} PNPase^{-} cells we observed the accumulation of a slightly shorter form of all the small RNAs (here designated respectively by MicA*, SgrS*, RyhB* and GlmY*) (Figure 1, central panel). These shorter sRNA are not detected or are barely perceptible in the wild-type strain. The shorter GlmY* (~140nt) is an exception, being the predominant RNA detected in the wild-type. This has been

previously observed as the full-length GlmY (~180nt) is rapidly processed in the 3'-end to originate the GlmY* species (Reichenbach et al., 2008; Urban & Vogel, 2008). Overexpression of PNPase in the *hfq pnp* strain was shown to reverse the accumulation of MicA* (Figure S1). This result confirmed that PNPase is responsible for the higher levels of small RNAs found in the Hfq$^-$ PNPase$^-$ cells.

We had previously demonstrated the involvement of PNPase in the degradation of MicA in stationary-phase cells harbouring Hfq (Andrade and Arraiano, 2008). To check the impact of PNPase in the regulation of all these small RNAs in the presence of Hfq, we have analysed the levels of the same small RNAs in the *pnp* single mutant and compared it to the wild-type strain (Figure 1, left panel). Inactivation of PNPase in cells expressing Hfq resulted in higher levels of two of these sRNAs, namely GlmY* and MicA. In contrast, two other small RNAs RyhB and SgrS showed decreased amounts in the PNPase mutant strain. The reduction in the levels of other sRNAs following PNPase inactivation in exponential-phase was recently observed and may potentially reflect an increase in the activity of other RNase(s) (De Lay & Gottesman, 2011), but the genetic pathways involved in this regulation have not yet been elucidated. Here we show that in cells without Hfq the inactivation of PNPase (Hfq$^-$ PNPase$^-$ cells) results in increasing levels of all the sRNAs analysed, but that this regulation is not universal in the presence of Hfq (Hfq$^+$ PNPase$^-$ cells). These results suggest that the binding of Hfq may impair the PNPase-dependent regulation of at least some small RNAs, but possibly many in stationary phase.

We also analysed the effect of RNase E in the control of these regulatory RNAs, in the absence of Hfq (Figure 1, right panel). Only RyhB* and both GlmY/GlmY* levels were increased in the Hfq$^-$ RNase E$^-$ mutant while MicA* and SgrS* levels did not change. This greatly contrasted with the strong stabilisation of all the small RNAs obtained in the Hfq$^-$ PNPase$^-$ mutant. Hence, our results

indicated that when these small RNAs are not associated with Hfq, they are clearly more vulnerable to degradation by PNPase than to cleavages by RNase E.

PNPase is a major factor in the rapid decay of the Hfq-free MicA*

Taking into account these results and our previous data on MicA (Andrade & Arraiano, 2008) we decided to use this sRNA as the main model for further investigation. MicA (previously SraD), is an antisense RNA that downregulates the expression level of outer membrane proteins OmpA (Rasmussen et al., 2005; Udekwu et al., 2005) and LamB (Bossi & Figueroa-Bossi, 2007) as well as the members of the PhoPQ regulon (Coornaert et al., 2010).

To evaluate whether the higher MicA* levels in the absence of PNPase were consequence of increased stability, we next analysed the decay rates of MicA* in the Hfq^{-} $PNPase^{-}$ cells (Figure 2). We also tested the potential role of RNase II and RNase R in the degradation of small RNAs in cells without Hfq.

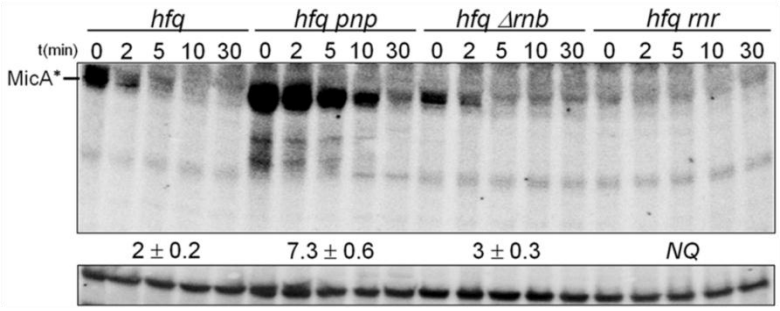


Figure 2. PNPase is the major exoribonuclease involved in the degradation of MicA*.
Samples from stationary-phase cultures of *hfq* and its derivative exoribonuclease mutants (*hfq pnp*, *hfq Δrnb* and *hfq rnr*) grown at 37^{o}C were withdrawn after inhibition of transcription (timepoints are shown in minutes) and total RNA was analysed by Northern blot. A specific riboprobe for MicA was used. A nonspecific band that cross-hybridised with the antisense MicA probe was used as loading control. This band migrates above MicA and disappears with a more stringent washing step of the membrane without affecting MicA signal (Andrade & Arraiano, 2008). Hybridisation with a 5S RNA riboprobe

gave identical results. Only the MicA* RNA species is detected in the absence of Hfq. Half-lives were determined after PhosphorImager densitometry quantification showing that PNPase is the major exoribonuclease involved in the degradation of the Hfq-unprotected MicA*. *NQ – not quantifiable.*

Stability measurements indicated that PNPase was found to be the only exoribonuclease significantly involved in the exo-degradation of MicA RNAs in stationary-phase cells lacking Hfq (Figure 2). Neither RNase II nor RNase R was shown to significantly affect this decay. The *hfq pnp* double mutant showed a nearly 4-fold stabilisation of MicA* when compared to *hfq* single mutant. Accordingly, the increasing levels of MicA* in Hfq$^-$ PNPase$^-$ cells are a consequence of its longer stability due to the inactivation of PNPase. These results indicate that PNPase has a major role in turning over MicA species that are not associated with Hfq.

Poly(A) polymerase I promotes the degradation of MicA*

Polyadenylation can promote RNA degradation by facilitating the exonucleolytic attack of an RNA substrate (Régnier & Hajnsdorf, 2009). Therefore, we decided to analyse the impact of polyadenylation in the degradation of MicA and compare it to PNPase.

In stationary-phase cells expressing Hfq, the lack of poly(A) polymerase I (PAP I/*pcn*B) resulted in a modest increase in MicA half-life (only a 1.5-fold upregulation) from 8.2 to 12.5 minutes, as detected by Northern blotting (Figure 3A). On the other hand and in the same conditions, inactivation of PNPase resulted in a stronger stabilisation of MicA (from 8.2 minutes in the wild-type to 27.5 minutes in the *pnp* mutant). This indicates that in the presence of Hfq,

PNPase activity against MicA surpasses the effect of PAP I polyadenylation-dependent pathways.

MicA is very unstable in the absence of Hfq; its half-life decreases from 8.2 minutes in the wild-type to 2 minutes in the *hfq* mutant (Figure 3). To check if PAP I could be involved in the rapid degradation of the MicA* in the absence of Hfq, we constructed the double *hfq pcnB* mutant. Two different *pcnB* mutations were used in this study: either a deletion Δ*pcnB* (O'Hara et al., 1995) or the *pcnB80* allele (Hajnsdorf et al., 1995) was transferred to the *hfq* mutant strain; measurement of MicA half-lives gave identical results for both strains. The double mutant *hfq pcn*B was found to have a significant 2.5-fold more stable MicA* when compared to the single *hfq* mutant (increasing half-life from 2 to 5 minutes). In the absence of Hfq, the MicA* RNA is more susceptible to poly(A)-mediated decay in stationary-phase cells. This was surprising as an Hfq^{-} mutant was reported to have low levels of polyadenylation (Hajnsdorf & Régnier, 2000; Le Derout et al., 2003; Mohanty et al., 2004). Notwithstanding the higher impact that poly(A) polymerase I displays in the degradation of the MicA* in the absence of Hfq, inactivation of PNPase still renders a more stable sRNA (Figure 3A, lower panel). Altogether, these results show that PNPase has a stronger effect than poly(A) polymerase I in the degradation of MicA RNAs, irrespective of the presence of Hfq.

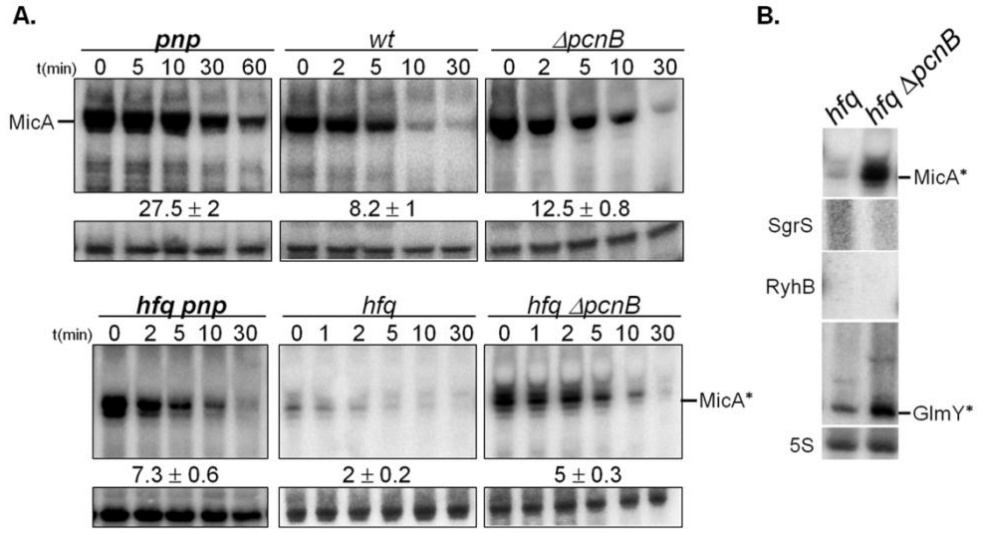


Figure 3. Lack of poly(A) polymerase I results in increasing levels of MicA*.
(A) Impact of poly(A) polymerase I (*pcn*B) in the degradation of the small MicA RNA in Hfq^+ or Hfq^- cells. Stationary-phase cultures of wild-type and its derivatives *pnp*, Δ*pcn*B, *hfq pnp*, *hfq* and *hfq* Δ*pcn*B strains were treated with rifampicin and total RNA was analysed by Northern blot. MicA was detected by use of a specific riboprobe. Only the shorter MicA* RNA is visible in the Hfq^- cells. An nonspecific band cross-reacting with MicA probe was used as loading control. (B) The steady-state levels of several small RNAs from stationary-phase cultures of *hfq* and *hfq* Δ*pcn*B mutants were evaluated by Northern blot.

We also determined the relative levels of other small RNAs in the *hfq* Δ*pcnB* double mutant compared to the *hfq* single mutant (Figure 3B). The levels of GlmY* were also increased by the lack of PAP I in the absence of Hfq. This was also confirmed to be consequence of the higher stabilisation of GlmY* in the *hfq pcn*B mutant (data not shown). GlmY* is known to be highly polyadenylated in cells harbouring Hfq (Reichenbach et al., 2008; Urban & Vogel, 2008). We have now shown that the lack of poly(A) polymerase I is an important factor affecting the sRNA decay in the absence of Hfq in stationary-phase cells. However, inactivation of PNPase in cells devoid of Hfq resulted in higher levels of all the small RNAs analysed, even the ones that were not affected by the lack of PAP I (namely RyhB* and SgrS*) (Figure 1 and Figure 3B). Hence, PNPase activity against

a small RNA that is not bound to Hfq does not necessarily require an active polyadenylation-dependent pathway.

In the absence of Hfq, MicA* is a substrate for PNPase but not for RNase E or RNase III

RNase E was thought to be responsible for the rapid degradation of small RNAs not protected by Hfq (Massé et al., 2003; Moll et al., 2003a). Surprisingly, we found that MicA* levels did not change substantially between the *hfq* single mutant and the *hfq rne*-1 double mutant (Figure 1, right panel). To analyse this observation further, we assayed MicA decay rates in both strains. As RNase E (*rne*) is essential in *E. coli*, we used a thermolabile allele (*rne*-1) and performed this set of experiments at the non-permissive temperature (Figure 4A).

From previous work, we have identified that RNase E is involved in the degradation of MicA in cells producing Hfq (Andrade and Arraiano, 2008). Surprisingly, our results revealed that MicA* is not stabilised significantly when RNase E is inactivated in the absence of Hfq (Figure 4A). This indicates that RNase E is not able to efficiently degrade MicA* unless Hfq is present in the cell. A similar RNase E/Hfq dependency was observed in OxyS turnover (Basineni et al., 2009).

To better assess the relative impact of RNase E and PNPase, we treated the culture of the *hfq pnp* mutant in the same conditions used to inactivate the thermosensitive RNase E (Figure 4B). No significant changes were detected when Hfq and RNase E were inactive, but MicA steady-state levels are substantially higher upon inactivation of both Hfq and PNPase (an 8-fold increase to the *hfq* mutant). This result clearly showed that in the absence of Hfq, PNPase is more

important than RNase E in the degradation of this sRNA. This result is also substantiated by data from Figure 1.

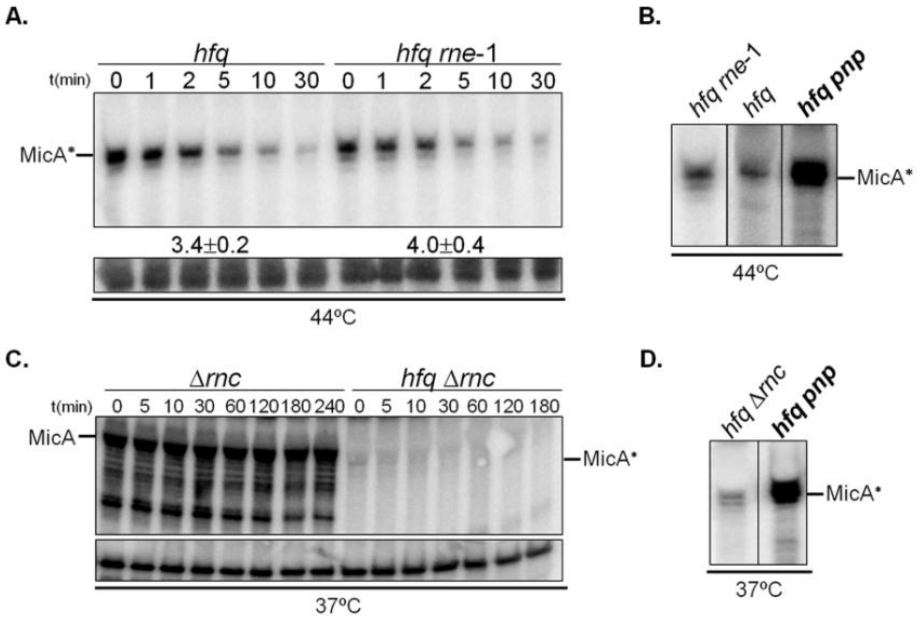


Figure 4. PNPase but not RNase E nor RNase III degrades the Hfq-free MicA* RNA.
(A) Northern blot detection of MicA RNA in Hfq$^-$ cells harbouring or not the *rne*-1 allele. Stationary-phase cultures were treated at 44ºC for inactivation of the thermosensitive RNase E (as mentioned before). MicA RNA stability was analysed by Northern blot with a specific riboprobe. (B) Northern blot analysis of MicA in Hfq$^-$ cells deficient in RNase E or PNPase. The double *hfq rne-1* mutant was grown at 30ºC until stationary-phase and then incubated at 44ºC to inactivate RNase E. For comparison, the *hfq* and *hfq pnp* were treated in the same conditions. (C) Northern blot detection of MicA in stationary-phase cultures of Hfq$^-$ cells harbouring or not RNase III (*rnc*), respectively. A loading control corresponding to a nonspecific band that cross-reacted with MicA probe is shown in the panel below. (D) Comparison of MicA* RNA steady-levels in Hfq$^-$ stationary-phase cells deficient in RNase III or PNPase grown at 37ºC.

The other main endoribonuclease involved in RNA degradation is RNase III (Arraiano et al., 2010). *In vitro* studies showed that *Salmonella* RNase III can cleave MicA when bound to its target *omp*A mRNA (Viegas et al., 2011). To further analyse the role of RNase III in cells without Hfq, we constructed and analysed double mutants lacking both Hfq and RNase III. RNA extracted from stationary-

phase cultures from the deletion mutant of *E. coli* RNase III (Δ*rnc*) and a double mutant lacking Hfq and RNase III (*hfq* Δ*rnc*) was analysed by Northern blotting (Figure 4C). Inactivation of RNase III in the presence of Hfq appeared to block the degradation of MicA (this RNA apparently did not decay even 240 min after transcription blocking). This clear showed that *E. coli* RNase III is important in the control of MicA stability. However, MicA RNAs were barely detected in the double mutant *hfq* Δ*rnc* reflecting the results obtained with the single *hfq* mutant. To confirm this result we also tested another allele of RNase III, the *rnc*105; the double mutant *hfq rnc*105 displayed identical results (not shown). The strong decrease in MicA levels typically found in the absence of Hfq obviously reduce the number of duplexes formed between this sRNA and its target mRNAs probably impairing RNase III activity against MicA. The MicA* levels found in the *hfq pnp* strain were higher (about a 6-fold increase to the *hfq* single mutant) than the MicA* levels found in the *hfq* Δ*rnc* mutant (Figure 4D). These results clearly indicated that PNPase was more important than RNase III in the elimination of MicA* from the cell. Overall, when MicA is not associated with Hfq, the 3'-5' exoribonucleolytic degradation pathway mediated by PNPase is found to be more important in this degradation than any of the main endoribonucleases involved in RNA turnover.

Hfq is required for the optimal expression of the full-length MicA

The slightly smaller MicA* is the predominant form in the *hfq* mutant in stationary-phase cultures, but it is barely detected in the wild-type (Figure 1). To examine if this RNA pattern was dependent on a growth phase specific regulation, we analysed both the wild-type and the *hfq* strain along the growth curve (Figure 5A and Figure S2). In exponential-phase, the *hfq* mutant exhibited not only the full-length MicA as well as additional shorter bands of similar intensity, apparently

differing of few nucleotides in size. This pattern was growth dependent. In stationary-phase there was a decrease in the amount of full-length MicA and what appeared to be a concomitant accumulation of the smaller MicA*. This contrasted sharply with the wild-type strain where the full-length MicA was the most prominent band irrespective of the growth phase analysed. Moreover, supplying Hfq *in trans* from a plasmid complemented *hfq* deficiency on MicA expression and resulted in the strong accumulation of the full-length RNA and in the elimination of the shorter sized RNAs (Figure 5A). These results indicated that Hfq determines MicA full-length expression along growth, particularly in the stationary-phase.

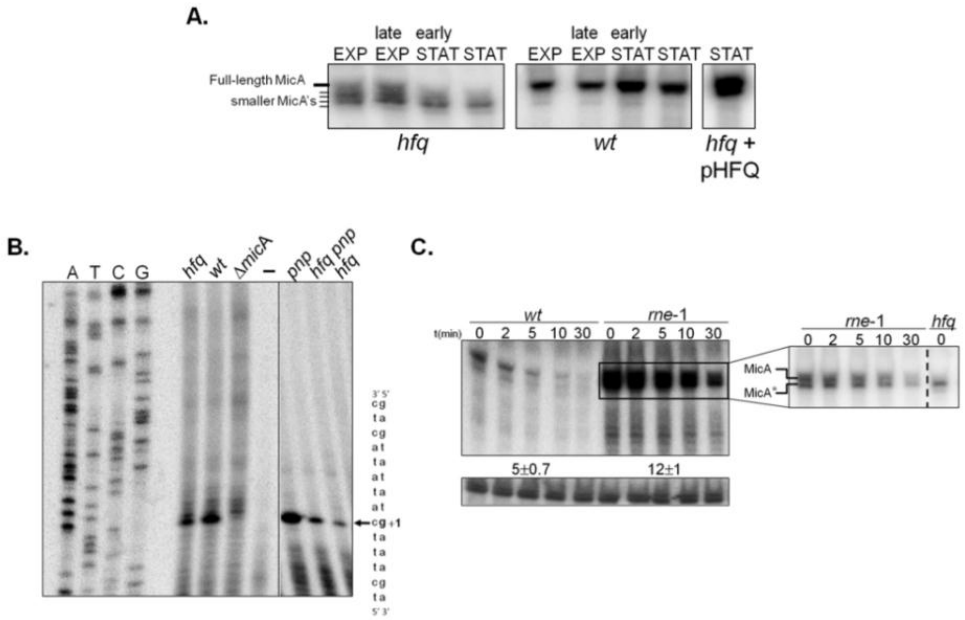


Figure 5. Hfq is required for the maintenance of the full-length MicA RNA.
(A) Steady-state levels of MicA RNA along the growth curve. Culture samples of wild-type or *hfq* mutant bacteria were collected at exponential (EXP), late exponential, early stationary and stationary-phase (STAT) (corresponding to OD_{600} values of ~0.3, ~1.7, ~2.5 and ~5.5 for the wild-type and ~0.3, ~0.8, ~1.6 and ~2.3 for the *hfq* mutant, respectively). The growth curves for the wild-type and the *hfq* mutant strain are given in Figure S2. A specific antisense MicA riboprobe was used to detect MicA. Stationary-phase cultures of the *hfq* mutant transformed with the overexpressing pHFQ plasmid shows complementation and do not exhibit the heterogeneous population of MicA's typically found in the *hfq* single mutant. (B) Determination of the 5'-end of MicA. Total RNA from stationary-phase cells of wild-type, *hfq*, *pnp* and *hfq pnp* strains was analysed by primer

extension with the [32P]-labelled primer MicA-PE. The same primer extension product (indicated by an arrow) is detected on all strains and absent from the deletion *micA* strain (Δ*micA*) and the negative control reaction (–) done without RNA. Part of the DNA sequence is indicated on the right. **The** transcription start site of MicA is indicated (+1) and is identical to the site described by Udekwu *et al*., 2005. The intensity of primer extension product obtained is higher in the wild-type rather than the *hfq* mutant, in agreement with the higher amount of MicA detected in the wild-type strain (see Figure 5A). (C) Northern blot detection of MicA in stationary-phase cultures of Hfq^+ cells upon inactivation of RNase E. Cultures of wild-type and an RNase E mutant strain were grown at 30ºC until reached stationary-phase and then shifted to the non-permissive temperature of 44ºC. After 5 min, transcription was blocked with addition of rifampicin and samples were withdrawn at times indicated. A specific riboprobe was used to detect MicA RNA. A nonspecific band that cross-hybridised with the antisense MicA probe was used as loading control. The insert corresponds to a shorter exposure of the membrane in which is visible that both the full-length MicA and the shorter MicA* RNA are detected and stabilised upon inactivation of RNase E in Hfq^+ cells. The *hfq* mutant was here used as a control to clearly identify MicA* RNA.

The difference in size between the full-length MicA and MicA* is small, apparently in the range of 3-4 nucleotides as visible on Northern blotting. Such small variation must lie at one of the RNA extremities. Primer extension analysis was performed to evaluate which extremity was shortened (Figure 5B). Stationary-phase cultures of both the wild-type (which expresses full-length MicA) and the *hfq* mutant strain (where MicA* is detected) showed accumulation of a band which matches the start of MicA sequence. Furthermore, this same band was identified when testing either the *pnp* mutant or the *hfq pnp* double mutant, showing that the MicA RNAs that accumulate upon PNPase inactivation retain the same 5' end than the wild-type MicA. Additional experimental approach using nuclease S1 mapping also determined the same 5'-end for both MicA species (Figure S3). Altogether, these findings supported that full-length MicA and MicA* have the same 5'-end and that the difference in size is located at the 3'-end. This suggests that the smaller RNA species probably arises from 3'-end processing of the full-length MicA.

The MicA* RNA is expressed at very low levels in the wild-type. This suggests that Hfq acts in order to prevent MicA* production or to ensure its rapid elimination. We decided to analyse the kinetics of decay and found that RNase E affected MicA* levels (Figure 5C). Inactivation of RNase E (in cells harbouring Hfq) resulted in the strong elevation of MicA levels in stationary-phase. However, a shorter exposure of this gel revealed the detection not only of the full-length MicA as well of the smaller MicA* (insert in Figure 5C). Both RNAs showed a two-fold stabilisation in the absence of RNase E. This indicated that even in the presence of Hfq, the shorter MicA* RNA fragment is produced in the cell.

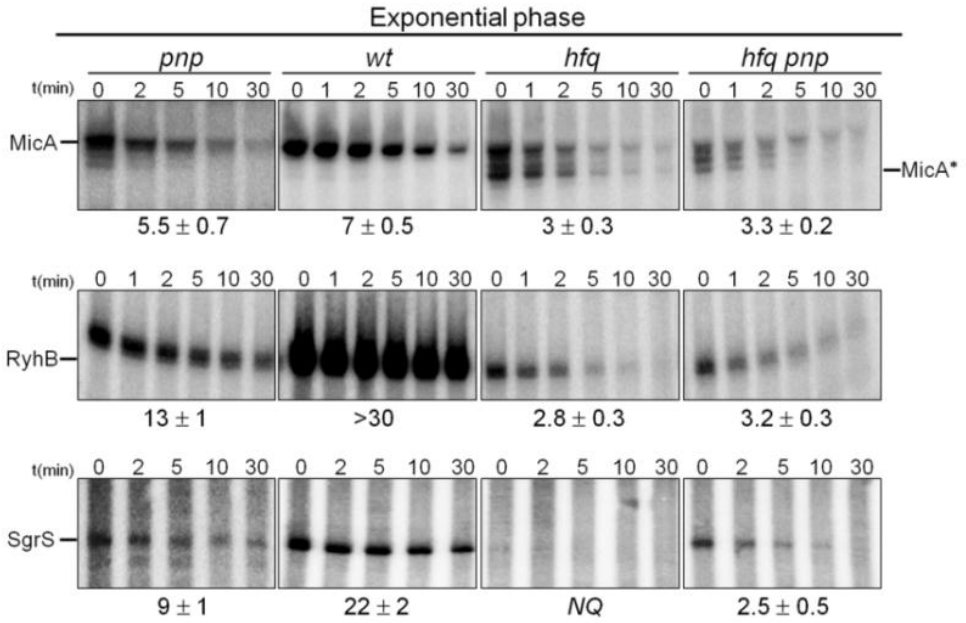
Growth phase regulation of small RNAs by PNPase

In the absence of Hfq, small RNAs are typically unstable and PNPase was found to be a major enzyme involved in the extensive degradation of MicA in stationary-phase cells. To check if this could be generalised to other small RNAs we extended this analysis to RyhB and SgrS. Since the RNA pattern of MicA changes along growth in Hfq^{-} cells (Figure 5A), is reasonable that different RNA degradation pathways might be involved in different stages of growth. To further analyse this, we decided to compare small RNA stability between exponential and stationary-phase cultures.

In the absence of Hfq, all the small RNAs analysed were highly unstable, regardless of the growth phase that was analysed (Figure 6). As consequence of the extensive degradation happening in the absence of Hfq, the MicA, RyhB and SgrS levels were strongly reduced in the *hfq* mutant when compared to wild-type, both in exponential and stationary-phase cultures. In contrast, all these small RNAs were markedly stabilised in stationary-phase cultures of the *hfq pnp* double mutant compared to *hfq* strain. Interestingly, this regulation is not as common in

exponentially growing cells. In fact, only SgrS was found to be stabilised in exponential-phase cultures of the *hfq pnp* mutant strain compared to *hfq* strain (although this is significantly lower than the stabilisation observed in stationary-phase cells). These results confirm PNPase as a major enzyme involved in the degradation of Hfq-free small RNAs in the cell.

Hfq-deficiency resulted in the detection of shorter small RNAs that are stabilised upon further inactivation of PNPase (Figure 1 and Figure 6). In exponential growing cells without Hfq, only MicA was found to exhibit a heterogeneous sized population (Figure 5A and Figure 6). From these fragments, MicA* is shown to be the most resistant and is even the predominant RNA species found in *hfq* mutants in stationary-phase. The smaller RyhB* and SgrS* RNAs were only detected in stationary-phase cells. Together, these results suggested a protection of the full-length sRNA by Hfq which seems particularly important for sRNA expression in the stationary-phase of growth.



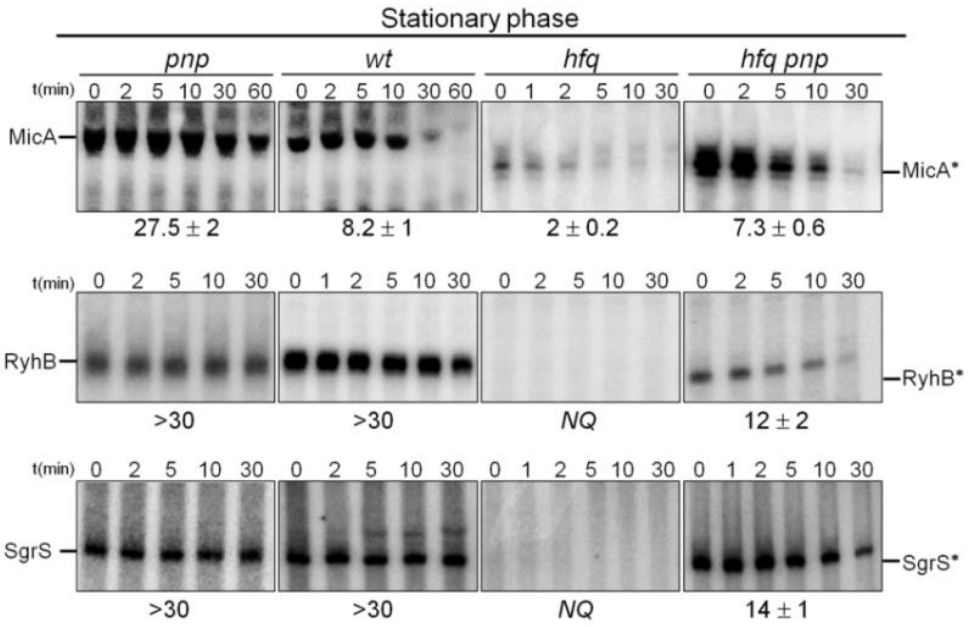


Figure 6. Growth phase regulation of Hfq-free small RNAs by PNPase.
Northern blot determination of MicA, RyhB and SgrS RNA stabilities between the wild-type and its isogenic *pnp*, *hfq* and *hfq pnp* mutants either in exponential-phase or stationary-phase cultures. Total RNA was extracted from culture samples withdrawn after inhibition of transcription with rifampicin (timepoints are shown in minutes). MicA, RyhB and SgrS RNAs were detected by the use of specific radiolabelled probes and quantified by PhosphorImager analysis. The full-length small RNAs or their respective shorter forms (where detected) are indicated on the gels. *NQ – not quantifiable*.

To analyse if PNPase is affecting the stability of small RNAs independently of Hfq, we further analysed the decay rates of MicA, RyhB and SgrS in the *pnp* single mutant. Inactivation of PNPase in exponential-phase cells producing Hfq resulted in reduced levels and decreased stability of the small RNAs. Similar results were reported with other small RNAs, suggesting that PNPase may somehow protect some sRNA in exponential-phase (De Lay & Gottesman, 2011). In fact, this is also observed in stationary-phase cultures, as inactivation of PNPase is also found to reduce RyhB and SgrS levels under this condition. Only MicA was shown to be a substrate for PNPase either in the absence or presence of Hfq. On the other hand, RyhB and SgrS were found to be preferably degraded by PNPase

in the absence of Hfq and were greatly stabilised in the *hfq pnp* double mutant, particularly in the stationary-phase of growth. Altogether, these results suggest that PNPase degrades small RNAs more efficiently in the absence of Hfq although this turnover pathway is clearly active in cells with Hfq. The fact that this occurs in cells expressing Hfq, may reflect the action of PNPase against small RNAs that are transiently in their Hfq-free state, as result of the dynamics of interaction with Hfq. PNPase-mediated degradation of small RNAs is suggested to be predominant in stationary-phase cells as this regulation apparently is not so common in exponential-phase cells. Therefore, these results demonstrated that the degradation pathways of a same small RNA can be different between exponential and stationary-phases and highlighted the role of PNPase in the growth phase regulation of small RNAs.

Discussion

This work demonstrated that the pool of small RNAs that are not associated with Hfq is preferably degraded by PNPase. Overall, our data highlights the impact of 3'-5' exonucleolytic RNA decay pathways re-evaluates the degradation mechanisms involved in the rapid decay of the Hfq-free small RNAs. The reduced levels of small RNAs typically found in the Hfq$^-$ strain were strongly increased upon inactivation of PNPase in stationary-phase cells (Figure 1). This seems to be a general feature since PNPase inactivation resulted in increasing levels of at least the MicA, SgrS, RyhB and GlmY sRNAs. We only detected the accumulation of slightly shorter sRNAs rather than the full-length species and this was shown to be consequence of the higher stability of these fragments (Figure 2).

The lack of poly(A) polymerase I was also found to impact the levels of small RNAs in the absence of Hfq although to a lesser extent than PNPase (Figure 3). These results were unexpected as no significant differences in mRNA stability were detected between *hfq* and *hfq* Δ*pcn*B mutants (Mohanty et al., 2004). In contrast, our results clearly showed that in the absence of Hfq the small RNA turnover can be affected by the lack of poly(A) polymerase I. The sRNAs found to be highly affected by polyadenylation (MicA* and GlmY*) were also found to be excellent substrates for PNPase. Nevertheless, PNPase activity against Hfq-unprotected small RNAs is not necessarily dependent on poly(A) polymerase I activity. RyhB* and SgrS* RNAs are not affected by polyadenylation although their levels were highly increased upon PNPase inactivation, as observed in the double *hfq pnp* mutant (Figure 1 and Figure 3B). Poly(A)-dependent pathways may thus not explain all the extraordinary impact of PNPase on sRNA turnover in the Hfq$^-$ cells**.**

Pioneer work on PNPase revealed its ability to synthesise RNA (Grunberg-Manago et al., 1955). Interestingly, it has been proposed that in the absence of Hfq, there is an increase in the biosynthetic activity of PNPase with heteropolynucleotide tails promoting RNA decay (Mohanty et al., 2004; Slomovic et al., 2008). Addition of these polynucleotide tails can potentially be responsible for PNPase notable impact on the degradation of sRNA in the absence of Hfq. RNase II (Marujo et al., 2000) and RNase R (Andrade et al., 2009a) are also major poly(A)-dependent exoribonucleases but they were not found to be involved in the degradation of MicA (Figure 2 and (Andrade & Arraiano, 2008). Similar results were obtained regarding the degradation of RyhB (data not shown). Surprisingly, despite RNase R intrinsic ability to easily degrade structured RNAs on its own and its affinity to poly(A) tails, RNase R was not shown to be part of these decay pathways. The absence of RNase R resulted in the reduction of MicA* levels in cells without Hfq (Figure 2). This might be consequence from an indirect effect in which the activity of a MicA repressor is increased when RNase R is not functional. Although the protection of RNA by a ribonuclease seems paradoxical, a similar effect has been described either for RNase II or PNPase (Marujo et al., 2000; De Lay & Gottesman, 2011). A major advantage feature of PNPase in the degradation of small RNAs might be its ability to form complexes with other proteins which can be particularly helpful in the elimination of such structured RNAs. However, we have already shown that PNPase activity on MicA can be independent of the degradosome assembly (Andrade & Arraiano, 2008).

RNase E has a role in sRNA degradation (Massé et al., 2003; Morita et al., 2005; Suzuki et al., 2006; Viegas et al., 2007; Andrade & Arraiano, 2008). However, our results demonstrated that its impact on Hfq^{-} cells may not be as general as previously believed. RNase E depletion did not affect the levels of SgrS and MicA RNA. While in the presence of Hfq both the full-length MicA and the MicA* RNAs are substrates for RNase E (Figure 5C), this regulation is lost when

Hfq is absent (Figure 4A). This indicates that RNase E requires Hfq in order to degrade MicA. A similar RNase E dependency of Hfq to act on sRNA turnover was also reported in the growth phase degradation of OxyS (Basineni et al., 2009). It is suggested that RNase E/Hfq cooperation (as observed in the mRNA decay mediated by sRNA) (Morita et al., 2005) can also be critical for the degradation of some small noncoding RNAs, like MicA. Nevertheless, we recognise that RNase E can also impact the levels of some sRNAs independently of Hfq. As observed, both RyhB* and GlmY are RNase E substrates even in cells lacking Hfq (Figure 1).

A possible RNase III/Hfq pathway was also analysed. RNase III inactivation results in extremely long-lived MicA; however, this is strictly dependent on the presence of Hfq as this stabilisation is completely lost in the *hfq* Δ*rnc* mutant (Figure 4C). The low levels of MicA found in *hfq* mutants strongly decrease the probability of base pairing with target mRNAs. The downregulation in sRNA-target mRNA duplexes probably explains the impairment in RNase III activity on MicA, in agreement with *in vitro* studies (Viegas et al., 2011). Data suggest that the degradation of small RNAs that are not associated with Hfq mainly occurs in a target-independent pathway, in which RNase III has a reduced impact. The free pool of small RNAs is then preferably degraded by PNPase.

Hfq was thought to mainly protect sRNA from RNase E cleavages as both proteins showed *in vitro* affinity for the same A/U-rich sequences in RNA (Moll et al., 2003a). However, it has been recently demonstrated that Hfq actually prefers to bind U-rich sequences at the 3'-end of small RNAs over internal A/U-rich sequences (Otaka, 2011; Sauer & Weichenrieder, 2011). Small RNAs, like MicA, usually display a short U-rich 3'-end sequence immediately downstream a stem-loop as a consequence of Rho-independent transcription termination (Rasmussen et al., 2005; Udekwu et al., 2005). The physiological meaning of the high affinity of Hfq to this U-rich sequence can be the protection of the 3'-end of the RNA against

degradation. Interestingly, our results showed that the 3' ends of the small RNAs are shortened in the absence of interaction with Hfq (Figure 1 and Figure 5B). Even though PNPase is observed to be the main exoribonuclease involved in the degradation of these shorter small RNAs, it does not seem to be the main responsible for the initial 3' end attack as this is not prevented in an *pnp* background. RNase II and RNase R inactivation did also not suppress the shortening of MicA. Data suggested that other (exo)nucleases would be responsible for the 3' end trimming of the small RNAs whey they are Hfq-free. The transcriptional terminator stem loop of the small RNAs may function as a physical barrier against exoribonucleases. PNPase may be favoured in this action and progress to degradation of the sRNA body while other RNases may be inhibited and therefore could only degrade few nucleotides before releasing the sRNA. In the presence of Hfq, the shorter sRNAs are barely detected probably because Hfq protects the 3' ends of the small RNAs.

Our results also indicate that small RNAs are subject to different degradation pathways depending on growth (Figure 6). In the stationary-phase, PNPase is shown to be the main enzyme in the degradation of small RNAs (this work and (Andrade & Arraiano, 2008). On the other hand, it has been proposed that in exponential-phase, PNPase can actually protect small RNAs from rapid degradation by other ribonucleases, namely from RNase E activity (De Lay & Gottesman, 2011). The growth phase regulation of sRNA turnover pathways may help explaining why RNase E was shown to affect sRNA decay in previous studies in which the exponential-phase of growth was analysed (Massé et al., 2003), while it is not found to be the predominant degradative enzyme in stationary-phase (this work). PNPase responds to environmental stimuli and has been suggested to be responsible for the addition of heteropolymeric tails to the 3' end of RNAs in the stationary-phase of growth (Cao & Sarkar, 1997; Mohanty & Kushner, 2000). PNPase could then use those tails to initiate RNA degradation. Accordingly, the

growth phase regulation of PNPase activities may thus help explaining the growth phase regulation of small RNAs driven by PNPase.

Variations in the levels of Hfq can most probably influence the degradation pathways of the small RNA. Interestingly, Hfq was reported to vary along the growth and decreased levels of this protein were found in the entry to stationary-phase (Ali Azam et al., 1999). Not only changes in the Hfq expression level but also variations in the pool of free Hfq can result in low amounts of this protein and consequently affect the sRNA-based regulatory pathways. Hfq binds very tightly the RNA molecules and this can result in the sequestration of Hfq. A model in which an increasing concentration of a competitor RNA promotes the dissociation of the Hfq-RNA complexes has recently been proposed to explain how it is possible to cycle the Hfq pool within the cell (Fender et al., 2010). In agreement, it was shown that induction of a sRNA without the concomitant overexpression of its target mRNA (or *vice versa*) can sequester Hfq and abolish the function of unrelated sRNAs (Hussein & Lim, 2011). Hence, Hfq depletion is likely to occur if transcription of sRNA and its target mRNAs is not coordinated. The rapid degradation of sRNA in the absence of interaction with Hfq may thus recycle any small RNAs that are produced in excess over Hfq. This reinforces the importance of studying the degradation of small RNAs when they are not associated with Hfq. Most of our work was performed in stationary-phase cells deleted for Hfq. However, we have shown that PNPase-mediated degradation of small RNAs is also an active regulatory pathway in cells expressing Hfq. This fact may reflect the action of PNPase against small RNAs that do not have their 3' ends protected by Hfq. Our results are in agreement with *in vitro* data showing that Hfq can protect an mRNA from the exonucleolytic activity of PNPase (Folichon et al., 2003).

A similar phylogenetic distribution may reflect functionally linked proteins (Pellegrini et al., 1999). A large number of bacteria encode both Hfq and PNPase

in their genomes (Zuo & Deutscher, 2001; Sun et al., 2002) while the presence of *E. coli* RNase E homologues is far more restricted (Condon & Putzer, 2002; Danchin, 2009). Interestingly, eukaryotes lack an RNase E but possess functional homologues of both PNPase and Hfq. The eukaryotic exosome adopts an PNPase-like conformation and is implicated in the processing and degradation of several RNAs, namely the small nucleolar RNAs (snoRNAs) and the small nuclear RNAs (snRNAs) (Houseley et al., 2006). The exosome activity is suggested to be modulated by the Lsm1-7 complex (whose subunits are homologous to bacterial Hfq) although this interplay is still unclear (Wilusz & Wilusz, 2008). The fact that Hfq and PNPase are more widespread than RNase E supports the interesting hypothesis that Hfq-protection of sRNA against degradation by PNPase is far more common than was previously envisioned.

Material and Methods

Growth conditions, strains and plasmids

Bacteria were grown at 37ºC unless stated otherwise, with shaking at 180 rpm in Luria-Bertani (LB) medium supplemented with thymine (50 μg ml^{-1}). SOC medium was used to recover cells after heat-shock in plasmid transformation steps. When required, antibiotics were present at the following concentrations: chloramphenicol, 50 μg ml^{-1}, kanamycin, 50 μg ml^{-1}; tetracycline, 20 μg ml^{-1}; ampicillin, 100 μg ml^{-1}. The *E. coli* strains used in this work are listed in Table 1. **Strain** MC4100 ***hfq*::cat** (kindly provided by S. Altuvia) was used as donor to move the mutant *hfq* allele into MG1693 **(wild-type)** and its derivative isogenic strains**.** Introduction of mutant alleles to different genetic backgrounds was done by P1 transduction and positive colonies were checked by PCR. A DNA sequence of Hfq was PCR-amplified with primers *hfq-Eco*RI (5'-GTG ACG AAG aAT TcC AGG TTG TTG-3') and *hfq-Hin*dIII (5'-CGG TCA AAC AAG CtT ATA ACC C-3') and following enzyme restriction it was cloned into pBAD24 yielding the overexpression pHFQ plasmid. Hfq expression is obtained even without addition of the arabinose inducer as the cloned DNA retains *hfq* own promoters. For plasmid pMicA, primers MicA-*PstI* (5'- TTT TCG CCA CCC GAA CTG CAG GC -3') and MicA-*Hin*dIII (5'- GGC TGG AAA AAC AaG CtT GAC AGA AAA GAA AAA GG -3') were used to amplify the *micA* gene. Following enzyme restriction the insert was ligated into pWSK29 in sites *Pst*I and *Hin*dIII. DNA polymerases and restriction enzymes were obtained from Fermentas and T4 DNA Ligase from Roche. All primers were obtained from StabVida (Portugal).

Table 1 - Bacterial strains used in this study

Strain	Relevant genotype	Reference
MC4100*hfq*	*hfq*	Soshy Altuvia
MG1693	*thyA715*	(Arraiano et al., 1988)
HM104	*thyA715 rnr*	(Andrade et al., 2006)
SK5665	*thyA715 rne-1*	(Arraiano et al., 1988)
SK5671	*thyA715 rne-1 pnp7*	(Arraiano et al., 1988)
SK5691	*thyA715 pnp7*	(Arraiano et al., 1988)
SK7988	*thyA715* Δ*pcnB*	(O'Hara et al., 1995)
SK7622	*thyA715* Δ*rnc38*	(Babitzke et al., 1993)
CMA201	*thyA715* Δ*rnb*	(Andrade et al., 2006)
CMA413	*thyA715* Δ*micA*	(Andrade & Arraiano, 2008)
CMA428	MG1693 *hfq*	This study
CMA429	MG1693 *hfq rnr*	This study
CMA430	MG1693 *hfq* Δ*rnb*	This study
CMA431	MG1693 *hfq pnp7*	This study
CMA436	MG1693 *rne*-1 *hfq*	This study
CMA441	MG1693 *hfq* Δ*rnc38*	This study
CMA448	MG1693 *hfq rnc105*	This study
CMA449	MG1693 *hfq* Δ*pcnB*	This study
CMA450	MG1693 *hfq pcnB80*	This study
CMA513	MG1693 *hfq* + pHFQ	This study

RNA extraction and Northern blot analysis

Overnight cultures from isolated colonies were diluted in fresh medium to an initial OD_{600}~0.03 and grown to exponential (OD_{600}~0.3) or stationary-phase (OD_{600}~5.5 to wild-type or OD_{600}~2.3 to *hfq* mutants). The growth curves for the wild-type strain and the *hfq* mutant are provided in Figure S2. For decay experiments, blocking of transcription was obtained by adding rifampicin to a final concentration of 500 μg ml^{-1}. Culture samples were withdrawn at defined timepoints and mixed with equal volume of RNA stop buffer (10 mM Tris pH 7.2, 5 mM $MgCl_2$, 25 mM NaN_3 and 500 μg ml^{-1} chloramphenicol). RNA was isolated following cell lysis and phenol:chloroform extraction. After precipitation step in ethanol and 300 mM sodium acetate, RNA was resuspended in MilliQ-water. The integrity of RNA samples was evaluated by agarose gel electrophoresis. When necessary, DNase RQ (Promega) treatment following new phenol:chloroform step was used to remove contaminant DNA. 10-40 μg of total RNA was used to analyse small RNA expression on 6%-12% polyacrylamide/7 M urea gels in TBE 1x. RNA was transferred onto Hybond-N+ membrane (Amersham Biosciences) using TAE 1x as transfer buffer. RNAs were UV crosslinked to the membrane with a UVC 500 apparatus (Amersham Biosciences). DNA templates carrying a T7 promoter sequence for *in vitro* transcription were generated by PCR using genomic DNA of MG1693 and primers listed in Table 2. GlmY was detected by 5'-end labelling of an antisense primer (Table 2). Radiolabelled probes were purified on G25 Microspin columns (GE Healthcare). Hybridisations were carried out overnight at 42-68ºC with the PerfectHyb Plus Hybridization Buffer (Sigma). RNA Decade markers (Ambion) or the 10 bp Step Ladder (Promega) were used for detection of small RNAs up to 150 nucleotides; for longer transcripts the 100-1000 bp Ladder (Biotools) was used. T7 RNA polymerase and T4 polynucleotide kinase were from Promega. All radiochemicals were purchased from Perkin-Elmer.

Table 2 – Oligonucleotides used in radiolabelling reactions

Probe	Sequence (5'–3')
MicA-T7	TAATACGACTCACTATAG GAA GGC CAC TCG TGA GTG GCC AA
MicA-F	GAA AGA CGC GCA TTT GTT ATC
SgrS-T7	TAATACGACTCACTATAGG CCA GCA GGT ATA AT C TGC
SgrS-F	GAT GAA GCA AGG GGG TGC CC
RyhB-T7	TAATACGACTCACTATAGG AAA AGC CAG CAC CCG GCT GG CTA A
RyhB-F	GCG ATC AGG AAG ACC CTC
5S-RNA-T7	TAATACGACTCACTATAGG ATG CCT GGC AGT TCC CTA CTC TCG C
5S-RNA-F	AAA CAG AAT TTG CCT GGC GGC AGT AG
GlmY	GCA CGT CCC GAA GGG GCT GAC ATA AG

The T7 promoter sequence in the oligos is underlined.

RNA half-life determination

Northern blot signals were visualised on PhosphorImager STORM 860 and bands intensities were quantified using the IMAGEQUANT software (Molecular Dynamics). Half-lives of RNA were determined by linear regression using the logarithm of the percentage of RNA remaining *versus* time, considering the amount of RNA at 0 min as 100%. A minimum of two independent RNA extractions from each strain were tested and half-lives correspond to average of at least three experiments.

Primer extension analysis

The MicA RNA was analysed by primer extension analysis using the MicA-PE primer (5'-CGT GAG TGG CCA AAA TTT CAT CTC TG-3'). 10 μg of each RNA sample was incubated with 1 pmol of 5'-end [γ-32P]ATP labelled primer. Sample denaturation was done for 5 min at 80ºC immediately followed by the annealing step (30min at 65ºC and 30min at 48ºC). cDNA synthesis was obtained using 200 units of SuperScript III Reverse Transcriptase following the manufacturer's instructions (Invitrogen). Incubation proceeded for 60 min at 55ºC and was terminated by heat inactivation of the samples for 15 min at 70ºC. The cDNA products were then ethanol precipitated with the addition of glycogen for 15 min in a -80ºC freezer. The cDNA pellet was dissolved in 2 μl 0.1 M NaOH/1 mM EDTA and 4 μl formamide loading buffer. Prior to loading, samples were denatured for 5 min at 80 ºC and then fractionated on 6% polyacrylamide/7 M urea gels. Plasmid pMicA was used in sequencing reactions with primer MicA-PE following the instructions of the Sequenase Version 2.0 DNA Sequencing Kit (USB). The gel was exposed on a PhosphorImager screen and the signal was detected on a PhosphorImager STORM 860.

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References

Afonyushkin T, Vecerek B, Moll I, Blasi U, Kaberdin VR. 2005. Both RNase E and RNase III control the stability of sodB mRNA upon translational inhibition by the small regulatory RNA RyhB. *Nucleic Acids Res 33*:1678-1689.

Aiba H. 2007. Mechanism of RNA silencing by Hfq-binding small RNAs. *Curr Opin Microbiol 10*:134-139.

Ali Azam T, Iwata A, Nishimura A, Ueda S, Ishihama A. 1999. Growth phase-dependent variation in protein composition of the *Escherichia coli* nucleoid. *Journal of bacteriology 181*:6361-6370.

Andrade JM, Arraiano CM. 2008. PNPase is a key player in the regulation of small RNAs that control the expression of outer membrane proteins. *RNA 14*:543-551.

Andrade JM, Cairrão F, Arraiano CM. 2006. RNase R affects gene expression in stationary phase: regulation of *ompA*. *Mol Microbiol 60*:219-228.

Andrade JM, Hajnsdorf E, Régnier P, Arraiano CM. 2009a. The poly(A)-dependent degradation pathway of *rps*O mRNA is primarily mediated by RNase R. *RNA 15*:316-326.

Andrade JM, Pobre V, Silva IJ, Domingues S, Arraiano CM. 2009b. The role of 3'-5' exoribonucleases in RNA degradation. *Prog Mol Biol Transl Sci 85*:187-229.

Arluison V, Hohng S, Roy R, Pellegrini O, Régnier P, Ha T. 2007. Spectroscopic observation of RNA chaperone activities of Hfq in post-transcriptional regulation by a small non-coding RNA. *Nucleic Acids Res 35*:999-1006.

Arraiano CM, Andrade JM, Domingues S, Guinote IB, Malecki M, Matos RG, Moreira RN, Pobre V, Reis FP, Saramago M, Silva IJ, Viegas SC. 2010. The critical role of RNA processing and degradation in the control of gene expression. *FEMS Microbiol Rev 34*:883-923.

Arraiano CM, Yancey SD, Kushner SR. 1988. Stabilization of discrete mRNA breakdown products in *ams pnp rnb* multiple mutants of *Escherichia coli* K-12. *J Bacteriol 170*:4625-4633.

Babitzke P, Granger L, Olszewski J, Kushner SR. 1993. Analysis of mRNA decay and rRNA processing in *Escherichia coli* multiple mutants carrying a deletion in RNase III. *Journal of bacteriology 175*:229-239.

Basineni SR, Madhugiri R, Kolmsee T, Hengge R, Klug G. 2009. The influence of Hfq and ribonucleases on the stability of the small non-coding RNA *OxyS* and its target *rpoS* in *E. coli* is growth phase dependent. *RNA Biol 6*:584-594.

Bossi L, Figueroa-Bossi N. 2007. A small RNA downregulates LamB maltoporin in *Salmonella*. *Mol Microbiol 65*:799-810.

Bouvier M, Sharma CM, Mika F, Nierhaus KH, Vogel J. 2008. Small RNA binding to 5' mRNA coding region inhibits translational initiation. *Mol Cell 32*:827-837.

Brennan RG, Link TM. 2007. Hfq structure, function and ligand binding. *Curr Opin Microbiol 10*:125-133.

Cao GJ, Sarkar N. 1997. Stationary phase-specific mRNAs in *Escherichia coli* are polyadenylated. *Biochem Biophys Res Commun 239*:46-50.

Chao Y, Vogel J. 2010. The role of Hfq in bacterial pathogens. *Curr Opin Microbiol 13*:24-33.

Condon C, Putzer H. 2002. The phylogenetic distribution of bacterial ribonucleases. *Nucleic acids research 30*:5339-5346.

Coornaert A, Lu A, Mandin P, Springer M, Gottesman S, Guillier M. 2010. MicA sRNA links the PhoP regulon to cell envelope stress. *Mol Microbiol 76*:467-479.

Danchin A. 2009. A phylogenetic view of bacterial ribonucleases. *Prog Mol Biol Transl Sci 85*:1-41.

De Lay N, Gottesman S. 2011. Role of polynucleotide phosphorylase in sRNA function in *Escherichia coli*. *RNA 17*:1172-1189.

Del Favero M, Mazzantini E, Briani F, Zangrossi S, Tortora P, Dehò G. 2008. Regulation of *Escherichia coli* polynucleotide phosphorylase by ATP. *J Biol Chem 283*:27355-27359.

Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, Eckert MR, Vogel J, Charpentier E. 2011. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature 471*:602-607.

Fender A, Elf J, Hampel K, Zimmermann B, Wagner EG. 2010. RNAs actively cycle on the Sm-like protein Hfq. *Genes Dev 24*:2621-2626.

Folichon M, Allemand F, Régnier P, Hajnsdorf E. 2005. Stimulation of poly(A) synthesis by *Escherichia coli* poly(A)polymerase I is correlated with Hfq binding to poly(A) tails. *Febs J 272*:454-463.

Folichon M, Arluison V, Pellegrini O, Huntzinger E, Régnier P, Hajnsdorf E. 2003. The poly(A) binding protein Hfq protects RNA from RNase E and exoribonucleolytic degradation. *Nucleic acids research 31*:7302-7310.

Geissmann TA, Touati D. 2004. Hfq, a new chaperoning role: binding to messenger RNA determines access for small RNA regulator. *EMBO J 23*:396-405.

Grunberg-Manago M, Oritz PJ, Ochoa S. 1955. Enzymatic synthesis of nucleic acidlike polynucleotides. *Science 122*:907-910.

Hajnsdorf E, Braun F, Haugel-Nielsen J, Régnier P. 1995. Polyadenylylation destabilizes the *rpsO* mRNA of *Escherichia coli*. *Proc Natl Acad Sci USA 92*:3973-3977.

Hajnsdorf E, Régnier P. 2000. Host factor Hfq of *Escherichia coli* stimulates elongation of poly(A) tails by poly(A) polymerase I. *Proc Natl Acad Sci USA 97*:1501-1505.

Houseley J, LaCava J, Tollervey D. 2006. RNA-quality control by the exosome. *Nature reviews 7*:529-539.

Hussein R, Lim HN. 2011. Disruption of small RNA signaling caused by competition for Hfq. *Proc Natl Acad Sci U S A 108*:1110-1115.

Le Derout J, Folichon M, Briani F, Dehò G, Régnier P, Hajnsdorf E. 2003. Hfq affects the length and the frequency of short oligo(A) tails at the 3' end of *Escherichia coli rps*O mRNAs. *Nucleic Acids Res 31*:4017-4023.

Lease RA, Woodson SA. 2004. Cycling of the Sm-like protein Hfq on the *DsrA* small regulatory RNA. *J Mol Biol 344*:1211-1223.

Link TM, Valentin-Hansen P, Brennan RG. 2009. Structure of *Escherichia coli* Hfq bound to polyriboadenylate RNA. *Proc Natl Acad Sci U S A 106*:19292-19297.

Marujo PE, Hajnsdorf E, Le Derout J, Andrade R, Arraiano CM, Régnier P. 2000. RNase II removes the oligo(A) tails that destabilize the *rpsO* mRNA of *Escherichia coli*. *RNA 6*:1185-1193.

Massé E, Escorcia FE, Gottesman S. 2003. Coupled degradation of a small regulatory RNA and its mRNA targets in *Escherichia coli*. *Genes Dev 17*:2374-2383.

Mikulecky PJ, Kaw MK, Brescia CC, Takach JC, Sledjeski DD, Feig AL. 2004. *Escherichia coli* Hfq has distinct interaction surfaces for *Dsr*A, *rpo*S and poly(A) RNAs. *Nat Struct Mol Biol 11*:1206-1214.

Mohanty BK, Kushner SR. 2000 Polynucleotide phosphorylase functions both as a 3' right-arrow 5' exonuclease and a poly(A) polymerase in *Escherichia coli*. *Proc Natl Acad Sci USA 97*:11966-11971.

Mohanty BK, Maples VF, Kushner SR. 2004. The Sm-like protein Hfq regulates polyadenylation dependent mRNA decay in *Escherichia coli*. *Mol Microbiol 54*:905-920.

Moll I, Afonyushkin T, Vytvytska O, Kaberdin VR, Blasi U. 2003a. Coincident Hfq binding and RNase E cleavage sites on mRNA and small regulatory RNAs. *RNA 9*:1308-1314.

Moll I, Leitsch D, Steinhauser T, Blasi U. 2003b. RNA chaperone activity of the Sm-like Hfq protein. *EMBO Rep 4*:284-289.

Morita T, Maki K, Aiba H. 2005. RNase E-based ribonucleoprotein complexes: mechanical basis of mRNA destabilization mediated by bacterial noncoding RNAs. *Genes Dev 19*:2176-2186.

Morita T, Mochizuki Y, Aiba H. 2006. Translational repression is sufficient for gene silencing by bacterial small noncoding RNAs in the absence of mRNA destruction. *Proc Natl Acad Sci U S A 103*:4858-4863.

Nurmohamed S, Vincent HA, Titman CM, Chandran V, Pears MR, Du D, Griffin JL, Callaghan AJ, Luisi BF. 2011. Polynucleotide Phosphorylase Activity May Be Modulated by Metabolites in *Escherichia coli*. *J Biol Chem 286*:14315-14323.

O'Hara EB, Chekanova JA, Ingle CA, Kushner ZR, Peters E, Kushner SR. 1995. Polyadenylylation helps regulate mRNA decay in *Escherichia coli*. *Proc Natl Acad Sci USA 92*:1807-1811.

Otaka *ea*. 2011. PolyU tail of rho-independent terminator of bacterial small RNAs is essential for Hfq action. *Proc Natl Acad Sci U S A in press*.

Pellegrini M, Marcotte EM, Thompson MJ, Eisenberg D, Yeates TO. 1999. Assigning protein functions by comparative genome analysis: protein phylogenetic profiles. *Proc Natl Acad Sci U S A 96*:4285-4288.

Prévost K, Desnoyers G, Jacques JF, Lavoie F, Massé E. 2011. Small RNA-induced mRNA degradation achieved through both translation block and activated cleavage. *Genes Dev 25*:385-396.

Rasmussen AA, Eriksen M, Gilany K, Udesen C, Franch T, Petersen C, Valentin-Hansen P. 2005. Regulation of *omp*A mRNA stability: the role of a small regulatory RNA in growth phase-dependent control. *Mol Microbiol 58*:1421-1429.

Régnier P, Hajnsdorf E. 2009. Poly(A)-assisted RNA decay and modulators of RNA stability. *Prog Mol Biol Transl Sci 85*:137-185.

Reichenbach B, Maes A, Kalamorz F, Hajnsdorf E, Gorke B. 2008. The small RNA *GlmY* acts upstream of the sRNA *GlmZ* in the activation of glmS expression and is subject to regulation by polyadenylation in *Escherichia coli*. *Nucleic Acids Res 36*:2570-2580.

Sambrook J, Russell DW. 2006. *The Condensed Protocols From Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York.

Sauer E, Weichenrieder O. 2011. Structural basis for RNA 3'-end recognition by Hfq. *Proc Natl Acad Sci U S A*.

Schumacher MA, Pearson RF, Moller T, Valentin-Hansen P, Brennan RG. 2002. Structures of the pleiotropic translational regulator Hfq and an Hfq-RNA complex: a bacterial Sm-like protein. *EMBO J 21*:3546-3556.

Slomovic S, Portnoy V, Yehudai-Resheff S, Bronshtein E, Schuster G. 2008. Polynucleotide phosphorylase and the archaeal exosome as poly(A)-polymerases. *Biochim Biophys Acta 1779*:247-255.

Soper T, Mandin P, Majdalani N, Gottesman S, Woodson SA. 2010. Positive regulation by small RNAs and the role of Hfq. *Proc Natl Acad Sci U S A 107*:9602-9607.

Sun X, Zhulin I, Wartell RM. 2002. Predicted structure and phyletic distribution of the RNA-binding protein Hfq. *Nucleic Acids Res 30*:3662-3671.

Suzuki K, Babitzke P, Kushner SR, Romeo T. 2006. Identification of a novel regulatory protein (CsrD) that targets the global regulatory RNAs *CsrB* and *CsrC* for degradation by RNase E. *Genes Dev 20*:2605-2617.

Tsui HC, Leung HC, Winkler ME. 1994. Characterization of broadly pleiotropic phenotypes caused by an *hfq* insertion mutation in *Escherichia coli* K-12. *Mol Microbiol 13*:35-49.

Tuckerman JR, Gonzalez G, Gilles-Gonzalez MA. 2011. Cyclic di-GMP activation of polynucleotide phosphorylase signal-dependent RNA processing. *J Mol Biol 407*:633-639.

Udekwu KI, Darfeuille F, Vogel J, Reimegard J, Holmqvist E, Wagner EG. 2005. Hfq-dependent regulation of *Omp*A synthesis is mediated by an antisense RNA. *Genes Dev 19*:2355-2366.

Updegrove TB, Correia JJ, Chen Y, Terry C, Wartell RM. 2011. The stoichiometry of the *Escherichia coli* Hfq protein bound to RNA. *RNA 17*:489-500.

Urban JH, Vogel J. 2008. Two seemingly homologous noncoding RNAs act hierarchically to activate *glm*S mRNA translation. *PLoS Biol 6*:e64.

Valentin-Hansen P, Eriksen M, Udesen C. 2004. The bacterial Sm-like protein Hfq: a key player in RNA transactions. *Mol Microbiol 51*:1525-1533.

Vecerek B, Moll I, Blasi U. 2007. Control of Fur synthesis by the non-coding RNA *Ryh*B and iron-responsive decoding. *EMBO J 26*:965-975.

Viegas SC, Pfeiffer V, Sittka A, Silva IJ, Vögel J, Arraiano CM. 2007. Characterization of the role of ribonucleases in *Salmonella* small RNA decay. *Nucleic Acids Res 35*:7651-7664.

Viegas SC, Silva IJ, Saramago M, Domingues S, Arraiano CM. 2011. Regulation of the small regulatory RNA MicA by ribonuclease III: a target-dependent pathway. *Nucleic Acids Res 39*:2918-2930.

Waters LS, Storz G. 2009. Regulatory RNAs in bacteria. *Cell 136*:615-628.

Wilusz CJ, Wilusz J. 2005. Eukaryotic Lsm proteins: lessons from bacteria. *Nat Struct Mol Biol 12*:1031-1036.

Wilusz CJ, Wilusz J. 2008. New ways to meet your (3') end oligouridylation as a step on the path to destruction. *Genes Dev 22*:1-7.

Zuo Y, Deutscher MP. 2001. Exoribonuclease superfamilies: structural analysis and phylogenetic distribution. *Nucleic Acids Res 29*:1017-1026.

Supplemental Data

Figure S1 – PNPase overexpression reduces MicA* levels in the *hfq pnp* mutant.

Figure S2 – Cell growth of wild-type and its isogenic *hfq* mutant.

Figure S3 - Nuclease S1 mapping of the 5' end of MicA.

Figure S1

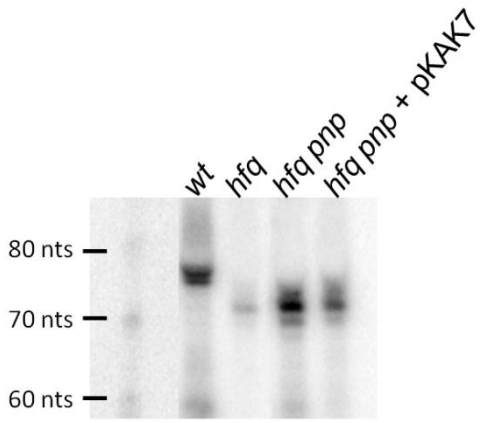

Figure S1. PNPase overexpression reduces MicA* levels in the *hfq pnp* mutant.
Northern blot detection of MicA from total RNA isolated from stationary phase cultures. Bacterial strains from left to right: wild-type, *hfq* mutant, *hfq pnp* double mutant without and carrying the plasmid pKAK7 (overexpressing PNPase cloned with its own promoters). The full-length MicA and the shorter MicA* are indicated on the gel. Ladder bands run along the gel are indicated on the left side.

Figure S2

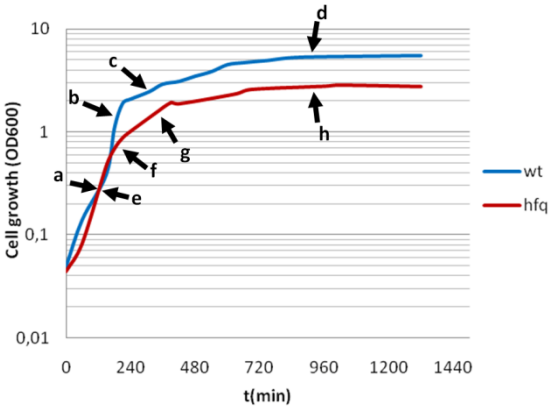

Figure S2. Cell growth of wild-type and its isogenic *hfq* mutant.
Cell cultures were grown in LB medium and incubated at 37°C with shaking. OD_{600} readings were determined over time. Strains displayed different growth curves and therefore exhibit different OD_{600} values for similar stages of growth. For Northern blot experiments in Figure 5A, total RNA was isolated from culture samples collected from four different stages: exponential (EXP), late exponential, early stationary and stationary phase (STAT). This corresponds on the graph to letters *a-d* for the wild-type (OD_{600} values of ~0.3, ~1.7, ~2.5 and ~5.5) and letters *e-h* for the *hfq* mutant (OD_{600} values of ~0.3, ~0.8, ~1.6 and ~2.3), respectively.

Figure S3

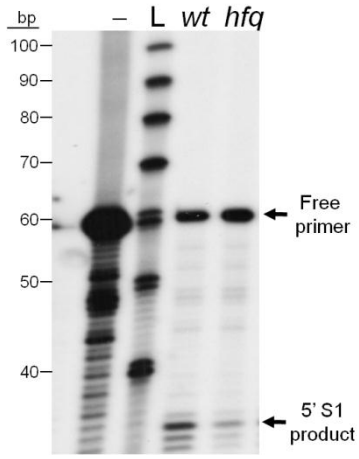

Figure S3. Nuclease S1 mapping of the 5' end of MicA.
S1 nuclease mapping was done according to the standard procedure (Sambrook & Russell, 2006). 30 μg of total RNA isolated from wild-type or *hfq* mutant was mixed with 0.1 pmol of 5'-end labelled MicA-S1 primer (5'- ttt ctc tct cta ttc agc tat ttt tct TTC AGG GAT GAT GAT AAC AAA TGC GCG TCT TTC -3'). This primer is a 60-mer DNA oligonucleotide complementary to the first 33 nucleotides of MicA (capital letters) and has an overhang extension of 27 nucleotides. For each sample, 100 units of S1 nuclease (Promega) were used. Incubation was performed at 37^{o}C for 75 minutes. Following S1 nuclease treatment, the protected DNA probes were loaded on 10% polyacrylamide gel containing 8 M urea. Detection of radioactive signals was done by use of PhosphorImager scanning. Ladder bands *(L)* are indicated on the left side of the gel. A negative control (–) corresponding to a RNA/primer sample not treated with S1 nuclease is shown on the first lane. Undigested labelled probe (with the expected size of 60-mer) and the protected S1 nuclease 5' end MicA fragment (with the expected size of 33 nucleotides) are identified by arrows on the right side of the gel.

Supplemental References

Sambrook J, Russell DW. 2006. *The Condensed Protocols From Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York.

Chapter 3

Small RNA Modules Confer Different Stabilities and Interact Differently With Multiple Targets

Keywords: MicA/ PNPase / RNA degradation/ RNA domains/ Small non-coding RNAs

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* Joint first authors

For this work I helped carried out the experiments, the analysis of the data and writing of the manuscript.

Table of Contents

Abstract

Bacterial Hfq-associated small regulatory RNAs (sRNAs) parallel eukaryotic microRNAs in their ability to control multiple target mRNAs. The small non-coding MicA RNA represses the expression of several genes, including major outer membrane proteins such as OmpA, Tsx and EcnB. In this study, we have characterised the RNA determinants involved in the stability of MicA and analysed how they influence the expression of its targets. Site-directed mutagenesis was used to construct MicA mutated forms. The 5'linear domain, the structured region with two stem-loops, the A/U-rich sequence or the 3' poly(U) tail were altered without affecting the overall secondary structure of MicA. The stability and the target regulation abilities of the wild-type and the different mutated forms of MicA were then compared. The 5' domain impacted MicA stability through an RNase III-mediated pathway. The two stem-loops showed different roles and disruption of stem-loop 2 was the one that mostly affected MicA stability and abundance. Moreover, STEM2 was found to be more important for the *in vivo* repression of both *omp*A and *ecn*B mRNAs while STEM1 was critical for regulation of *tsx* mRNA levels. The A/U-rich linear sequence is not the only Hfq-binding site present in MicA and the 3' poly(U) sequence was critical for sRNA stability. PNPase was shown to be an important exoribonuclease involved in sRNA degradation. In addition to the 5´ domain of MicA, the stem-loops and the 3´ poly(U) tail are also shown to affect target-binding. Disruption of the 3´U-rich sequence greatly affects all targets analysed. In conclusion, our results have shown that it is important to understand the "sRNA anatomy" in order to modulate its stability. Furthermore, we have demonstrated that MicA RNA can use different modules to regulate its targets. This knowledge can allow for the engineering of non-coding RNAs that interact differently with multiple targets.

Introduction

Small RNA-mediated networks control a wide variety of cellular processes. The development of new experimental strategies has contributed enormously to the increasing number of small RNAs identified in bacteria (Sharma & Vogel, 2009). About 100 small RNAs have been experimentally confirmed in *Escherichia coli* and many more have been predicted (Shinhara et al., 2011). Comparative profiling of strains has contributed to the identification of novel non-coding RNAs in other bacteria (Ferrara et al., 2012). Small RNAs are distinct amongst themselves and their structural diversity makes it difficult to unify this class of cell regulators. sRNAs are diverse in size and do not display a common sequence that can be used as a signature (Liu & Camilli, 2010). They present diverse modes of action, exerting either a positive or a negative effect on the expression of the target mRNAs.

The interactions between sRNA and mRNAs contribute to the differential modulation of gene expression. Bacterial *trans*-encoded small RNAs bind to their target mRNAs through the establishment of short and imperfect antisense base pairing interactions in a close parallel to the action of eukaryotic miRNAs (Beisel & Storz, 2010). The base pairing of sRNAs can take place at different sites on the target but they usually occur within the 5' end of the mRNA (Sharma et al., 2007; Bouvier et al., 2008; Guillier & Gottesman, 2008; Prévost et al., 2011). It was shown that sRNA-mRNA pairs can be subject to endonucleolytic degradation, in which RNase E and RNase III play major roles (Afonyushkin et al., 2005; Morita et al., 2005). However, as result of the base pairing dynamics not all the population of a small RNA is going to be bound to its targets. Degradation of the free fraction may unbalance the pool of available small RNAs. The 3'-5' exoribonucleolytic degradation plays an important role in this regulation and PNPase was shown to be a major enzyme in the small RNAs turnover (Andrade & Arraiano, 2008;

Andrade et al., 2012). Therefore, the study of the elements controlling the sRNA stability is critical to better understand the regulation of the sRNA-based pathways.

The well characterised small RNA MicA was used as model for our study. MicA was initially identified to repress the synthesis of several major outer membrane proteins (OMPs) (Rasmussen et al., 2005; Udekwu et al., 2005; Bossi & Figueroa-Bossi, 2007). The list of target mRNAs for MicA was recently expanded through the use of microarray studies (Gogol et al., 2011). MicA belongs thus to the increasing cluster of sRNAs that regulate multiple targets. The architecture of a small RNA greatly contributes to its stability and may define its ability to interact with different target mRNAs. Enzymatic and chemical probing was used to map the structure of MicA which was essentially consistent with the conformation predicted by the mfold algorithm (Zuker, 2003; Udekwu et al., 2005). Based on the sequence and secondary structure, we have defined the following domains in MicA: a 5' linear sequence; a Hfq-binding A/U-rich sequence; two structured elements (stem-loops) and finally a U-rich linear stretch in the 3' end (Figure 1 and Figure S1). A similar modular structure was proposed to other small RNAs, like RybB and SgrS (Balbontín et al., 2010; Papenfort et al., 2010; Otaka et al., 2011; Rice & Vanderpool, 2011).

The 5' end of MicA was suggested to be the principal target recognition domain, as found for many other small RNAs (Papenfort & Vogel, 2009). For the RybB RNA it was even possible to define a short "seed" sequence located in the 5' end that is responsible for interaction with multiple targets in *Salmonella* (Balbontín et al., 2010; Papenfort et al., 2010). However, this does not seem to be the common rule and the nucleotides and the length of the 5' sRNA sequence involved in the regulation of the different targets may differ (Guillier & Gottesman, 2008).

All the *E. coli trans*-encoded sRNA such as MicA bind to RNA chaperone Hfq, a protein homologous to Sm and Sm-like proteins involved in RNA processing in eukaryotes (Olejniczak, 2011; Vogel & Luisi, 2011). Hfq plays multiple roles in the cell but mostly facilitates the sRNA-mRNA annealing (Valentin-Hansen et al., 2004; Hajnsdorf & Boni, 2012). It was found to bind linear A/U-rich sequences in RNA and was recently shown to preferably interact with the 3' end of small RNAs (Schumacher et al., 2002; Sauer & Weichenrieder, 2011; Sauer et al., 2012). Hfq is also very important for sRNA stabilization and was shown to protect from RNase E- and PNPase-mediated degradation (Moll et al., 2003; Andrade et al., 2012).

Although sRNAs are usually structured molecules, the degree of RNA folding varies according to the GC-content of the sRNA analysed. A stem-loop followed by a short U-rich sequence corresponding to the *rho*-independent transcriptional terminator is ubiquitous amongst small RNAs (Reynolds & Chamberlin, 1992; Abe & Aiba, 1996). Very recently, Hfq was shown to bind to this sequence which makes this a potential domain for interaction with mRNA (Otaka et al., 2011; Sauer & Weichenrieder, 2011; Sauer et al., 2012). The presence of additional stems is frequent in many sRNAs and MicA harbours a total of two stem-loops. These structures can potentially act as stabilizer elements as they can hinder the 3'-5' exonucleolytic degradation pathway of many RNA substrates (Andrade et al., 2009). The structure of the sRNA can also be critical for interaction with the target mRNA and conformational rearrangements can lead to disruption of sRNA-mRNA base pairing. This is well illustrated in many studies such as the OxyS interaction with the *fhlA* mRNA (Altuvia et al., 1998; Argaman & Altuvia, 2000) or the base pairing between RyhB and the *iscRSUA* policistronic transcript (Desnoyers et al., 2009).

Through use of mutational studies we analysed the modular domain organization of MicA. Several nucleotide changes were introduced in the *mic*A

gene in order to disrupt independently each predefined domain without affecting the overall conformation of the molecule. We demonstrate that several elements present in the 3' end of MicA act as stabilizers of this small RNA and this region is also suggested to play important roles in the MicA-dependent riboregulation of different target mRNAs. In addition to the well characterised role of the 5' end of MicA in the interaction with targets we suggest that elements present in the 3' end of MicA can also contribute to the differential regulation of the mRNA targets.

Results

1. Engineering synthetic MicA RNAs

Base pairing with target mRNAs is a determinant of small RNA function and stability. The 5' region of MicA was predicted to be the major region involved in the interaction with the target mRNAs. However, additional elements present in the small RNA molecule can potentially influence MicA activity and/or stability. From the analysis of MicA sequence and its secondary structure (as reported in (Udekwu et al., 2005)) we have defined the following small RNA modules: a 5'-end linear stretch, a structured region harbouring two stem-loops (STEM1 and STEM2) separated by an A/U-rich sequence and finally the transcriptional termination U-rich sequence located at the 3'-end (Figure 1). The 5' end and the far most 3' end nucleotides of MicA are highly conserved while the regions encompassing the stem-loops can comprise some variability amongst enterobacteria, as observed in the sequence alignment in Figure 1.

In order to get insights into the relationship between the architecture, the function and stability of a small RNA, we constructed several variants of MicA using the technique of overlapping PCR. The nucleotide changes introduced are easily identified in the schematic representation of MicA variants (Figure 1 and Figure S1). RNA secondary structure might be critical for the function of a small RNA. The chemical mapping of the structure of MicA RNA was previously performed and essentially agreed with the mfold algorithm analysis (Zuker, 2003; Udekwu et al., 2005). Accordingly, we used mfold to predict nucleotide changes that would not alter the overall secondary structure of the MicA molecule. The wild-type and the variant forms of MicA were cloned in a low copy number plasmid and were expressed from MicA own promoter. The synthetic MicA sRNAs were expressed *in trans* from a plasmid in cells deleted for the chromosomal *mic*A gene.

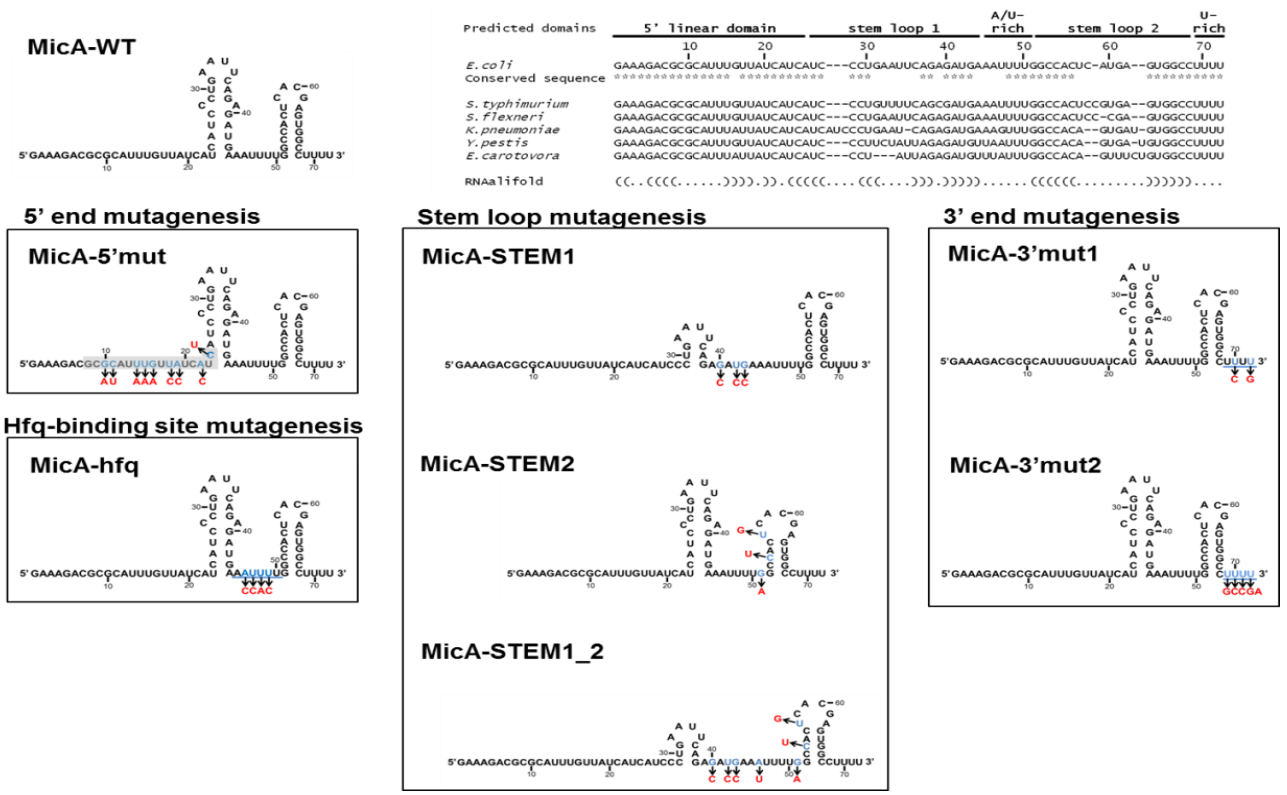

Figure 1. Construction of MicA mutants. Nucleotide sequence and secondary structure of the E. coli wild-type MicA is shown on top. The proposed modular domain organization of MicA is indicated. MicA sequence and structure is conserved in several enterobacteria as analysed by the CLUSTALW (Chenna et al., 2003) and the RNAalifold software (Bernhart et al., 2008). The model structures predicted with mfold (Zuker, 2003) for the different MicA mutants are schematized. Mutagenesis of the different domains was designed to not disturb the overall secondary structure of the MicA RNA. Nucleotides changes are indicated by arrows.

The 5’ linear region of MicA is involved in the interaction with multiple target mRNAs (Gogol et al., 2011). MicA lacks a base pairing “seed” and the nucleotides involved in interactions with mRNAs can vary according to the target analysed. For this reason, we introduced extensive mutations in this 5’ linear sequence (MicA-5’mut) in order to maximize the disruption of MicA binding with its targets (Figure 1: 5’ end mutagenesis).

The proper RNA folding can also be crucial for interaction with mRNAs and MicA exhibits two stable stem-loops (Figure 1). Furthermore, such structured features can potentially act as stabilizing elements as they may serve as physical barriers against 3’-5’ exonucleolytic degradation (Andrade et al., 2009). These elements may thus play multifunctional roles and we constructed several mutants to study them. MicA-STEM1 mutant harbours mutations that almost disrupt completely the first stem-loop (located more closely to the 5’ end) without affecting the global conformation of the molecule. On the other hand, the second stem-loop (closer to the 3’ end) was shown to be thermodynamically stronger and we could not disrupt it as this greatly changed the secondary structure of MicA; hence mutations introduced in MicA-STEM2 were chosen to allow stem-loop relaxation while the overall secondary structure of the molecule was not disturbed. In addition to these single mutants we also constructed the double MicA-STEM1_2 mutant harbouring mutations in both stem-loops (Figure 1: Stem-loop mutagenesis).

Another module present in MicA RNA is the short linear A/U-rich region between the two stem-loops that is a predicted *in vitro* binding site for Hfq (Rasmussen et al., 2005). Base pairing between the sRNA and its target mRNA is facilitated in the presence of the RNA chaperone Hfq (Valentin-Hansen et al., 2004). In order to analyse the *in vivo* importance of this region for the Hfq-dependent regulation of MicA we mutated the A/U-rich (5’-AAUUU-3’) to a C-rich

stretch (5'-ACCAC-3') as these mutations are predicted to almost disrupt Hfq-binding to the RNA (Figure 1: Hfq-binding site mutagenesis).

The last unit to be analysed here is the short poly(U) tail of the *rho*-independent terminator, a general feature of bacterial small RNAs. This was recently shown to be a key sequence for riboregulation and Hfq action on small RNAs (Otaka et al., 2011; Sauer & Weichenrieder, 2011; Sauer et al., 2012). Two mutants were designed for this region; MicA-3'mut1 exhibits two nucleotide substitutions in the poly(U) sequence while MicA-3'mut2 harbours more nucleotide changes (Figure 1: 3' end mutagenesis). All the mutations described above were then analysed for their impact on MicA stability and capability to regulate its targets. All the work was performed with cultures in the stationary phase of growth, a condition in which MicA levels are increased (Argaman et al., 2001; Udekwu et al., 2005).

2. Experimental determination of the secondary structure of MicA mutants

Although mfold prediction was previously shown to agree with the structure of the wild-type MicA (Zuker, 2003; Udekwu et al., 2005), we decided to experimentally validate the structural models of two of our most relevant mutants, MicA-5'mut and MicA-STEM1_2 (Figure 2). To characterise *in vitro* and *in vivo* the RNA secondary structures of these two mutants, we have used a broad range of enzymatic and chemical probes. Namely, the techniques performed were dimethyl sulphate (DMS) modification of RNA nucleotides, the use of RNase A (cuts C and U unpaired residues) or RNase T1 (identifies unpaired G residues) and detection of single-stranded residues by lead acetate (PbAc) and in line probing.

First we carried out *in vitro* and *in vivo* probing with DMS, which methylates unpaired adenosines and cytidines. After DMS treatment, a specific antisense primer to the 3' end of MicA was used to perform primer extension reactions. However, this amplification revealed to be highly problematic probably due to the strong STEM2 present in MicA secondary structure that prevented annealing of a primer in this region. In fact, we only succeeded in obtaining cDNA from cells expressing the MicA-STEM1_2 variant; this result seems to support the relaxation of the STEM2 secondary structure. Only the use of a modified LNA primer allowed amplification with good resolution from nucleotides 1 to 50. Complementary studies using additional methods allowed then a better resolution of the most 3' end nucleotides.

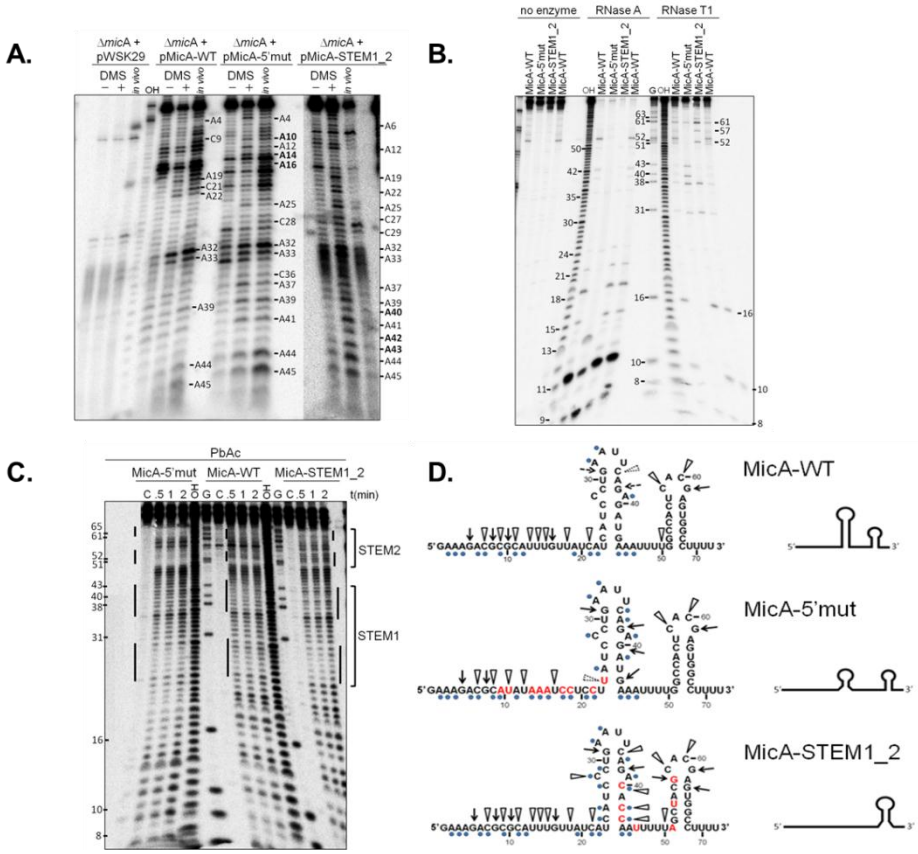

Figure 2. Determination of the secondary structure of MicA-5'mut and MicA-STEM1_2 RNAs.

(A) Results from *in vitro* and *in vivo* dimethyl sulphate (DMS) modification assay. cDNA obtained from total RNA samples treated (+) or untreated (-) with DMS *in vitro*, as indicated on top of the gel. In vivo denotes the reactions from DMS added directly to cell cultures. The position of some adenosine and cytidine residues that reacted with DMS is given. RNA extracted from cells transformed with the empty pWSK29 vector or expressing the MicA-WT, MicA-5'mut RNA and MicA-STEM1_2 RNAs was tested. MicA-STEM1_2 is expressed to lower levels than MicA-WT (see Figure 7). Therefore, the panel with MicA-STEM1_2 RNA corresponds to the same gel but the image was slightly contrasted to better visualise the bands. The 3' end of the MicA-DMS primer (Table III) used in the reverse transcription reaction is complementary to nucleotide 58 in MicA sequence.
(B) Enzymatic probing of the different MicAs using RNase A and RNase T1. The position of several nucleotides is given. Controls with no addition of enzyme are shown on the left side of the gel. Alkaline ladders of MicA-WT are denoted as OH. A G-specific ladder generated by RNase T1 digestion of MicA-WT RNA under denaturing conditions is shown. Please note that in each series, MicA-WT was tested in duplicate.
(C) Lead acetate probing. All reactions were done in native conditions, with addition of 5mM PbAc. Incubation proceeded for 0.5, 1 or 2 minutes, as indicated. C is an untreated control, and G is a T1 ladder obtained under denaturing conditions. OH represents an alkaline ladder prepared with MicA-WT RNA. Some nucleotides are given for orientation. Thick lines on the side of the lanes represent the position of stem-loop arms.
(D) Representation of MicA-WT, MicA-5'mut and MicA-STEM1_2 sequences, showing the enzymatic cleavages by RNase T1 (arrows) and RNase A (triangles) and the reactivity of nucleotides to DMS (blue dots below the nucleotides). Broken lines indicate weaker cleavage sites. Mutated nucleotides are shown in red. On the right side, is a schematic representation of the conformation of these RNAs.

The mfold prediction of the MicA-WT RNA structure was very accurate compared to our experimental data (Figure 2) and to what was previously reported to the chromosomally encoded wild-type MicA (Zuker, 2003; Udekwu et al., 2005). A very good validation of the proposed computational model was also observed with the structure mapping of the MicA-STEM1_2. Mutations in the STEM1_2 MicA impaired the formation of STEM1 although mfold would predict the existence of a very weak stem-loop in this region; both methods agreed in the relaxation of the STEM2. Surprisingly, DMS probing and RNase cleavage assays of the MicA-5'mut RNA suggested that mutations introduced in the 5' end resulted in the partial disruption of STEM1, a feature that was not predicted by the computational analysis. Using lead acetate (Figure 2C) and in line probing (Figure S2) we observed that disruption of STEM1 in the MicA-5'mut did not seem as

stronger as observed in the MicA-STEM1_2 variant. Altogether, these results suggested that a relaxed STEM1 is still probably detected in the conformation of the MicA-5'mut RNA while no changes were detected on STEM2 conformation (Figure 2D).

3. The 5' domain impacts MicA stability through an RNase III-mediated pathway

In order to study the impact of the different MicA modules on the stability of this sRNA, we measured the decay rate for the MicA-WT and compared it with the altered forms of MicA (represented in Figure 1).

We first focused on the analysis of the role of the 5' end of MicA in the stability of this small RNA. Mutagenesis of the 5' end of MicA was previously shown to have an effect in the regulation of its target mRNAs (Udekwu et al., 2005; Bossi & Figueroa-Bossi, 2007; Coornaert et al., 2010). The 5' mutant that we constructed harbours more extensive modifications (9 nucleotide changes) than the other reported mutants. The structure mapping of this RNA (Figure 2) revealed that modifications introduced in the 5' end could also affect the conformation of the STEM1. However, the MicA-5'mut RNA is shown to act differently than the MicA-STEM1 variant that harbours mutations that disrupt the STEM1 but maintain the 5' linear end intact (as presented in the following set of results). This indicates that mutations introduced in the 5' end have an effect that can be separated from the presence of an intact STEM1.

We found that MicA-5'mut is more stable than MicA-WT (half-lives of 25 min and 11 min, respectively as determined by Northern blotting) (Figure 3, upper panels). This result suggested that the degradation of MicA RNA is dependent on its 5' end sequence. Since 5' end of MicA interacts with target mRNAs, we could

also infer from this result that the stability of MicA could also be linked to its ability to base pair with mRNAs.

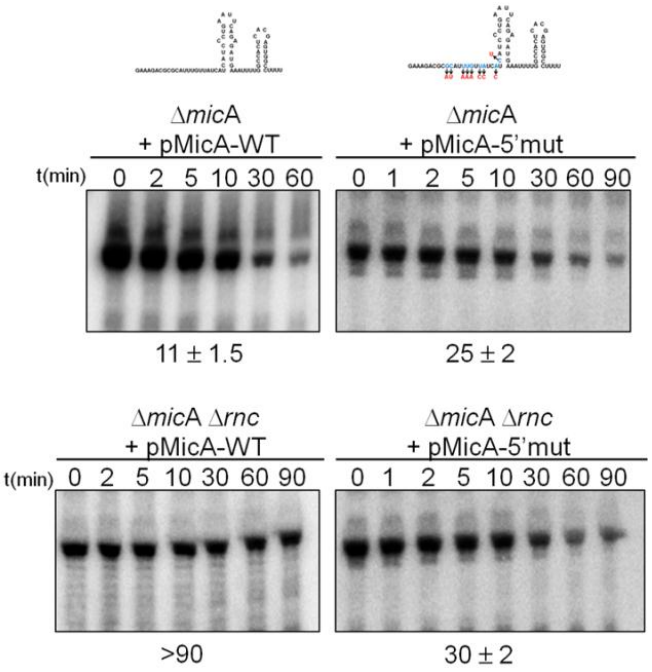

Figure 3. Mutagenesis of the 5' linear domain of MicA. Northern blot analysis of MicA in Δ*mic*A cells or its isogenic derivative lacking RNase III (Δ*mic*A Δ*rnc*), expressing *in trans* either the wild-type MicA (from the pMicA-WT plasmid) or the 5' mutated MicA variant (from the pMicA-5'mut plasmid). RNA was extracted from stationary phase cultures and MicA stability was measured as described in *Material and Methods*.

RNase III is a double-stranded RNA endoribonuclease (Arraiano et al., 2010). RNase III impact on MicA stability could result from the action on the small RNA itself (once it exhibits double-stranded regions) or from the activity of RNase III on the sRNA-mRNA hybrid. To test this, we performed an *in vitro* activity assay using purified *E. coli* RNase III and radioactive labelled wild-type MicA RNA as substrate and we found that this sRNA was not cleaved (Figure S3). This is in agreement with recent findings from our laboratory where it was shown that *Salmonella* RNase III (*rnc*) also does not cleave wild-type MicA *in vitro* but is able to cleave it

when it is bound to its target *omp*A mRNA (Viegas et al., 2011). Consequently, the activity of RNase III against MicA can potentially be used as an indirect approach to indicate when MicA is base pairing with its target mRNAs.

To test the *in vivo* impact of *E. coli* RNase III in this regulation, we constructed the double mutant Δ*mic*A Δ*rnc* and measured the stability of the *trans*-encoded MicAs. As expected, MicA-WT was highly stabilized in the absence of RNase III (MicA barely decayed even after 90 min after blocking of transcription) (Figure 3, lower panels). The introduction of several mutations in the 5' end of MicA strongly impaired the RNase III-mediated degradation of this sRNA; in the absence of RNase III, MicA-5'mut showed only a stability of 30 min *versus* the >90 min obtained for the MicA-WT. Hence, the 5' end domain of MicA modulates this sRNA stability through an RNase III-dependent pathway.

Even though in the Δ*mic*A strain the MicA-5'mut is stabilised compared to the MicA-WT, we consistently observed that the MicA-5'mut is less abundant (Figure 3). The increasing stability that is observed is probably related to impaired RNase III activity against this molecule, as formation of MicA-mRNA target duplexes that corresponds to the RNase III substrate is suggested to be reduced. The lower abundance is more difficult to explain, but similar observations have been made (Bernstein et al., 2002; Le Derout et al., 2010).

4. Distinct roles of stem-loops in promoting MicA stability

MicA displays two GC-rich stem-loops, STEM1 (immediately after the 5' linear sequence) and STEM2 (closer to 3' end) (Figure 1). Computational analysis using the mfold algorithm (Zuker, 2003) predicts that STEM2 is thermodynamically stronger ($\Delta G = -12.0$) than STEM1 ($\Delta G = -8.2$). In agreement, our experimental structure mapping of the MicA variants showed that STEM1 conformation was

strongly affected by the nucleotide changes introduced (Figure 2). Hence, we also wanted to analyse the importance of these structural elements in the control of MicA stability.

Plasmids expressing either the wild-copy of MicA (pMicA-WT) or the altered STEM1 (pMicA-STEM1) or STEM2 (pMicA-STEM2) versions were used to transform Δ*mic*A cells. The expression and stability of these MicA variants were tested by Northern blotting (Figure 4A). Results showed that these structured elements play different roles in protecting MicA from degradation. Disruption of STEM1 barely affected MicA stability (MicA-STEM1 exhibit an half-life of 9 min) when compared to the MicA-WT (half-life of 11 min). Actually, the levels of the MicA-STEM1 variant are even slightly higher than the MicA-WT (Figure 4A). In contrast, perturbation of STEM2 considerably reduced the stability of this small RNA (MicA-STEM2 has a half-life of 4.6 min) as well as its abundance when compared to wild-type MicA (Figure 4A). In *Salmonella* two other point mutations located in STEM2 have also been shown to affect the ability of MicA to downregulate *lam*B mRNA levels (Bossi & Figueroa-Bossi, 2007). The MicA-STEM1_2 (harbouring mutations in both stem-loops) followed the results obtained with the single disruption of STEM2 (Figure 4B). Relaxation of the transcriptional terminator is likely to result in transcriptional read-through (Reynolds & Chamberlin, 1992; Abe & Aiba, 1996). However, as no major transcriptional termination read-through products were detected on these gels, the low levels of the MicA-STEM2 or the MicA-STEM1_2 variants are suggested to be consequence of their low stabilities. The 3'-5' exoribonuclease PNPase was previously shown to degrade the wild-type MicA (Andrade & Arraiano, 2008) and was the likely candidate for the rapid degradation of these MicA variants. A new Δ*mic*A *pnp* strain was then constructed and transformed with the adequate plasmid. Clearly, inactivation of PNPase resulted in a more long-lived MicA-STEM1_2 RNA and its levels were strongly increased

(Figure 4B). The other major exoRNases (RNase II and RNase R) did not significantly participate in the degradation of this sRNA.

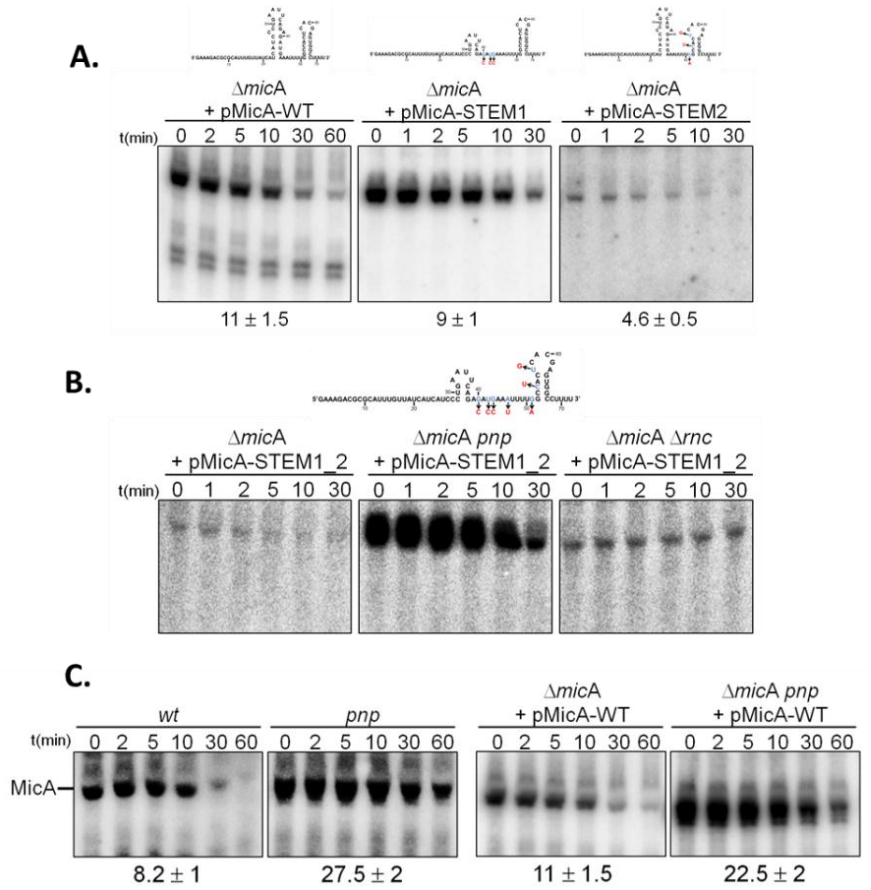

Figure 4. Mutagenesis of MicA stem-loops. (A) Northern blot analysis of MicA in Δ*mic*A cells expressing *in trans* either the wild-type MicA (from the pMicA-WT plasmid) or the stem-loops mutated MicA variants (from the pMicA-STEM1 or pMicA-STEM2 plasmids). When expressing the MicA-WT it is possible to visualise two lower molecular weight bands (<60 nts) that were previously identified in work performed in *Salmonella* to correspond to breakdown products of the duplex MicA-target mRNA (Viegas et al., 2011).
(B) Impact of the disruption of both stem-loops (MicA-STEM1_2 variant) on MicA stability. Plasmid pMicA-STEM1_2 was used to transform Δ*mic*A cells and its isogenic derivatives lacking PNPase (Δ*mic*A *pnp*) or RNase III (Δ*mic*A Δ*rnc*).
(C) Northern blot analysis of the chromosomally encoded MicA or the MicA-WT expressed from plasmid, comparing the stability pattern in cells expressing or not PNPase. RNA was extracted from stationary phase cultures.

We also compared the activity of RNase III against the MicA-STEM1_2 RNA. This altered MicA variant with mutations on both hairpins was found to be stabilized in the Δ*mic*A Δ*rnc* when compared to the Δ*mic*A strain (Figure 4B). This was expected as MicA-STEM1_2 retains an intact 5' end domain that is known to direct RNase III cleavage. Strikingly, the MicA-STEM1_2 expression level is much higher upon inactivation of PNPase rather than RNase III. This may suggest that a large fraction of MicA-STEM1_2 RNA might not be bound to its targets and therefore the free population of this sRNA is preferably degraded exonucleolytically by PNPase. As expected, PNPase was also confirmed to be important in the decay of the plasmid encoded MicA-WT (Figure 4C). Overall, these results indicate that STEM1 plays a minor role in protecting MicA while STEM2 functions as an effective stabilizer element protecting MicA from degradation.

5. The A/U-rich linear sequence is not the only Hfq-binding site present in MicA

Another structural domain in MicA is an A/U-rich single-stranded region flanked by the two stem-loop structures. Previous work showed that Hfq has *in vitro* affinity for this region (Rasmussen et al., 2005). However, the role of this module in regulation of MicA stability has not been addressed. In order to study this sequence, we constructed a plasmid expressing a MicA variant in which we changed the linear A/U-rich tail between the stem-loops to a C-rich sequence (Figure 1: Hfq-binding site mutagenesis). This is predicted to impair the binding of Hfq to this sequence (Link et al., 2009). The pMicA-hfq plasmid was then used to transform Δ*mic*A cells and RNA extracted from stationary phase cultures was analysed by Northern blotting and compared to Δ*mic*A harbouring pMicA-WT.

The mutated MicA-hfq is less stable (<50%) than the MicA-WT (Figure 5A). This clearly showed that the internal A/U-rich sequence plays relevant roles in determining MicA stability *in vivo*. If this sequence was the only Hfq-binding site present in MicA, the stabilities of both MicA-hfq in cells harbouring Hfq and the MicA-WT in cells lacking Hfq were expected to be similar. However, the MicA-WT in Δ*hfq* cells is less stable than the MicA-hfq variant in the presence of Hfq (Figure 5A).

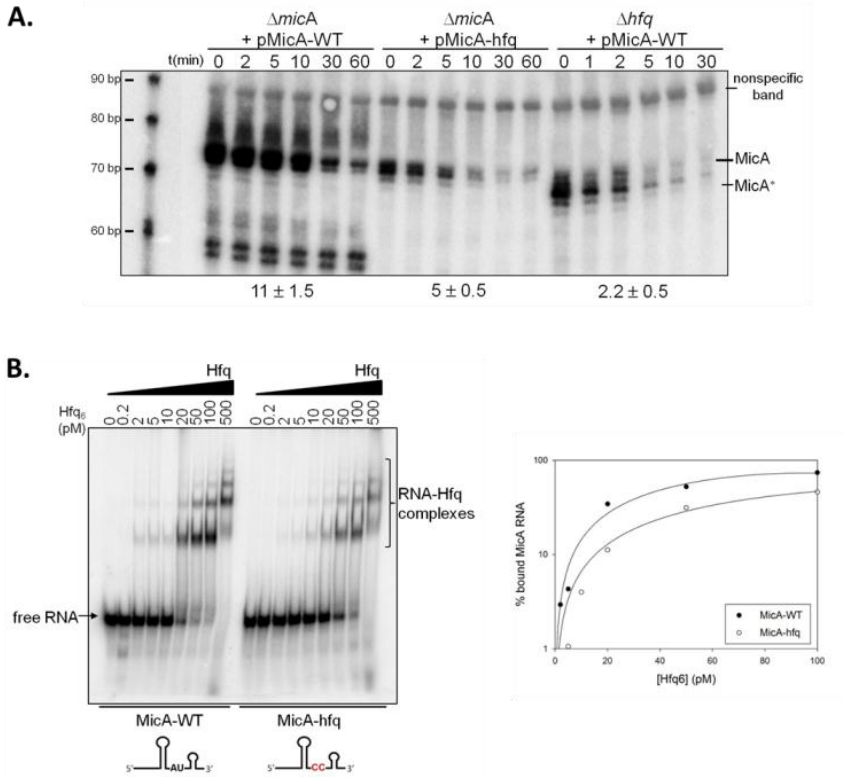

Figure 5. Mutagenesis of the A/U-rich domain, a Hfq-binding site in MicA.
(A) Northern blot analysis of MicA in Δ*mic*A cells expressing *in trans* either the wild-type MicA (from the pMicA-WT plasmid) or a MicA variant with the A/U-rich domain mutated to a C-rich sequence (from the pMicA-hfq plasmid). Plasmid pMicA-WT was also used to transform a deletion strain of *hfq*. A smaller form of MicA (designated MicA∗) is only clearly observed in the absence of Hfq; this fragment had been previously identified (Andrade et al., 2012). A size marker is shown on the left of the gel. The riboprobe used to detect MicA, cross-reacts with a nonspecific band, (that is also detected on the Δ*mic*A strain, Figure S6) that was here used as loading control. A more stringent washing step eliminates this band without affecting the MicA signal, as previously described (Andrade & Arraiano, 2008). RNA was extracted from stationary phase cultures.
(B) Mutagenesis of the A/U-rich domain of MicA to a C-rich sequence affects the Hfq binding ability to this small RNA. The gel mobility shift assay was performed with a

constant amount of radiolabelled MicA-WT or MicA-hfq variant as RNA substrates and increasing amounts of purified Hfq protein, as indicated in the figure. The free RNA and the Hfq-RNA complexes are indicated. The gels were then dried and exposed to a PhosphorImager screen and quantified using ImageQuant software. The results were plot using SigmaPlot software and binding curves were fit. Filled circles represent MicA-WT and open circles represent MicA-hfq variant.

Furthermore, the lack of Hfq results in the detection of a slightly smaller MicA species (MicA*) (previously identified in (Andrade et al., 2012)) that is not detected in the Δ*mic*A transformed with the pMicA-hfq plasmid (Figure 5A). Hfq is thus suggested to protect the 3' end of MicA against nucleolytic degradation that originates the shorter and rather unstable MicA* species. Altogether, these results lead to the conclusion that the internal A/U-rich sequence is probably not the only Hfq-binding site in MicA. To confirm this, we have performed gel mobility shift experiments with a constant amount of radiolabelled MicA-WT or MicA-hfq variant as RNA substrates and increasing amounts of purified Hfq (Figure 5B). Hfq was able to form the same complexes with MicA-WT or MicA-hfq RNAs although binding with MicA-hfq was less efficient (with Kd values of 65 and 380 pM, respectively). Since Hfq could still form complexes with the MicA-hfq variant, this confirmed that this single-stranded A/U-rich sequence between the two stem-loops is not the only Hfq-binding site present in MicA.

6. The 3' U-rich terminator sequence is critical for MicA stability

Hfq binds preferably A/U-rich sequences but was also shown to interact with other U-rich elements present in mRNAs (Folichon et al., 2003). The poly(U) tail from the transcriptional terminator was thus an excellent candidate to interact with Hfq (Figure 1). Recent findings demonstrated that the poly(U) sequence downstream the terminator is involved in the Hfq-dependent regulation

of small RNAs (Otaka et al., 2011; Sauer & Weichenrieder, 2011). We further analysed the role of this domain in the control of MicA stability.

We constructed two MicA mutants in which we changed the nucleotides in the 3' poly(U) tail. In the MicA-3'mut1 variant, the 5'-UUUU-3' linear sequence immediately after the terminator was changed to a 5'-UCUG-3' sequence while in the MicA-3'mut2 this modification was more extensive (to 5'-GCCGA-3') (Figure 1: 3' end mutagenesis). The corresponding plasmids harbouring these nucleotide changes in the 3' end of MicA were used to transform the Δ*mic*A strain. In order to attenuate inefficient transcription termination that could arise from modification of the 3' poly(U) sequence, an additional stretch of 8 T's was included immediately after the 4U residues in both cloning strategies. The MicA-3'mut1 variant showed decreased stability when compared to the wild-type (half-life from 11 min to 6 min) (Figure 6A). Extended mutagenesis of the 3' RNA sequence even resulted in a more drastic reduction (to 2.3 min for MicA-3'mut2).

The 3' altered MicA's are slightly longer than the wild-type MicA (Figure 6A). However, the elongated RNA species was also detected (in low level) when expressing the MicA-WT (Figure 6A, upper arrow on side of the gel). The wild-type MicA terminates in a linear stretch of 4 U's which functions as efficient termination site (Argaman et al., 2001; Udekwu et al., 2005; Gogol et al., 2011). Nevertheless, an alternative transcriptional termination site located next to the this sequence can be found in the *mic*A DNA (5'-TTTTCTTTT-3') which can lead to slightly longer MicA species, as we observe in Figure 6A. Mutagenesis of the poly(U) sequence most probably results in the relaxation of the transcriptional termination leading to increasing amounts of this read-through RNA. However, even after the extensive modification of the 3' poly(U) sequence in the MicA-3'mut2 we did not detect in these gels the accumulation of other major transcriptional read-through bands when compared to the MicA-WT (Figure 6A).

This suggests that the reduced levels of the 3' altered MicA RNAs are mainly consequence of its rapid turnover, as observed with the mutants in STEM2 (Figure 4). In fact, we found that inactivation of PNPase resulted in the strong accumulation and stabilization of both the 3' MicA modified RNAs (Figure 6B).

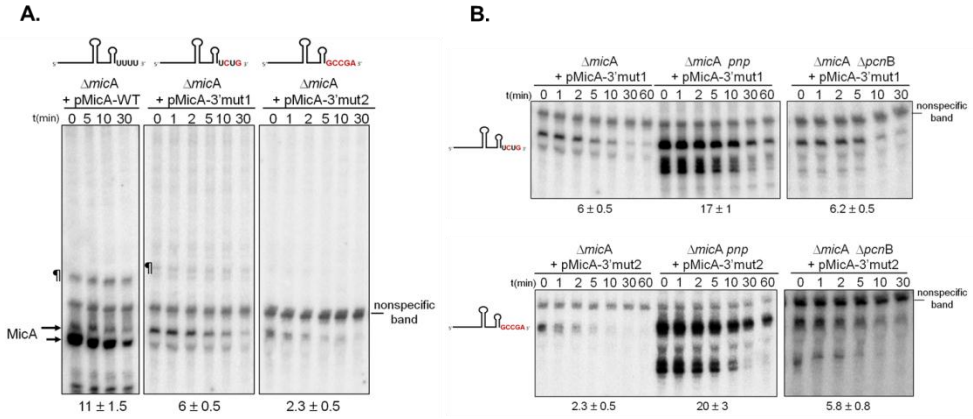

Figure 6. Mutagenesis of the 3' end U-rich domain of MicA.
(A) Effect of mutations in the 3'end U-rich linear sequence in the stability of the MicA RNA. Northern blot analysis of MicA in Δ*mic*A cells expressing *in trans* the wild-type MicA (from the pMicA-WT) or the mutated 3' end variants (from the pMicA-3'mut1 or pMicA-3'mut2 plasmids). Read-through bands are indicated by the symbol (¶). Two different sized forms of MicA can be detected and are marked with arrows on the side of the gel.
(B) Northern blot analysis of MicA in Δ*mic*A cells or its derivative isogenic mutants lacking either PNPase (Δ*mic*A *pnp*) or Poly(A) polymerase I (Δ*mic*A Δ*pcn*B) expressing *in trans* either the mutated MicA-3'mut1 or the MicA-3'mut2 variant. RNA was extracted from stationary phase cultures. Upon hybridization of the membrane, a nonspecific band is observed and was here used as loading control (Andrade & Arraiano, 2008).

In contrast, inactivation of the poly(A) polymerase (encoded by the *pcn*B gene) (Régnier & Hajnsdorf, 2009) did not show a great effect in the degradation of these full-length 3' end MicA mutants (Figure 6B) although MicA-3'mut2 RNA was more affected than the MicA-3'mut1 variant. Yet, polyadenylation seemed to affect the degradation of an intermediate breakdown product (Figure 6B). Inactivation of PNPase resulted in much stronger stabilisation of these sRNAs, an

indication that PNPase is not dependent on an active poly(A)-dependent pathway to actively degrade these small RNAs.

7. Differential control of target mRNAs by MicA variants

We wanted to investigate whether our different MicA mutants were functional in riboregulation. To test this, we analysed the RNA levels of three different targets: *omp*A, *tsx* and *ecn*B mRNAs. OmpA is a major outer membrane protein and was the first identified target of MicA. EcnB (entericidin B membrane lipoprotein) and Tsx (nucleotide transporter) were recently shown to be specifically regulated by MicA in *Salmonella* (Rasmussen et al., 2005; Udekwu et al., 2005; Bossi & Figueroa-Bossi, 2007; Gogol et al., 2011). Our work extends the list of known target mRNAs in *E. coli* as for the first time *tsx* and *ecn*B transcripts are also shown to be regulated by MicA.

A deletion strain of *mic*A was transformed either with the empty plasmid (pWSK29), the plasmid expressing the wild-type copy of MicA or with the plasmids harbouring the different MicA variants and the levels of the target mRNAs were then evaluated. This provided a simple approach to test how the different mutations were affecting MicA repressor activity. Overexpression of the wild-type MicA is very efficient in the downregulation of its target mRNAs as compared to Δ*mic*A transformed with the empty vector (compare Figure 7, lane 1 and lane 2). On the other hand, MicA mutants exhibited different levels of repression (Figure 7A, lanes 3-8).

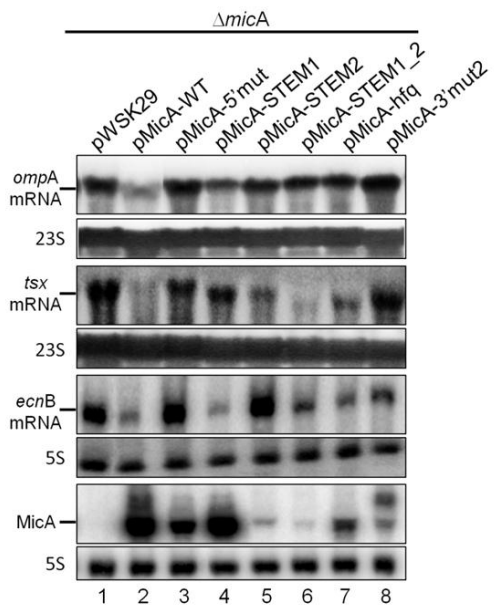

Figure 7. Differential control of target mRNAs by the synthetic MicA variants.
Northern blot analysis of *omp*A, *tsx*, *ecn*B and MicA transcripts. Total RNA was extracted from stationary phase cultures of Δ*mic*A cells transformed with a low copy number based plasmid (pWSK29) expressing either the wild-type copy of MicA or one of the mutated variants described in this work. Plasmids used were: pWSK29 (lane 1), pMicA-WT (lane 2), pMicA-5'mut (lane 3), pMicA-STEM1 (lane 4), pMicA-STEM2 (lane 5), pMicA-STEM1_2 (lane 6), pMicA-hfq (lane 7) and pMicA-3'mut2 (lane 8). 23S was used as loading control for the *omp*A and *tsx* transcripts (analysis from agarose Northern blots) while 5S was used as loading control for *ecn*B and MicA RNAs (analysis from polyacrylamide Northern blots).

Compared to the MicA-WT expression, the mutated forms of MicA (with exception of MicA-STEM1) present in Figure 7 have reduced concentrations as result of their lower stabilities (Figure 7, lower panel) and this could contribute to different levels of repression observed. Nevertheless, despite this different expression levels, we verified that a same MicA variant can differentially affect the levels of different target mRNAs. Even though the levels of MicA-5'mut were lower than the MicA-WT, all the target mRNAs accumulated showing that modification of the 5' linear sequence is critical to downregulate the expression of these targets (compare Figure 7, lane 2 and lane 3). In addition, the levels of

MicA-STEM1 were identically to MicA-WT but we could detect different regulatory effects as MicA-STEM1 RNA was still able to repress *ecn*B expression (identically to MicA-WT) while it did not function as well to downregulate *omp*A or *tsx* mRNA levels (compare Figure 7, lanes 2 and 4). Conversely, the levels of MicA-STEM2 were strongly reduced but MicA-STEM2 was found to be much more important in the regulation of *ecn*B and *omp*A mRNAs than STEM1 (compare Figure 7, lane 2 with lanes 4-5).

The stem-loops are shown to have different effects on the regulation of different targets. STEM2 seems to have a more generalised effect, affecting all the tested mRNAs while STEM1 was shown to particularly affect the *tsx* mRNA (compare Figure 7, lane 2 with lanes 4-5). MicA-STEM1_2 variant (harbours mutations in both MicA stem-loops) was not found to simply add the effects of STEM1 and STEM2 mutations (compare Figure 7, lanes 4-6). Despite the low levels of MicA-STEM1_2 (that follows the expression levels found to MicA-STEM2), this sRNA is still able to repress the expression of *tsx* mRNA but unlike MicA-STEM1 it did not seems to repress *omp*A and *ecn*B expressions (compare Figure 7, lanes 4-6).

The mutants for the two high affinity Hfq-binding sites in MicA were also analysed for their riboregulatory activity (Figure 1 and Figure 7, lanes 7-8). Mutagenesis of either the internal A/U-rich or the 3' poly(U) tail reduces the ability of Hfq to interact with the sRNA and this is expected to affect the base pairing ability of the sRNA with the target mRNAs (Sauer & Weichenrieder, 2011; Sauer et al., 2012). MicA-hfq variant was less functional than the MicA-WT in the downregulation of all the targets, showing that this domain is required for riboregulation. However, this was shown to be more important for regulation of *omp*A mRNA than for the others transcripts tested (Figure 7, compare lanes 2 and 7). On the other hand, the MicA-3'mut2 variant (harbouring mutations in the 3'

poly(U) tail) resulted in the accumulation of all the targets analysed (Figure 7, compare lanes 2 and 8), to a level higher than the one obtained when overexpressing the MicA-hfq variant (Figure 7, compare lanes 7 and 8). These results suggest that the 3’ poly(U) sequence plays more relevant roles than the internal A/U-rich sequence in promoting interactions with the different target mRNAs; it is also possible that 3' poly(U) sequence can affect specificity of a sRNA.

Discussion

The architecture of a small RNA is critical for its stability, influences the formation of sRNA-protein complexes and can affect base pairing with target mRNAs. The knowledge of the important factors controlling the sRNA abundance in the cell is of utmost importance for the manipulation of sRNA-based pathways. Through our mutagenic studies we have defined distinct modules in MicA that were shown to play distinct roles in protecting MicA from degradation. Mutations in the 5' end domain resulted in the stabilisation of this sRNA, presumably by impairing RNase III activity against MicA. On the other hand, mutations in several 3' end elements resulted in unstable MicA's and we showed that the 3'-5' exoribonuclease PNPase was a major player in this degradation. Our data also suggest that different domains of MicA can be involved in the riboregulation of target mRNAs. Moreover, we have shown that the effect of these sRNA mutations in their regulatory pathways cannot be directly deduced from the levels or stability of the small RNAs.

The free form of MicA is not cleaved by RNase III and only when it is bound to a target mRNA it becomes a substrate to RNase III (Figure S3 and (Viegas et al., 2011)). This also helps explaining why mutagenesis of the 5' end domain of MicA resulted in the stabilisation of this sRNA (Figure 3). This implies that the free (not bound to its targets mRNAs) population of MicA is degraded by a distinct pathway that does not necessarily involve RNase III. In fact, our data suggest that PNPase is actively involved in this decay. Therefore, alternative degradation pathways are used to control either the free MicA or the target-bound MicA. In an apparent paradox, even though MicA-5'mut was found to be more stable it is less abundant than MicA-WT (Figure 3). Similar results can be found in the literature for other RNAs. In fact, in *E. coli* the levels of RNAs not always have a direct correspondence to their half-lives as shown by a microarray analysis (Bernstein et al., 2002). We

may speculate that mutagenesis of the 5' linear sequence of MicA could affect the production of this sRNA. Interestingly, it has been suggested that mutations inserted in the 5' UTR sequence can influence the transcription rate in non-bacterial systems (Kudla et al., 2006).

The linear 5' end sequence was shown to be the main domain that many small RNAs use to bind their targets (for example RybB, OmrA, OmrB and MicA) (Guillier & Gottesman, 2008; Balbontín et al., 2010; Papenfort et al., 2010; Gogol et al., 2011). We confirmed that the 5' linear sequence of MicA (corresponding to the first 23 nts) is essential for repression of all the targets analysed (*omp*A, *tsx* and *ecn*B mRNAs). From these, *tsx* mRNA was the less affected by the mutations in the 5' end of MicA, probably because the nucleotide changes still resulted in an extensive complementation with this target, as predicted by computational analysis (Figure S4). However, we have found that the 5' linear sequence of MicA does not seem to be the only domain involved in the regulation of the targets (Figure 7).

We observed that MicA stem-loops could affect differently the amounts of the distinct target mRNAs. STEM2 was found to be much more important for the *in vivo* repression of both *omp*A and *ecn*B mRNAs than STEM1, unlike we could expect from the RNAhybrid prediction (Figure S4). In contrast, STEM1 was critical for regulation of *tsx* transcript levels while disruption of STEM2 had a considerably less impact on this mRNA. MicA-STEM1_2 is clearly less efficient in the repression of *ompA or ecnB* mRNA levels (Figure 7). Therefore, these results suggest that the structure of both MicA stem-loops is important for interaction with such targets.

The main function of stem-loops is usually considered the protection of RNA against degradation. The formation of a double-stranded region within the sRNA can sequester sequences susceptible to RNase E endonucleolytic cleavages and efficiently act as physical barriers against 3'-5' exonucleolytic degradation

(Arraiano et al., 2010). The two stem-loops present in MicA were found to play distinct roles. Surprisingly, the extensive disruption of STEM1 did not significantly affect the stability of MicA (Figure 4A). The main role of STEM1 seems thus not to be the protection of the sRNA against degradation, unlike it was shown to happen with STEM2. This has implications in the relative abundance of these variants as MicA-STEM1 shows identical levels to MicA-WT whereas MicA-STEM2 is strongly downregulated (Figure 7). However, as showed here, some targets like *omp*A and *ecn*B mRNAs are more strongly affected by MicA-STEM2 than by the MicA-STEM1 variant, which supports that the effects of these mutations in the regulatory pathways are not simply the result from changes in sRNA stability. The Hfq-binding sites in MicA are in two separate domains: the internal A/U-rich sequence and the 3' U-rich tail after the transcriptional terminator. The Hfq-binding site mutants of MicA were expected to be less efficient in the interactions with target mRNAs (because Hfq is known to accelerate the rate of sRNA-mRNA duplex formation) (Valentin-Hansen et al., 2004). Surprisingly, our results showed that these sequences can play distinct roles in the regulation of the different targets. Mutation of the 3' U-rich sequence of MicA was shown to have a more generalised effect in all the mRNAs tested while mutation of the A/U-rich sequence had a more pronounced impact in the regulation of *omp*A mRNA levels than on the other targets. RNAhybrid predicted that mutations affecting the 3' poly(U) sequence of MicA would affect more the base pairing with target mRNAs but failed to predict the impact of disrupt the internal A/U-rich sequence of MicA in the regulation of *omp*A mRNA levels (Figure S4).

These results suggest that interaction of Hfq with the sRNA seems to greatly depend on the target itself or might require the interaction with additional factors. Mutants in one of the high affinity Hfq-binding sites of MicA (MicA-hfq and MicA-3'mut variants) were shown to accumulate at levels inferior to the MicA-WT (Figure 7). However, for each mutation we can observe distinct effects

for the different targets. For example, changes introduced in the 3' U-rich linear sequence of MicA affected more strongly the levels of *omp*A and *tsx* mRNA in comparison to *ecn*B mRNAs. Again we conclude that this differential response shows that the effect of the mutations on target expression cannot be simply deducted from the sRNA levels.

Hfq is known to protect sRNA from RNase E and PNPase-mediated degradation (Moll et al., 2003; Andrade et al., 2012). Mutations that disrupted the Hfq-binding sites were found to result in more labile MicA's probably because these MicA variants become more accessible to the action of RNases. Modification of the 3' U-rich linear tail of the transcriptional terminator (Figure 6) was shown to destabilize MicA to a higher extension than the mutations introduced in the internal A/U-rich sequence (Figure 5). Stability measurements revealed that PNPase was the main enzyme involved in the degradation of these MicA variants. We also examined the effect of modifying the 3' poly(U) tail of the RybB, a small RNA that shares multiple targets with MicA (Balbontín et al., 2010; Papenfort et al., 2010; Gogol et al., 2011). Modification of the nucleotides immediately after the terminator stem-loop (from poly(U) to a 5'-CCGUC-3' sequence) resulted as well in a more labile sRNA (Figure S5). Recent findings showed that shortening of the 3' U-rich tail of other sRNAs also resulted in the instability of these sRNAs (Otaka et al., 2011). Therefore, Hfq binding to the uridylated 3' end of MicA agrees with the protection of this protein against the 3'-5' exonucleolytic degradation by PNPase (this work and (Andrade et al., 2012)).

The modular structure of MicA helps explaining the dynamics of interaction with its multiple targets. The 5' end of MicA is critical for repression of some targets (such as *omp*A expression) while the 3' end of MicA harbours elements that may be more relevant for regulation of other targets (such as *tsx* mRNA). These findings can most probably be extended to other regulatory RNAs, judging

by the results of a computational search which predicted that several other small RNAs can potentially use different regions to establish base pairing interactions with their targets (Peer & Margalit, 2011). FnrS and Spot42 are good examples of this, as both were shown to use different single-stranded regions for base pairing with different set of targets (Durand & Storz, 2010; Beisel et al., 2012).

Our work confirms the importance of the 5' end domain both in riboregulation and in the stabilisation of the sRNA. However, we expand this view by showing that 3' end elements not only are critical for the stability of the sRNA but are also suggested to be involved in the regulation of some target mRNAs. As a matter of fact, the 3' end is shown to harbour different stabilizer elements, namely stem-loops and high affinity Hfq-binding sites. Actually, Hfq has even a higher affinity for the 3'U-rich sequence rather than for internal A/U-rich sequences typically found in small RNAs (Sauer & Weichenrieder, 2011; Sauer et al., 2012). The 3' end terminal nucleotides of MicA are highly conserved (as observed in the sequence alignment in Figure 1) and most likely our findings with *E. coli* MicA can be extrapolated across species. Moreover, the modular structure of MicA is commonly found among small RNAs, supporting that our results may be generalized to other non-coding RNAs (Balbontín et al., 2010; Papenfort et al., 2010; Otaka et al., 2011; Rice & Vanderpool, 2011). There has been growing interest in the use of synthetic regulatory RNAs to program gene expression networks (Davidson & Ellington, 2005; Lioliou et al., 2010; Liang et al., 2011). We believe that mutations that alter the 3'end region, namely the 3'U-rich sequence of the sRNA can be a useful strategy to manipulate the networks regulated by small RNAs.

Materials and Methods

Strains and growth conditions

E. coli K-12 strain MG1693 (Arraiano et al., 1988) or its derivatives were used in this work (Table I). Deletion mutant of *ryb*B was constructed by the one step inactivation of chromosomal genes method (Datsenko & Wanner, 2000). Bacteria were grown at 37ºC in Luria-Bertani (LB) medium supplemented with thymine (50 μg ml^{-1}). SOC medium was used to recover cells after heat-shock in plasmid transformation steps. When required, antibiotics were present at the following concentrations: chloramphenicol, 50 μg ml^{-1}, kanamycin, 50 μg ml^{-1}; ampicillin, 100 μg ml^{-1}.

Table I. Strains used in this work

Strain	Relevant genotype	Reference
GSO80	MC4100*hfq*	(Zhang et al., 2002)
MG1693	*thy*A715	(Arraiano et al., 1988)
SK5691	*thyA715 pnp*7	(Arraiano et al., 1988)
SK7622	*thyA715* Δ*rnc*38	(Babitzke et al., 1993)
CMA413	*thyA715* Δ*mic*A	(Andrade & Arraiano, 2008)
CMA428	MG1693 *hfq*	(Andrade et al., 2012)
CMA514	*thyA715* Δ*mic*A + pMicA-WT	This study
CMA515	*thyA715* Δ*mic*A + pMicA-5'mut	This study
CMA516	*thyA715* Δ*mic*A + pMicA-STEM1	This study
CMA517	*thyA715* Δ*mic*A + pMicA-STEM2	This study
CMA518	*thyA715* Δ*mic*A + pMicA-STEM1_2	This study
CMA519	*thyA715* Δ*mic*A + pMicA-hfq	This study
CMA520	*thyA715* Δ*mic*A + pMicA-3'mut1	This study
CMA521	*thyA715* Δ*mic*A + pMicA-3'mut2	This study
CMA522	*thyA715* Δ*mic*A Δ*rnc*38 + pMicA-WT	This study
CMA523	*thyA715* Δ*mic*A Δ*rnc*38 + pMicA-5'mut	This study
CMA524	*thyA715* Δ*mic*A *pnp*7 + pMicA-WT	This study
CMA525	*thyA715* Δ*mic*A *pnp*7 + pMicA-STEM1_2	This study
CMA526	*thyA715* Δ*mic*A Δ*rnc*38 + pMicA-STEM1_2	This study
CMA527	*thyA715* Δ*mic*A Δ*pcn*B + pMicA-3'mut1	This study
CMA528	*thyA715* Δ*mic*A Δ*pcn*B + pMicA-3'mut2	This study
CMA529	MG1693 *hfq* + pMicA-WT	This study

CMA530	*thyA715* Δ*mic*A *pnp*7 + pMicA-3'mut1	This study
CMA531	*thyA715* Δ*mic*A *pnp*7 + pMicA-3'mut2	This study
CMA532	*thyA715* Δ*ryb*B + pRybB-WT	This study
CMA533	*thyA715* Δ*ryb*B + pRybB-3'mut	This study

Construction of plasmids

All plasmids used in this work are based on the very low copy number pWSK29 (Wang & Kushner, 1991) and are indicated in Table II. DNA fragments containing the mutagenic micA variants (MicA-5'mut, MicA-STEM1, MicA-STEM2, MicA-STEM1_2, MicA-hfq, MicA-3'mut1, MicA-3'mut2) were amplified by PCR overlapping using the oligonucleotides indicated in Table III. Partial fragments were amplified with MicA-HindIII and the respective mutagenic forward primer or MicA-PstI and the respective mutagenic reverse primer (Table III). PCR bands were gel eluted using the gel extraction NucleoSpin Extract II kit (Macherey-Nagel). For each mutation, the partial PCRs carry an overlapping region of 20 nucleotides. Approximately equal amounts of each partial PCR (for a given construct) were added to Pfu reaction mix containing dNTPs but lacking primers. The extension step (30 s at 95^{o}C, 60 s at 55^{o}C and 30 s at 72^{o}C) proceeded for 15 cycles. The external primers MicA-HindIII and MicA-PstI were then added and the PCR reactions run for 20 cycles. A DNA fragment (274 bp) encompassing the entire wild-type MicA (MicA-WT) was directly amplified using primers MicA-HindIII and MicA-PstI. All DNA inserts include the MicA natural promoter (previously identified in (Udekwu & Wagner, 2007) and were HindIII/PstI cloned into pWSK29. Competent DH5α cells were used in the cloning procedure. Positive clones were selected by colony PCR. The nucleotide sequences of all constructs were confirmed by DNA sequencing (Stab Vida).

Table II. Plasmids used in this work

Plasmid	Comments	Reference
pWSK29	very low copy number plasmid; ampicilin resistance	(Wang & Kushner, 1991)
pMicA-WT	wild-type copy of MicA	This study
pMicA-5'mut	MicA variant harbouring mutations in the 5' linear sequence	This study
pMicA-STEM1	MicA variant harbouring mutations in stem-loop 1	This study
pMicA-STEM2	MicA variant harbouring mutations in stem-loop 2	This study
pMicA-STEM1_2	MicA variant harbouring mutations in stem-loops 1 and 2	This study
pMicA-hfq	MicA variant harbouring mutations in the internal A/U-rich linear sequence (an high affinity Hfq-binding site)	This study
pMicA-3'mut1	MicA variant harbouring 2 nucleotides changes in the 3' end U-rich terminator sequence	This study
pMicA-3'mut2	MicA variant harbouring 5 nucleotides changes in the 3' end U-rich terminator sequence	This study
pRybB-WT	wild-type copy of RybB	This study
pRybB-3'mut	MicA variant harbouring 5 nucleotides changes in the 3' end U-rich terminator sequence	This study

RNA extraction and Northern analysis

For decay experiments, blocking of transcription was obtained by adding rifampicin to a final concentration of 500 mg ml^{-1}. Culture samples were withdrawn at defined timepoints thereafter and mixed with an equal volume of RNA stop buffer (10 mM Tris at pH 7.2, 5 mM $MgCl_2$, 25 mM NaN_3, and 500 mg ml^{-1} chloramphenicol). Total RNA was extracted by the phenol:chloroform method from stationary phase cultures as previously described (Andrade et al., 2012). Genomic DNA was removed from samples using the Turbo DNase (Ambion), as described by supplier. For Northern analysis, 10-30 μg of total RNA was fractionated under denaturing conditions either in 6% (for detection of the *ecn*B mRNA) or 10% polyacrylamide/7 M urea gels in TBE (for detection of the sRNAs) or by 1.2% agarose formaldehyde-denaturing gel in MOPS buffer (for detection of *omp*A and *tsx* mRNAs). RNAs were transferred onto Hybond-N^+ membrane (GE Healthcare) and U.V. crosslinked by UV irradiation using a UVC 500 apparatus (Amersham Biosciences). Membranes were hybridized with radiolabelled specific probes overnight in PerfectHyb Plus Hybridization Buffer (Sigma Aldrich) at 42-68ºC. Specific probes were obtained either by 5' end-labelling of antisense oligonucleotides using [γ-^{32}P]-ATP and T4 polynucleotide Kinase (Fermentas) or by *in vitro* transcription reactions with PCR DNA templates carrying a T7 promoter sequence through the use of [α-^{32}P]-UTP and T7 RNA polymerase (Promega). Radiolabelled probes were purified on G25 Microspin columns (GE Healthcare). The probe used to detect MicA in the Northern blot experiments correspond to an antisense riboprobe complementary to the entire wild-type MicA sequence (about 74 nucleotides in length). RNA was analysed by Phosphorimaging (Storm860) using the ImageQuant software (Molecular Dynamics). The half-lives of RNA were determined by linear regression using the logarithmic of the percentage of RNA remaining versus time, considering the amount of RNA at 0 min as 100%. Primers used in this work were obtained from Stab Vida (Portugal)

and are described in Table III. All radiochemicals were purchased from PerkinElmer.

Table III. Primers used in this work

PRIMER	Sequence (5'-3')
Overlapping PCR	
MicA-HindIII	AATGGAAGCttCTGATACCGAACCG
MicA-PstI	TTTTCGCCACCCGAACTGCAGGC
MicA-5'mut F	GCATATAAATCCTCCTTATCCCTGAATTCAGAGATGAAATTTTGGC
MicA-5'mut R	GATAAGGAGGATTTATATGCGTCTTTCATATACTCAGACTCGCCT
MicA-STEM1 F	GAcAccAAaTTTTgGCcACtCACGAGTGGCCTTTTTCTTTTCTGTCAGG
MicA-STEM1 R	aGTgGCcAAAAtTTggTgTCTGAATTCAGGGATGATGATAACAAATGCGC
MicA-STEM2 F	GAgAtgAAaTTTTaGCtACgCACGAGTGGCCTTTTTCTTTTCTGTCAGG
MicA-STEM2 R	cGTaGCtAAAAtTTcaTcTCTGAATTCAGGGATGATGATAACAAATGCGC
MicA-STEM1_2 R	cGTaGCtAAAAaTTggTgTCTGAATTCAGGGATGATGATAACAAATGCGC
MicA-STEM1_2 F	GAcAccAAtTTTTaGCtACgCACGAGTGGCCTTTTTCTTTTCTGTCAGG
MicA-hfq F	CAGAGATGAAccacTGGCCACTCACG
MicA-hfq R	GTGGCCAGTGGTTCATCTCTGAATTCAGGGATG
MicA-3'mut1 F	aaaaAAAAGtcggcAGGCCACTCGTGAG TGGCC
MicA-3'mut1 R	TgccgaCTTTTttttCTGTCAGGCGTGTTTTTCCAG
MicA-3'mut2 F	aaaaAAAAGcAgAAGGCCACTCGTGAGTGGCC
MicA-3'mut2 R	TTCTGCTTTTttttCTGTCAGGCGTGTTTTTCCAG
RybB-PstI	CGTCCTGCaGACGCTGGCAGGGACAATC
RybB-HindIII	GACCGTAAGCttCTATCGCGCGAGGAG
RybB-3mut F	GTTGATGGGTgccTcTTTTTTTTGTTATCTAAAACTTATC
RybB-3mut R	GATAACAAAAAAAAgAggcACCCATCAACCTTGAACCG
RNA substrate (*in vitro* transcription)	
T7-MicA	TAATACGACTCACTATAGAAAGACGCGCATTTGTTATCATC
***in vitro* MicA-wt**	AAAAGGCCACTCGTGAGTGGC
***in vitro* MicA-mut2**	TCGGCAGGCCACTCGTGAGTGGCC
cDNA synthesis (reverse transcriptase reaction)	
MicA-DMS LNA	AAA+A+G+AA+A+A+AGGCCACTCGTG

Gene deletion	
RybB-delFw	CACAACCGCAGAACTTTTCCGCAGGGCATCAGTCTTAATTAGTATTGTGT AGGCTGGAGCTGCTTC
RybB-delR	TGGTTGAGAGGGTTGCAGGGTAGTAGATAAGTTTTAGATAACGGTCCAT ATGAATATCCTCCTTAG
Northern probes	
MicA-T7	TAATACGACTCACTATAGGAAGGCCACTCGTGAGTGGCCAA
MicA-Fw	GAAAGACGCGCATTTGTTATC
RybB-T7	TAATACGACTCACTATAGGAACAAAAAACCCATCAACCTTGAACCG
RybB-Fw	ACTGCTTTTCTTTGATGTCCC
***omp*A-T7**	TAATACGACTCACTATAGGAAAAAAAACCCCGCAGCAGC
***omp*A-Fw**	TTGTAGACTTTACATCGCCAGGG
***ecn*B-T7**	TAATACGACTCACTATAGGTTATTGCTGCGCTTTCGTTGC
***ecn*B-Fw**	ATGGTGAAGAAGACAATTGCAGCG
***tsx*-T7**	TAATACGACTCACTATAGGGCTCATCGGCAGGCCAGTGTCG
***tsx*-Fw**	GCGGTACTGGCGCTCTCTTCG
23S-RNA	CCT ACA CGC TTA AAC CGG GAC
5S-RNA	CAT CGG CGC TAC GGC GTT TCA CTT C

Nucleotide changes are indicated in small capitals;

T7 promoter sequence is underlined.

"+" precedes LNA-modified nucleotides

Electrophoretic mobility shift assays (EMSA)

Binding assays were performed in 10 mM Tris–HCl (pH 8), 1 mM EDTA, 80 mM NaCl and 1% glycerol (v/v) (Ziolkowska et al., 2006) with increasing concentrations of His_6-tagged Hfq purified protein (kindly provided by Eliane Hajnsdorf) and a constant amount of radiolabeled [α-^{32}P]-UTP MicA as substrate. Reactions were incubated at 37°C for 30 min. EMSA samples were then electrophoresed on native 5% polyacrylamide gels in 1× TBE buffer in a cold room. Dried gels were then exposed on Phosphorimager screens and the corresponding signals were analysed using the ImageQuant software (Molecular Dynamics).

Binding data were fit to a Polynomial Quadratic curve and Kd values were calculated from the fit of the curve using SigmaPlot software (Systat Software).

5'-end labelling of RNA

MicA-WT and MicA-mutant RNA variants were transcribed with T7 RNA polymerase from PCR products obtained with primers described on Table III and relevant plasmid eluted from agarose gel as DNA templates. After dephosphorylation with Calf intestine alkaline phosphatase (Fermentas), RNA was 5'-end labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Fermentas). Labelled RNAs were then purified by 10% polyacrylamide/7M urea/ 1x TBE gel electrophoresis, eluted and precipitated with ethanol.

Chemical probing

The dimethyl sulphate (DMS) modification of unpaired adenosine and cytidine nucleotides was carried out essentially as described (Tijerina et al., 2007). For *in vitro* reactions, a 25 µl of total RNA renatured in Na-Cacodylate/EDTA buffer supplemented with 10mM $MgCl_2$ was treated with 1 µl freshly prepared DMS (Sigma-Aldrich) solution (diluted 1:7 in ethanol) for 10 min at 37^{o}C. Reaction was stopped with addition of 475 µl of quenching solution (4.3 M β-mercaptoethanol/0.3 M sodium acetate) and RNAs were precipitated overnight at -80^{o}C. The in vivo DMS modification of RNA from stationary phase cultures was performed essentially as described (Benito et al., 2000; Brunel & Romby, 2000). Primer extension reactions were carried out using a [γ-^{32}P]-5'-end labelled MicA-DMS LNA^{TM} primer (Exiqon) and the Transcriptor reverse transcriptase (Roche). After RNA alkaline hydrolysis, cDNA was resuspended in 6µl formamide loading

buffer. Samples were analysed on 6% or 8% polyacrylamide/7M urea gels run in TBE 1x buffer. The lead acetate cleavages were carried out as described (Salvail et al., 2010) with addition of 5mM PbAc (Sigma-Aldrich) to renatured 5'-end-labelled MicA RNAs in structure buffer 1x (Ambion) supplemented with 0.1mg/ml of yeast RNA. Samples were collected after incubation for 0.5, 1 or 2 min and reactions were stopped by addition of 10 µl of loading buffer II (Ambion). In line probing was performed as described (Regulski & Breaker, 2008). Samples were fractionated on 10% polyacrylamide/7M urea gels run in TBE 1x buffer. Gels were dried and exposed on the phosphor screen.

Enzymatic probing

Ribonucleases T1 (0.01U) and RNase A (0.01U) (Ambion) were incubated with the RNA for 15 min at 37^{o}C following manufacturer's instructions. Before use, 5'-end labelled RNAs were renatured in structure buffer 1x (Ambion). Unfolded RNAs were prepared in sequencing buffer 1x and a ladder of G-specific cleavages was obtained upon RNase T1 incubation. Alkaline ladders correspond to incubation of the RNA in the alkaline hydrolysis buffer for 15 min at 90^{o}C. Reactions were stopped by adding 10 µl of loading buffer II (Ambion). Samples were then fractionated on 10% polyacrylamide/7M urea gels run in TBE 1x buffer. Gels were dried and exposed on the phosphor screen.

RNase III cleavage assay of MicA RNA

Reactions were performed using 1000 µM RNase III purified protein (kindly provided by Allen Nicholson) and radiolabeled [α-^{32}P]-UTP MicA-WT RNA as substrate. RNase III reaction buffer consisted of 160 mM NaCl, 30 mM Tris–HCl

(pH 8), 0.1 mM EDTA, 0.1 mM DTT and 10mM $MgCl_2$ (Amarasinghe et al., 2001). Addition of the enzyme started the reaction and samples were collected at different timepoints. Incubation was performed at 37ºC. Reactions were stopped by the addition of formamide loading buffer supplemented with 20 mM EDTA. Reaction products were resolved in a 15% polyacrylamide/7 M urea gel. Signals were visualized by PhosphorImaging and analysed using the ImageQuant software (Molecular Dynamics).

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References

Abe H, Aiba H. 1996. Differential contributions of two elements of rho-independent terminator to transcription termination and mRNA stabilization. *Biochimie 78*:1035-1042.

Afonyushkin T, Vecerek B, Moll I, Bläsi, Kaberdin VR. 2005. Both RNase E and RNase III control the stability of *sod*B mRNA upon translational inhibition by the small regulatory RNA RyhB. *Nucleic Acids Res 33*:1678-1689.

Altuvia S, Zhang A, Argaman L, Tiwari A, Storz G. 1998. The *Escherichia coli* OxyS regulatory RNA represses *fhl*A translation by blocking ribosome binding. *EMBO J 17*:6069-6075.

Amarasinghe AK, Calin-Jageman I, Harmouch A, Sun W, Nicholson AW. 2001. *Escherichia coli* ribonuclease III: affinity purification of hexahistidine-tagged enzyme and assays for substrate binding and cleavage. *Methods Enzymol 342*:143-158.

Andrade JM, Arraiano CM. 2008. PNPase is a key player in the regulation of small RNAs that control the expression of outer membrane proteins. *RNA 14*:543-551.

Andrade JM, Pobre V, Matos AM, Arraiano CM. 2012. The crucial role of PNPase in the degradation of small RNAs that are not associated with Hfq. *RNA 18*:844-855.

Andrade JM, Pobre V, Silva IJ, Domingues S, Arraiano CM. 2009. The role of 3'-5' exoribonucleases in RNA degradation. *Prog Mol Biol Transl Sci 85*:187-229.

Argaman L, Altuvia S. 2000. *fhl*A repression by *OxyS* RNA: kissing complex formation at two sites results in a stable antisense-target RNA complex. *J Mol Biol 300*:1101-1112.

Argaman L, Hershberg R, Vogel J, Bejerano G, Wagner EG, Margalit H, Altuvia S. 2001. Novel small RNA-encoding genes in the intergenic regions of *Escherichia coli*. *Curr Biol 11*:941-950.

Arraiano CM, Andrade JM, Domingues S, Guinote IB, Malecki M, Matos RG, Moreira RN, Pobre V, Reis FP, Saramago M, Silva IJ, Viegas SC. 2010. The critical role of RNA processing and degradation in the control of gene expression. *FEMS Microbiol Rev 34*:883-923.

Arraiano CM, Yancey SD, Kushner SR. 1988. Stabilization of discrete mRNA breakdown products in *ams pnp rnb* multiple mutants of *Escherichia coli* K-12. *J Bacteriol 170*:4625-4633.

Babitzke P, Granger L, Olszewski J, Kushner SR. 1993. Analysis of mRNA decay and rRNA processing in *Escherichia coli* multiple mutants carrying a deletion in RNase III. *Journal of bacteriology 175*:229-239.

Balbontín R, Fiorini F, Figueroa-Bossi N, Casadesus J, Bossi L. 2010. Recognition of heptameric seed sequence underlies multi-target regulation by RybB small RNA in *Salmonella enterica*. *Mol Microbiol 78*:380-394.

Beisel CL, Storz G. 2010. Base pairing small RNAs and their roles in global regulatory networks. *FEMS Microbiol Rev 34*:866-882.

Beisel CL, Updegrove TB, Janson BJ, Storz G. 2012. Multiple factors dictate target selection by Hfq-binding small RNAs. *EMBO J 31*:1961-1974.

Benito Y, Kolb FA, Romby P, Lina G, Etienne J, Vandenesch F. 2000. Probing the structure of RNAIII, the Staphylococcus aureus agr regulatory RNA, and identification of the RNA domain involved in repression of protein A expression. *RNA 6*:668-679.

Bernhart SH, Hofacker IL, Will S, Gruber AR, Stadler PF. 2008. RNAalifold: improved consensus structure prediction for RNA alignments. *BMC bioinformatics 9*:474.

Bernstein JA, Khodursky AB, Lin PH, Lin-Chao S, Cohen SN. 2002. Global analysis of mRNA decay and abundance in *Escherichia coli* at single-gene resolution using two-color fluorescent DNA microarrays. *Proc Natl Acad Sci U S A 99*:9697-9702.

Bossi L, Figueroa-Bossi N. 2007. A small RNA downregulates LamB maltoporin in *Salmonella*. *Mol Microbiol 65*:799-810.

Bouvier M, Sharma CM, Mika F, Nierhaus KH, Vogel J. 2008. Small RNA binding to 5' mRNA coding region inhibits translational initiation. *Mol Cell 32*:827-837.

Brunel C, Romby P. 2000. Probing RNA structure and RNA-ligand complexes with chemical probes. *Methods Enzymol 318*:3-21.

Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD. 2003. Multiple sequence alignment with the Clustal series of programs. *Nucl Acids Res 31*:3497-3500.

Coornaert A, Lu A, Mandin P, Springer M, Gottesman S, Guillier M. 2010. MicA sRNA links the PhoP regulon to cell envelope stress. *Mol Microbiol 76*:467-479.

Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A 97*:6640-6645.

Davidson EA, Ellington AD. 2005. Engineering regulatory RNAs. *Trends in biotechnology 23*:109-112.

Desnoyers G, Morissette A, Prévost K, Massé E. 2009. Small RNA-induced differential degradation of the polycistronic mRNA *iscRSUA*. *EMBO J 28*:1551-1561.

Durand S, Storz G. 2010. Reprogramming of anaerobic metabolism by the *FnrS* small RNA. *Mol Microbiol 75*:1215-1231.

Ferrara S, Brugnoli M, De Bonis A, Righetti F, Delvillani F, Deho G, Horner D, Briani F, Bertoni G. 2012. Comparative profiling of *Pseudomonas aeruginosa* strains reveals differential expression of novel unique and conserved small RNAs. *PloS One 7*:e36553.

Folichon M, Arluison V, Pellegrini O, Huntzinger E, Régnier P, Hajnsdorf E. 2003. The poly(A) binding protein Hfq protects RNA from RNase E and exoribonucleolytic degradation. *Nucleic acids research 31*:7302-7310.

Gogol EB, Rhodius VA, Papenfort K, Vogel J, Gross CA. 2011. Small RNAs endow a transcriptional activator with essential repressor functions for single-tier control of a global stress regulon. *Proc Natl Acad Sci U S A 108*:12875-12880.

Guillier M, Gottesman S. 2008. The 5' end of two redundant sRNAs is involved in the regulation of multiple targets, including their own regulator. *Nucleic Acids Res 36*:6781-6794.

Hajnsdorf E, Boni IV. 2012. Multiple activities of RNA-binding proteins S1 and Hfq. *Biochimie 94*:1544-1553.

Kudla G, Lipinski L, Caffin F, Helwak A, Zylicz M. 2006. High guanine and cytosine content increases mRNA levels in mammalian cells. *PLoS Biol 4*:e180.

Le Derout J, Boni IV, Régnier P, Hajnsdorf E. 2010. Hfq affects mRNA levels independently of degradation. *BMC Mol Biol 11*:17.

Liang JC, Bloom RJ, Smolke CD. 2011. Engineering biological systems with synthetic RNA molecules. *Mol Cell 43*:915-926.

Link TM, Valentin-Hansen P, Brennan RG. 2009. Structure of *Escherichia coli* Hfq bound to polyriboadenylate RNA. *Proc Natl Acad Sci U S A 106*:19292-19297.

Lioliou E, Romilly C, Romby P, Fechter P. 2010. RNA-mediated regulation in bacteria: from natural to artificial systems. *New biotechnology 27*:222-235.

Liu JM, Camilli A. 2010. A broadening world of bacterial small RNAs. *Curr Opin Microbiol 13*:18-23.

Moll I, Afonyushkin T, Vytvytska O, Kaberdin VR, Blasi U. 2003. Coincident Hfq binding and RNase E cleavage sites on mRNA and small regulatory RNAs. *RNA 9*:1308-1314.

Morita T, Maki K, Aiba H. 2005. RNase E-based ribonucleoprotein complexes: mechanical basis of mRNA destabilization mediated by bacterial noncoding RNAs. *Genes Dev 19*:2176-2186.

Olejniczak M. 2011. Despite similar binding to the Hfq protein regulatory RNAs widely differ in their competition performance. *Biochemistry 50*:4427-4440.

Otaka H, Ishikawa H, Morita T, Aiba H. 2011. PolyU tail of *rho*-independent terminator of bacterial small RNAs is essential for Hfq action. *Proc Natl Acad Sci U S A 108*:13059-13064.

Papenfort K, Bouvier M, Mika F, Sharma CM, Vogel J. 2010. Evidence for an autonomous 5' target recognition domain in an Hfq-associated small RNA. *Proc Natl Acad Sci U S A 107*:20435-20440.

Papenfort K, Vogel J. 2009. Multiple target regulation by small noncoding RNAs rewires gene expression at the post-transcriptional level. *Research in microbiology 160*:278-287.

Peer A, Margalit H. 2011. Accessibility and evolutionary conservation mark bacterial small-RNA target-binding regions. *Journal of bacteriology 193*:1690-1701.

Prévost K, Desnoyers G, Jacques JF, Lavoie F, Massé E. 2011. Small RNA-induced mRNA degradation achieved through both translation block and activated cleavage. *Genes Dev 25*:385-396.

Rasmussen AA, Eriksen M, Gilany K, Udesen C, Franch T, Petersen C, Valentin-Hansen P. 2005. Regulation of *omp*A mRNA stability: the role of a small regulatory RNA in growth phase-dependent control. *Mol Microbiol 58*:1421-1429.

Régnier P, Hajnsdorf E. 2009. Poly(A)-assisted RNA decay and modulators of RNA stability. *Prog Mol Biol Transl Sci 85*:137-185.

Regulski EE, Breaker RR. 2008. In-line probing analysis of riboswitches. *Methods Mol Biol 419*:53-67.

Rehmsmeier M, Steffen P, Hochsmann M, Giegerich R. 2004. Fast and effective prediction of microRNA/target duplexes. *RNA 10*:1507-1517.

Reynolds R, Chamberlin MJ. 1992. Parameters affecting transcription termination by *Escherichia coli* RNA. II. Construction and analysis of hybrid terminators. *J Mol Biol 224*:53-63.

Rice JB, Vanderpool CK. 2011. The small RNA *SgrS* controls sugar-phosphate accumulation by regulating multiple PTS genes. *Nucleic Acids Res 39*:3806-3819.

Salvail H, Lanthier-Bourbonnais P, Sobota JM, Caza M, Benjamin JA, Mendieta ME, Lepine F, Dozois CM, Imlay J, Masse E. 2010. A small RNA promotes siderophore production through transcriptional and metabolic remodeling. *Proc Natl Acad Sci U S A 107*:15223-15228.

Sauer E, Schmidt S, Weichenrieder O. 2012. Small RNA binding to the lateral surface of Hfq hexamers and structural rearrangements upon mRNA target recognition. *Proc Natl Acad Sci U S A 109*:9396-9401.

Sauer E, Weichenrieder O. 2011. Structural basis for RNA 3'-end recognition by Hfq. *Proc Natl Acad Sci U S A*.

Schumacher MA, Pearson RF, Moller T, Valentin-Hansen P, Brennan RG. 2002. Structures of the pleiotropic translational regulator Hfq and an Hfq-RNA complex: a bacterial Sm-like protein. *EMBO J 21*:3546-3556.

Sharma CM, Darfeuille F, Plantinga TH, Vogel J. 2007. A small RNA regulates multiple ABC transporter mRNAs by targeting C/A-rich elements inside and upstream of ribosome-binding sites. *Genes Dev 21*:2804-2817.

Sharma CM, Vogel J. 2009. Experimental approaches for the discovery and characterization of regulatory small RNA. *Curr Opin Microbiol 12*:536-546.

Shinhara A, Matsui M, Hiraoka K, Nomura W, Hirano R, Nakahigashi K, Tomita M, Mori H, Kanai A. 2011. Deep sequencing reveals as-yet-undiscovered small RNAs in *Escherichia coli*. *BMC genomics 12*:428.

Tijerina P, Mohr S, Russell R. 2007. DMS footprinting of structured RNAs and RNA-protein complexes. *Nature protocols 2*:2608-2623.

Udekwu KI, Darfeuille F, Vogel J, Reimegard J, Holmqvist E, Wagner EG. 2005. Hfq-dependent regulation of *Omp*A synthesis is mediated by an antisense RNA. *Genes Dev 19*:2355-2366.

Udekwu KI, Wagner EG. 2007. Sigma E controls biogenesis of the antisense RNA *Mic*A. *Nucleic acids research 35*:1279-1288.

Valentin-Hansen P, Eriksen M, Udesen C. 2004. The bacterial Sm-like protein Hfq: a key player in RNA transactions. *Mol Microbiol 51*:1525-1533.

Viegas SC, Silva IJ, Saramago M, Domingues S, Arraiano CM. 2011. Regulation of the small regulatory RNA MicA by ribonuclease III: a target-dependent pathway. *Nucleic Acids Res 39*:2918-2930.

Vogel J, Luisi BF. 2011. Hfq and its constellation of RNA. *Nature reviews Microbiology 9*:578-589.

Wang RF, Kushner SR. 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. *Gene 100*:195-199.

Zhang A, Wassarman KM, Ortega J, Steven AC, Storz G. 2002. The Sm-like Hfq protein increases *OxyS* RNA interaction with target mRNAs. *Mol Cell 9*:11-22.

Ziolkowska K, Derreumaux P, Folichon M, Pellegrini O, Régnier P, Boni IV, Hajnsdorf E. 2006. Hfq variant with altered RNA binding functions. *Nucleic Acids Res 34*:709-720.

Zuker M. 2003. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res 31*:3406-3415.

Supplemental Data

Supplemental References

Figure S1

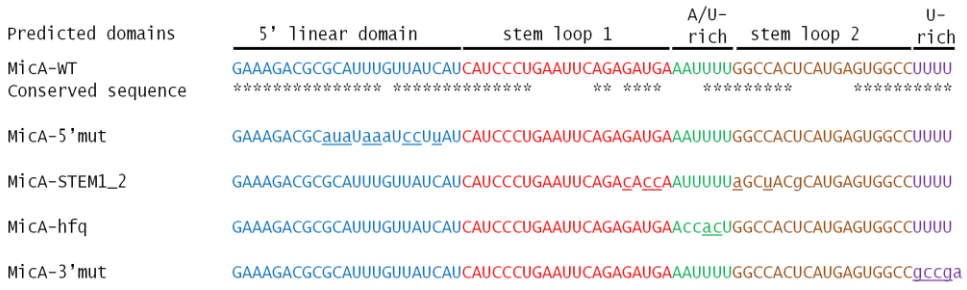


Figure S1. Identification of the nucleotide changes introduced in the synthetic MicA variants

E. coli MicA wild-type sequence is indicated on top and the mutated MicA variants are shown below. Designation of each MicA variant is indicated on the left of each sequence. A multiple alignment of MicA in several eubacteria (see Figure 1) identified the conserved nucleotides (*) in MicA sequence. A color-code was used to better scheme the domains of MicA: the 5' linear domain (blue), the stem-loop 1 (red), the Hfq-binding site A/U-rich sequence (green), the stem-loop 2 (brown) and the 3' poly(U) terminator tail (purple). Mutated nucleotides are shown in lowercase; if conserved, the residue is also underlined.

Figure S2

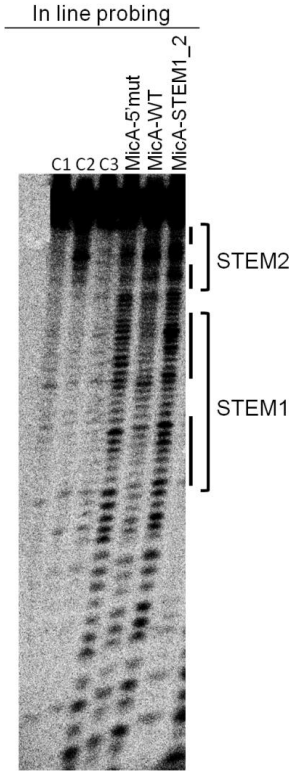


Figure S2. In line probing analysis of MicA RNAs
5'-end labelled MicA-RNA was prepared in 50 mM Tris pH8, 20 mM MgCl2 and 100 mM KCl. In line probing reactions (Regulski & Breaker, 2008) were carried out for 48h at room temperature and were stopped with addition of loading buffer II (Ambion). Untreated controls (C1: MicA-5'mut; C2: MicA-WT; C3: MicA-STEM1_2). Alkaline ladders and RNase T1 ladders were run on the same gel (data not shown). Thick lines on the side of the lanes represent the position of stem-loop arms. Samples were fractionated on 10% polyacrylamide/7M urea gels run in TBE 1x buffer.

Figure S3

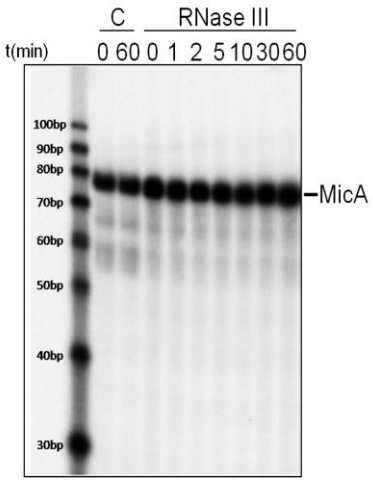


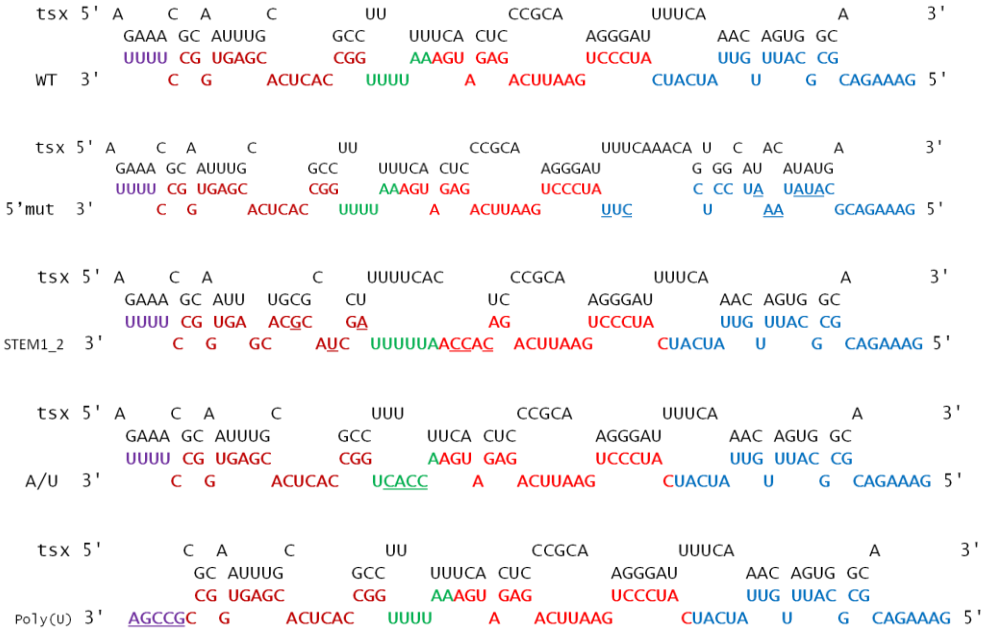
Figure S3. *In vitro* RNase III cleavage assay

In vitro activity assay (Amarasinghe et al., 2001) with 1000 μM purified RNase III and radioactive labelled wild-type MicA RNA as substrate. Addition of RNase III started the reaction and samples were taken across time. A parallel reaction without the addition of enzyme was used as control. A size marker is shown on the left of the gel.

Figure S4

A. MicA interactions with *ompA* mRNA

B. MicA interactions with *tsx* mRNA



C. MicA interactions with *ecn*B mRNA

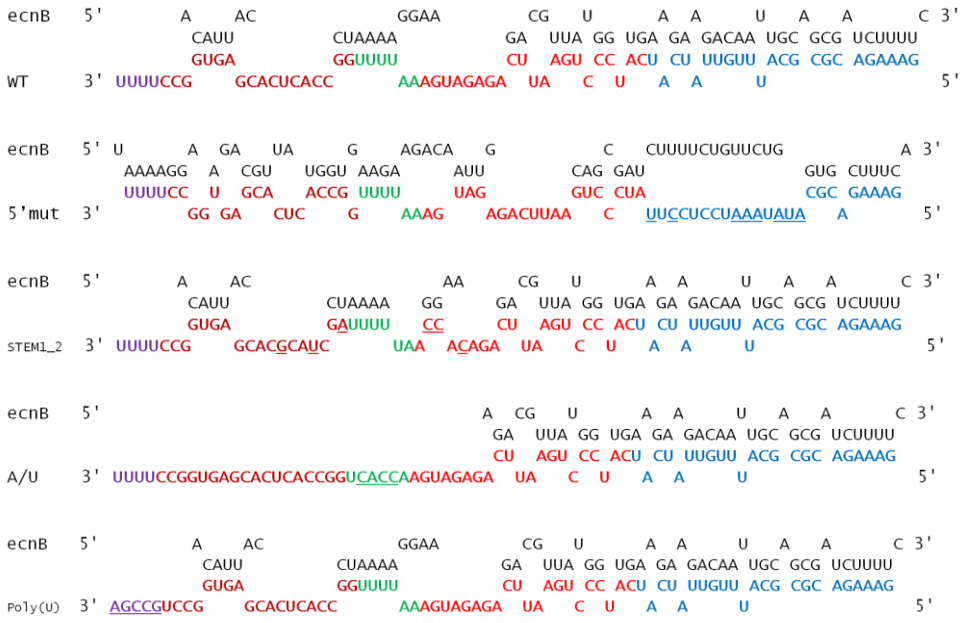


Figure S4. Predicted Interactions between MicA-WT and the synthetic MicA variants with *omp*A, *tsx* and *ecn*B mRNAs

The RNAhybrid software (Rehmsmeier et al., 2004) was used to predict interactions between MicA forms and target mRNAs, using the default parameters. A segment of the 5' end of each target mRNA was chosen as previously described (Gogol et al., 2011). The complete sequences of all MicA variants were used. Nucleotide changes are shown underlined. For representative purposes, the predicted domains of MicA are color-coded: the 5' linear domain (blue), the stem-loop 1 (red), the Hfq-binding site A/U-rich sequence (green), the stem-loop 2 (brown) and the 3' poly(U) terminator tail (purple).

Figure S5

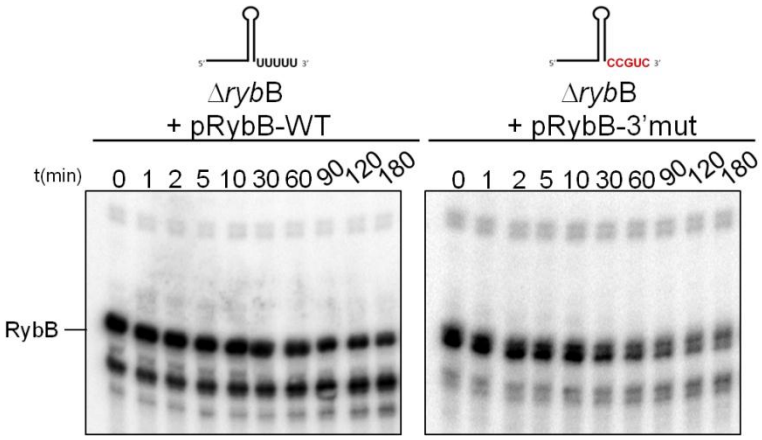


Figure S5. Mutagenesis of the 3' end U-rich tail of RybB
Decay measurement of the RybB. Deleted *ryb*B cells (Δ*ryb*B) were transformed with a plasmid expressing either the wild-type copy (pRybB-WT) or a RybB variant in which the 3' U-rich tail was modified to a CG-rich sequence (pRybB-3'mut). Total RNA was extracted from stationary phase cultures.

Figure S6

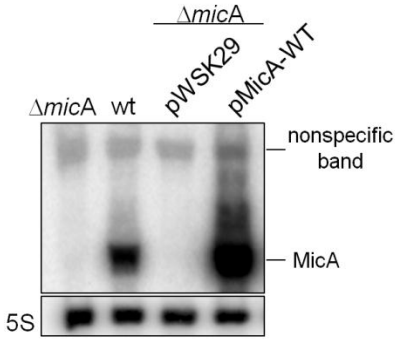


Figure S6. Northern blot analysis of MicA RNA
A band denoted nonspecific is detected on Northern blot analysis from RNA extracted from the wild-type (wt) and Δ*mic*A strains (transformed or not with plasmid pMicA-WT) when using the MicA riboprobe described in *Materials and Methods.*

Supplemental References

Amarasinghe AK, Calin-Jageman I, Harmouch A, Sun W, Nicholson AW (2001) *Escherichia coli* ribonuclease III: affinity purification of hexahistidine-tagged enzyme and assays for substrate binding and cleavage. Methods Enzymol 342: 143-158.

Gogol EB, Rhodius VA, Papenfort K, Vogel J, Gross CA (2011) Small RNAs endow a transcriptional activator with essential repressor functions for single-tier control of a global stress regulon. Proc Natl Acad Sci U S A 108: 12875-12880.

Regulski EE, Breaker RR (2008) In-line probing analysis of riboswitches. Methods Mol Biol 419: 53-67.

Rehmsmeier M, Steffen P, Hochsmann M, Giegerich R (2004) Fast and effective prediction of microRNA/target duplexes. RNA 10: 1507-1517.

Chapter 4

Analysis of the global role of Escherichia coli exoribonucleases by RNA-Seq

Keywords: RNase II/ RNase R/ PNPase/ RNA-Seq/ Exoribonucleases/ RNA degradation

This chapter contains unpublished data.

For this chapter I compiled several RNA-Seq results that are not published. Moreover, these results are not yet experimentally validated so this chapter only has results from bioinformatics analysis.

I planned the experiments and did the bioinformatics analysis of the data and wrote the chapter.

The analysis of RNA-Seq data is computationally very demanding and for this reason I did several bioinformatics courses:

EMBO Practical course – "**Computational RNA Biology**", 2010
The Gulbenkian Training Programme in Bioinformatics – "**RNA Bioinformatics**", 2010
The Gulbenkian Training Programme in Bioinformatics – "**Bioinformatics using Python for Biologists**", 2011
EMBO Practical Course - "**MicroRNA-profiling: From in-situ hybridization to next-generation sequencing**", 2011
The Gulbenkian Training Programme in Bioinformatics – "**Transcriptome Assembly, Automatic Functional Annotation and Data Mining**", 2012
The Gulbenkian Training Programme in Bioinformatics – "**Bioinformatics and Functional Genomics using R**", 2012

With these courses I learned the basic on bioinformatics adapted to the field of RNA, acquired some programming skills and the knowledge to analyse the Next-Generation Sequencing data.

Table of Contents

Abstract

Exoribonucleases are crucial for RNA degradation. In *E. coli* there are three main exoribonucleases responsible for the exodegradative activity in the cell, RNase II, RNase R and PNPase. RNase II and RNase R are 3'-5' hydrolytic exoribonucleases and belong to the RNase II family of enzymes. PNPase is a 3'-5' phosphorolytic exoribonuclease from the PDX family of enzymes. In this work we analysed the roles of these exoribonucleases in both exponential and stationary phase, using RNA-Seq, the currently most advanced technology for whole-transcriptome analysis. The adaptation to stationary phase involves major rearrangements of the *E. coli* transcriptome. Therefore we also analysed the transcriptomic changes between the exponential and stationary phase cells.

We identified more than 1000 transcripts that were differentially expressed when we compared exponential with stationary growth phase. Most of these transcripts can be clustered into functional categories related to *E. coli* membrane and transport. We also found that the three exoribonucleases have different roles depending on the growth phase.

In exponential phase, the deletion of RNase II significantly affects 187 transcripts, deletion of RNase R affects 202 transcripts and deletion of PNPase affects 226 transcripts. Interestingly most of the transcripts affected by RNase II belong to flagellar assembly and motility. On the other hand, the transcripts affected by RNase R can be clustered into many different functional categories but it seems that RNase R might have an important role in anaerobic respiration. PNPase seems to overlap both RNase II and RNase R roles. In addition, PNPase also appears to have a more relevant role in the control of stable RNAs.

In stationary phase, RNase R is the exoribonuclease that seems to have the predominant role. The RNase R deletion significantly affects almost 700

transcripts while RNase II deletion only affects 117 transcripts and PNPase deletion affects 228 transcripts. Nevertheless, PNPase seems to be the most important exoribonuclease involved in the degradation of sRNAs.

Analysis of an RNase II and RNase R double mutant shows that the cell is somehow able to compensate the double mutation. The transcripts affected by the double mutation are similar to the ones affected by the single mutants, but the double mutant is not an “addition” of the single mutants.

In conclusion, our data suggests that the three exoribonucleases have different roles in the cell, even though there is some overlap between their functions. Moreover they have different roles depending on the growth phase.

Introduction

In stationary phase, *E. coli* cells undergo several physical and morphological adaptations (Hengge-Aronis, 1999). This adaptation requires an extensive adjustment of the gene expression. Therefore, many genes expressed in exponential phase are repressed in stationary phase, while other set of genes becomes highly expressed in stationary phase (Ishihama, 1997). These changes from exponential to stationary phase depend not only on the transcription, but also on the degradation of RNAs. In *E. coli* there are three main exoribonucleases (RNase II, RNase R and PNPase) involved in RNA degradation (Andrade et al., 2009b; Arraiano et al., 2010).

RNase II is a hydrolytic exoribonuclease that processively degrades RNA in the 3'-5' direction. RNase II is sensitive to secondary structures, and the enzyme is known to stall before it reaches a double-stranded region (Cannistraro & Kennell, 1999; Spickler & Mackie, 2000). Although RNase II degrading activity is sequence-independent, its favourite substrate is poly(A) tails. RNase II rapidly degrades poly(A) tails but it halts if it finds secondary structures such as the Rho-independent terminators. Therefore, the degradation of polyadenylated stretches by RNase II can paradoxically protect some RNAs because the other exoribonucleases (PNPase and RNase R) need a short poly(A) tail as a "toehold" in order to degrade secondary structures (Hajnsdorf et al., 1994; Pepe et al., 1994; Coburn & Mackie, 1996; Marujo et al., 2000; Mohanty & Kushner, 2000; Folichon et al., 2005).

RNase R is another 3'-5' hydrolytic exoribonuclease from the RNase II family of exoribonucleases (Cheng & Deutscher, 2002; Vincent & Deutscher, 2006). RNase R can easily degrade highly structured RNAs and was shown to be a key enzyme involved in the degradation of polyadenylated RNA (Cheng & Deutscher,

2002, 2003; Andrade et al., 2009a; Awano et al., 2010). RNase R is also a critical enzyme involved in RNA and protein quality control, namely in the degradation of defective tRNAs and rRNAs and is involved in degradation during *trans*-translation (Cairrão et al., 2003; Cheng & Deutscher, 2003; Vincent & Deutscher, 2006; Awano et al., 2010). The activity of RNase R is modulated according to the growth conditions of the cell and is induced under several stress conditions (Cairrão et al., 2003; Andrade et al., 2006). RNase R is a highly unstable protein in exponential phase, however this protein is stabilized in stationary phase and other stress conditions, leading to its relative increase (Chen & Deutscher, 2010).

Contrarily to RNase II and RNase R, PNPase is a 3'-5' phosphorolytic enzyme. PNPase activity is blocked by double-stranded RNA structures (Spickler & Mackie, 2000), but it can form complexes with other proteins allowing it to degrade through extensive structured RNA (Arraiano et al., 2010). PNPase is not only a degradative enzyme, but is also capable of adding heteropolymeric tails (Mohanty & Kushner, 2000 ; Slomovic et al., 2008). In exponentially growing *E. coli*, more than 90% of the transcripts are polyadenylated and Rho-dependent transcription terminators were suggested to be modified by the polymerase activity of PNPase (Mohanty & Kushner, 2006).

The role of exoribonucleases has been extensively studied, but there are still many unanswered questions (Arraiano et al., 2010). What is the exact role of these exoribonucleases in the RNA metabolism, which RNAs are substrates for each one of them and how the different exoribonucleases select their substrates are just some of the questions that are still under debate. To try to obtain some answers of which are the preferential targets of each RNase we have used RNA-Seq, the current state-of-the-art technology.

RNA-Seq uses recently developed deep-sequencing technologies. In general, a population of RNA (total or fractionated, for instance the selection of

poly(A)$^+$ species) is converted to a library of cDNA fragments with adaptors attached to one or both ends (Figure 1). Each molecule, with or without amplification, is then sequenced in a high-throughput manner to obtain short sequences from one end (single-end sequencing) or both ends (pair-end sequencing) (Wang et al., 2009). With this technique it is possible to obtain information about the entire transcriptome of any organism or even from microbial communities (metatranscriptomics) (Guell et al., 2011).

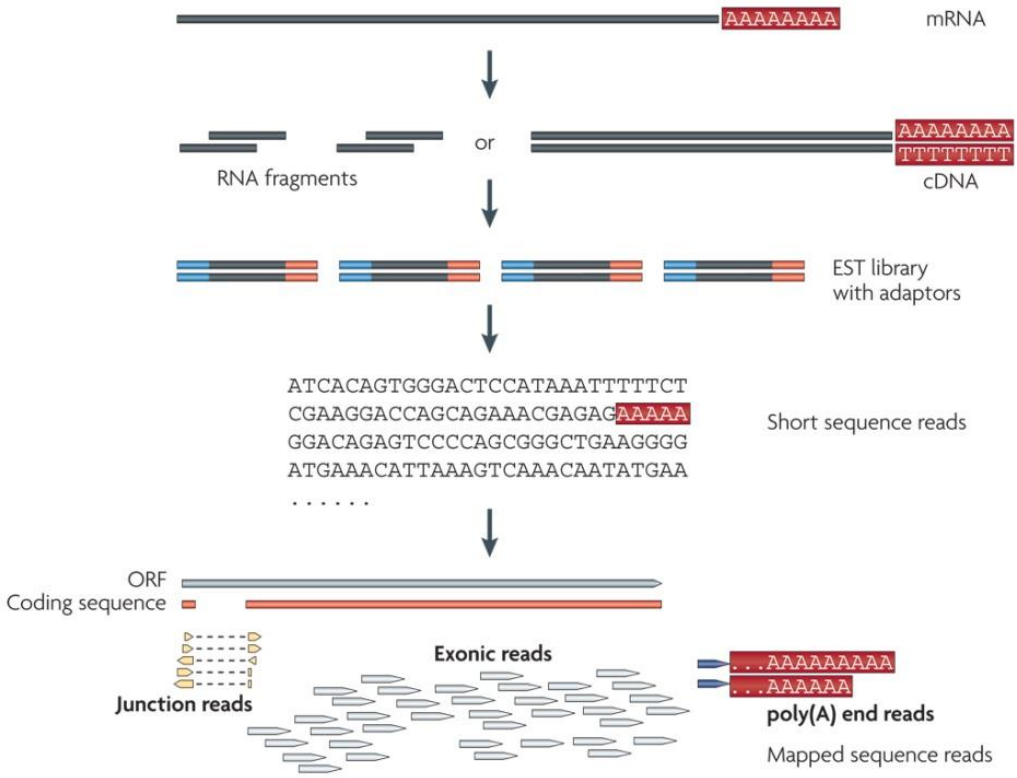


Figure 1 - RNA-Seq experiment
RNAs are first converted into a library of cDNA fragments through RNA fragmentation. Sequencing adaptors (blue) are subsequently added to each cDNA fragment and a short sequence is obtained from each cDNA using high-throughput sequencing technology. The resulting sequence reads are aligned with the reference genome or transcriptome. Figure adapted from (Wang et al., 2009).

RNA-Seq has overcome some of the drawbacks of tiling arrays that has been an extensively used technique for whole-transcriptome profiling (Table 1). The major advantages of RNA-Seq when compared with tiling arrays are the capability to provide single-base resolution and a better signal-to-noise ratio due to a reduced background (Guell et al., 2011). Another major advantage is the fact that RNA-Seq does not require previous knowledge of the genomic sequence and therefore can be used for transcriptome profiling of organisms that are still not sequenced.

Table 1 - Advantages of RNA-Seq compared with other transcriptomics methods

Technology	**Tiling microarray**	**RNA-seq**
Principle	Hybridization	High-throughput sequencing
Resolution	From several to 100 bp	Single base
Throughput	High	High
Reliance on genomic sequence	Yes	No
Background noise	High	Low
Simultaneously map transcribed regions and gene expression	Yes	Yes
Required amount of RNA	High	Low

Nevertheless, RNA-Seq also presents some disadvantages, most of them related with the fact that RNA-Seq is still a very recent technique. The monetary cost of RNA-Seq is still a major limitation although the price is becoming more accessible. An important issue of RNA-Seq is the high amount of information that it provides. Consequently analysing RNA-Seq data is computationally very demanding and requires extensive bioinformatics knowledge and resources. In spite of these, RNA-Seq is a very powerful tool for transcriptome studies. For this reason, in this work we used RNA-Seq to explore the whole transcriptome of *E. coli* mutants for the three exoribonucleases (RNase II, RNase R and PNPase) at exponential and stationary phases to clarify their roles in RNA metabolism.

Results

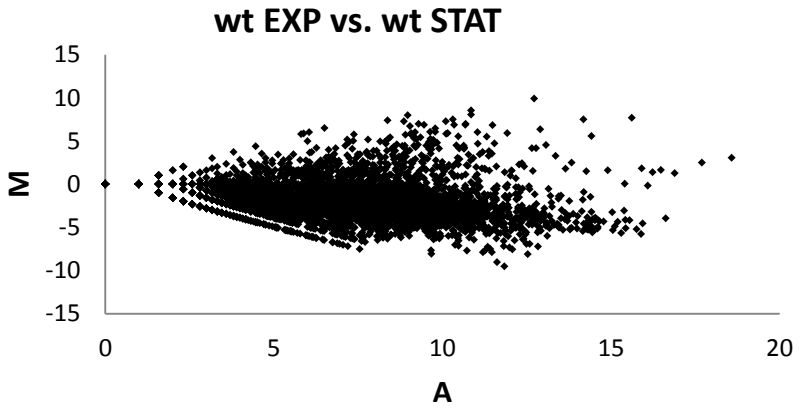
Transcriptome-wide analysis

The RNA levels in the cell are a balance between transcription and degradation rates, therefore when studying transcriptomics is important to understand the degradation pathways involved in the establishment of the RNA levels. Exoribonucleases are at the center of RNA degradation pathways and respond to environmental stimuli, being for example modulated by many factors, such as stress or metabolites (Cairrão et al., 2003; Chen & Deutscher, 2010; Gatewood & Jones, 2010). Interestingly, work from this lab and others have shown that exoribonucleases can be growth-phase regulated. As examples, RNase R levels are increased in stationary phase (Andrade et al., 2006) and PNPase is particularly important in the degradation of sRNAs in stationary phase (Andrade & Arraiano, 2008; Andrade et al., 2012). In general, the RNA decay machinery is suggested to vary along the growth phase. Usually most of the *E. coli* studies are done in exponential phase, however in its natural environment bacteria stay mostly in the stationary phase due to nutrient limitations and several stresses (Kolter et al., 1993). In this work, RNA-seq was used to compare the transcriptome of wild-type cells with mutants for the exoribonucleases RNase II (Δ*rnb*), RNase R (Δ*rnr*) and PNPase (Δ*pnp*) in exponential and stationary phases of growth.

We analysed the RNA-Seq results of all the transcripts in the cell. We plot the fold-change of the transcripts (Figure 2) to obtain an overview of the transcriptomic changes when comparing two samples. Each point in the MA scatterplots corresponds to a transcript. The transcripts with M equal to zero do not change between the two samples that are being compared. On the other hand, transcripts with M above zero are up-regulated while transcripts with M below zero are down-regulated. We observed a high range of the fold-change of the transcripts when comparing wild-type exponential cells with wild-type

stationary cells (Figure 2A). This suggests that many of the transcripts are differentially expressed between exponential and stationary phase. As for the different exoribonucleases mutants the dispersion of the log fold-change is not that high for most of the transcripts (Figure 2B and 2C). However, there are some differences between the different mutants and the wild-type cells. In both exponential and stationary phase the PNPase mutant is the one that presents higher dispersion of the fold change values followed by RNase R mutant, while RNase II scatterplots show low dispersions for most of the transcripts (Figure 2B and 2C). This indicates that PNPase and RNase R have broader effects in the transcripts expression than RNase II.

A



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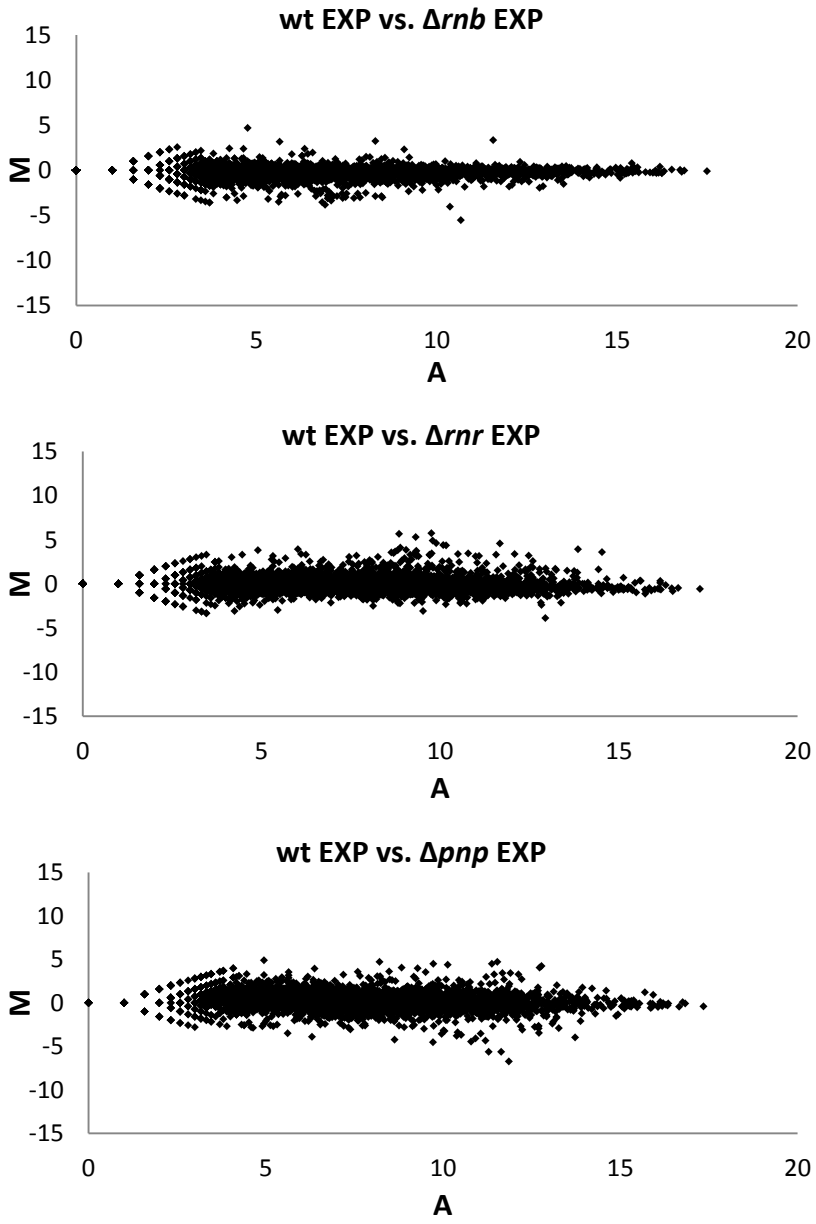

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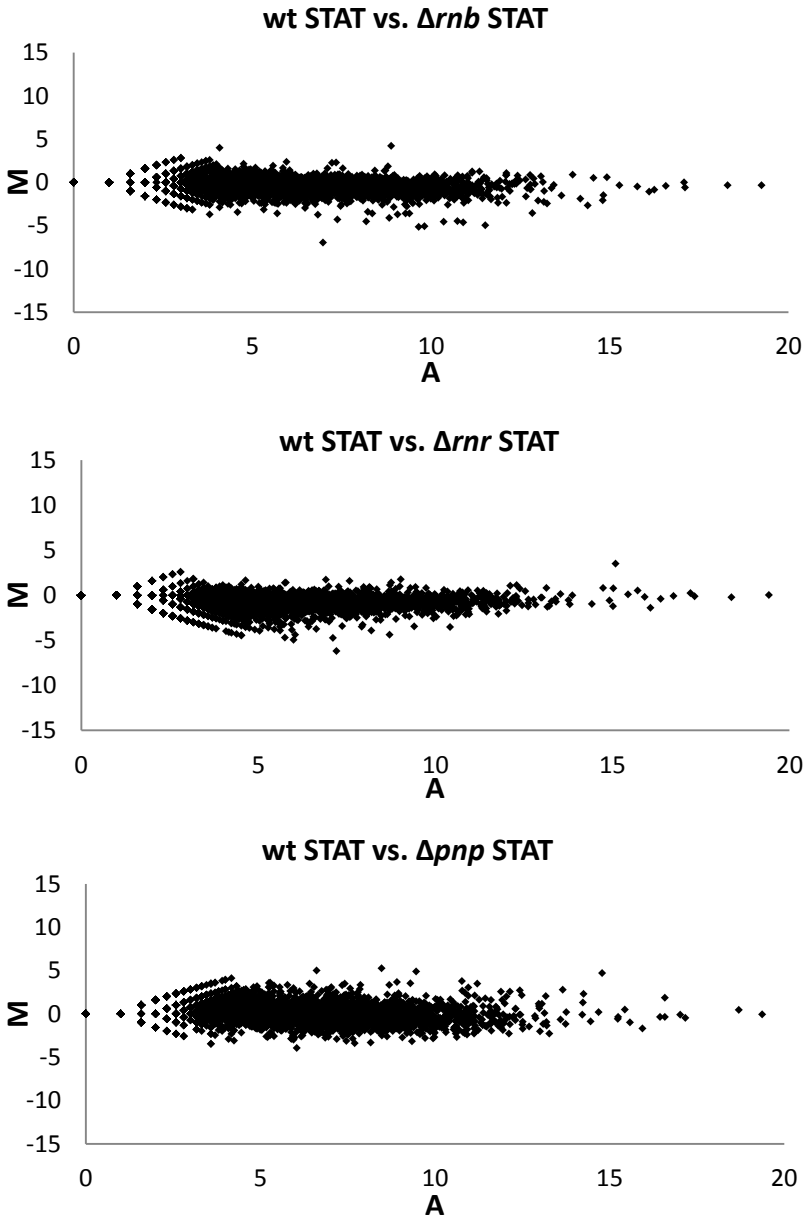


Figure 2 – Overall RNA expression levels. A) MA scatterplot comparing wild-type (wt) in exponential with wild-type in stationary phase. B) MA scatterplot comparing wild-type (wt) with the different exoribonucleases mutants in exponential. C) MA scatterplot comparing wild-type (wt) with the different exoribonucleases in stationary. M is the Log2 of the number of reads of condition 1 divided by the number of reads of condition 2, while A is the Log2 of the sum of the two conditions. For example, M = log2(wt STAT/wt EXP), A = log2(wt STAT + wt EXP).

We also calculated the number of transcripts that were up or down-regulated when comparing the different samples (Table 2). Comparing wild-type Δexponential phase with wild-type stationary phase cells we observed that the vast majority of the transcripts are down-regulated in stationary phase (approximately 85%), comparatively only approximately 14% of transcripts are up-regulated (Table 2). The exoribonucleases are involved in the degradation of RNAs, therefore when comparing an exoribonuclease mutant with the wild-type we would expect to have more up than down-regulated transcripts. Surprisingly, we found many more down-regulated transcripts in all the exoribonucleases mutants when compared to the wild-type, being particularly visible in the Δ*rnb* and Δ*rnr* mutants in stationary phase (Figure 2B and 2C). In exponential phase, the percentage of transcripts that are up-regulated when comparing the Δ*rnb* mutant with the wild-type is lower than the percentage of transcripts that are down-regulated (~29% and ~67% respectively, Table 2). The percentage of down-regulated transcripts is also higher in the Δ*rnr* mutant in both exponential (~54%) and stationary phase (~85%). Only PNPase deletion resulted in more up-regulated than down-regulated transcripts in exponential and stationary phases, but even in the Δ*pnp* mutant there are still a considerable percentage of down-regulated transcripts (Table 2).

Table 2 – Percentage of up and down-regulated transcripts in each of the conditions analysed.

Condition		%Up-regulated transcripts	% Down-regulated transcripts
Wild-type (wt) EXP *vs.* wt STAT		14,19	84,52
Exponential phase	wt *vs.* Δ*rnb*	29,05	66,90
	wt *vs.* Δ*rnr*	41,78	54,49
	wt *vs.* Δ*pnp*	58,95	38,62
Stationary phase	wt *vs.* Δ*rnb*	26,66	67,02
	wt *vs.* Δ*rnr*	9,74	85,42
	wt *vs.* Δ*pnp*	47,85	47,40

The high percentage of down-regulated transcripts in the exoribonucleases mutants might be an indirect consequence of the exoribonucleases deletion, although there are some evidences that some transcripts can be protected instead of degraded by the exoribonucleases (Marujo et al., 2000; Mohanty & Kushner, 2003; De Lay & Gottesman, 2011). These set of results indicate that the role of the exoribonucleases in RNA metabolism is much more complex than previously believed.

Exponential *versus* stationary phase of growth

Next, we used the algorithm Cufflinks to determine the relative abundance of the transcripts and afterwards we use the algorithm Cuffdiff to find significant changes in transcript expression, when comparing two samples (Trapnell et al., 2010). With this approach we were able to identify more than 1000 transcripts that were significantly different between the exponential and stationary wild-type samples (Table S1). These transcripts belong to different functional categories. Most of them are related to *E. coli* membrane and transport, but some transcripts are linked to more specific functional categories like anaerobic respiration, regulation of cell shape and flagellum organization (Figure 3). These results were expected as *E. coli* cells undergo several physical and morphological adaptations when entering the stationary phase (Hengge-Aronis, 1999).

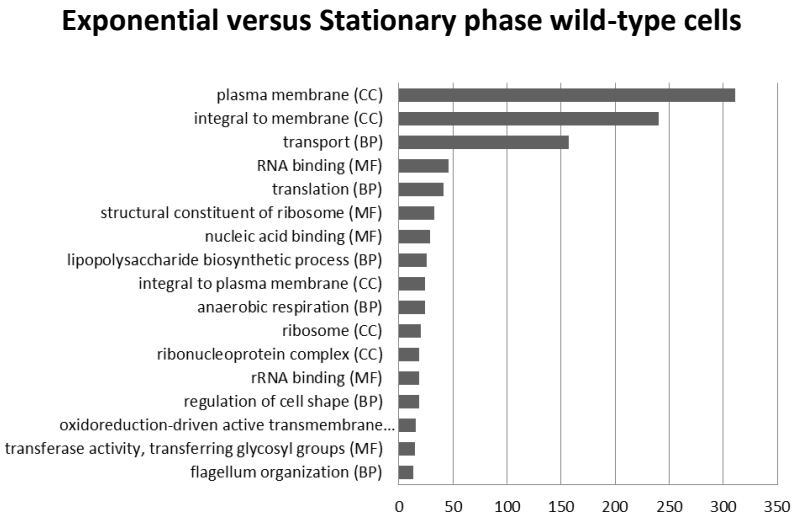


Figure 3 – Transcripts distribution in different functional categories. The differentially expressed transcripts between wild-type exponential cells and wild-type stationary cells are grouped into different functional categories (CC – cellular component, BP – Biological process and MF – metabolic function). Not all the transcripts are represented in this figure. These results were obtained using GeneCodis (Tabas-Madrid et al., 2012).

Small RNAs are important regulators in the cell and their roles are particular important in stationary phase. The GeneCodis program used to cluster the transcripts into the different functional categories does not cluster small non-coding RNAs. Therefore, we analysed the differentially expressed sRNA between exponential and stationary phase. Most of the sRNAs are known to be up-regulated in stationary phase and in fact, the transcript with the biggest fold-change is the *arr*S small RNA (increases from 7 reads in exponential to 6760 reads in stationary). *Arr*S is a sRNA that is an antisense RNA of the *gadE* transcript (central activator of the acid resistance system in *E. coli*). Transcription of *arr*S is induced by acidic growth conditions and in stationary phase (Aiso et al., 2011). This is not the only sRNA that becomes up-regulated in stationary phase. Of all the 1095 transcripts that are differentially expressed 22 are sRNA, of these 18 are up-regulated (Table 3). Although the fold-change of the *ssr*S sRNA (6S RNA) is not as

high as compared with other transcripts, this sRNA is the most expressed sRNA in stationary phase (199689.02 RPKM – Reads Per Kilobase per Million mapped reads). This result is in agreement with other reports that demonstrated that 6S is essential for growth adaptation (Wassarman & Storz, 2000; Geissen et al., 2010) .

Table 3 – sRNAs differentially expressed when comparing exponential with stationary wild-type cells.

	sRNA	sRNA description	RPKM		Reads		Log2 fold change
			wt EXP	wt STAT	wt EXP	wt STAT	
Up-regulated in stationary phase	***arr*S**	Antisense to *gad*E transcript	10,20	7553,18	7	6760	**9,92**
	***rye*A**	SraC small RNA	186,15	22375,34	247	50225	**7,67**
	***rpr*A**	Required for wt production of RpoS in response to osmotic shock	71,13	3268,18	64	3835	**5,91**
	***omr*A**	Involved in regulation of the outer membrane proteins	70,36	2404,54	56	2496	**5,48**
	***mic*F**	Regulates *omp*F; is implicated in resistance to antibiotic drugs	71,54	1218,80	59	1311	**4,47**
	***glm*Y**	Specifically increases synthesis of GlmS	252,67	3484,36	341	6133	**4,17**
	***ryh*B**	Involved in iron homeostasis	64,40	859,38	52	905	**4,12**
	***omr*B**	Involved in regulation of the outer membrane proteins	103,77	1380,79	79	1370	**4,12**
	***ryb*B**	Expression is dependent on the σE	245,97	2081,74	183	2020	**3,46**
	***rdl*B**	Antisense regulatory RNA part of a toxin-antitoxin pair	32,88	232,65	16	173	**3,43**

	***rdl*C**	Antisense regulatory RNA part of a toxin-antitoxin pair	24,98	161,11	13	132	**3,34**
	***gad*Y**	positive regulator of gadX and gadW	67,12	520,62	60	607	**3,34**
	***ssr*S**	6S RNA; is involved in stationary phase regulation of transcription by the σ70	31932,32	199689,02	42911	349981	**3,03**
	***isr*A**	McaS sRNA; it positively regulates flagellar motility and biofilm formation	95,86	499,18	115	781	**2,76**
	***dic*F**	Inhibits cell division	26,93	136,82	16	106	**2,73**
	***rdl*A**	Antisense regulatory RNA part of a toxin-antitoxin pair	66,69	220,44	33	175	**2,41**
	***chi*X**	MicM sRNA; negatively regulates expression of the DpiA/DpiB two-component system	1085,61	4388,11	839	4423	**2,40**
	***ryd*B**	Regulator of RpoS	22,04	54,08	15	48	**1,68**
Up-regulated in Exponential phase	***tff***	T44 predicted small RNA or attenuator	1709,64	83,63	1834	117	**3,97**
	***ryf*A**	Unknown function	45,06	1,50	92	4	**4,52**
	***psr*O**	Unknown function	829,78	19,59	1072	33	**5,02**
	***rtt*R**	Unknown function	314,61	3,61	137	2	**6,10**

RPKM – Reads Per Kilobase per Million, Units used for Next-generation sequencing data.
Reads – Number of reads of a transcript in the different samples.
Fold-change – Calculated from the number of reads of the transcripts (log2 (reads wt STAT/ reads wt EXP)).

Surprisingly, there are 4 sRNAs up-regulated in exponential phase. Not much is known about these sRNAs and more attention should be given to the study of the role of sRNAs in exponential phase.

Considering the importance of ribonucleases in RNA metabolism we also analysed their expression comparing exponential and stationary phases of growth. Surprisingly, the exoribonucleases transcripts here studied were all found to be down-regulated in stationary phase (Table 4).

Table 4 – Ribonuclease RNA levels and fold-change in exponential and stationary wild-type cells.

mRNA	RPKM		Reads		Log fold change
	wt EXP	wt STAT	wt EXP	wt STAT	
rne	253,60	11,59	4733	282	-4,07
rnc	247,87	19,82	1045	109	-3,26
rnb	139,58	8,44	1597	125	-3,68
rnr	513,14	21,29	7373	399	-4,21
pnp	1011,22	39,96	12747	657	-4,28

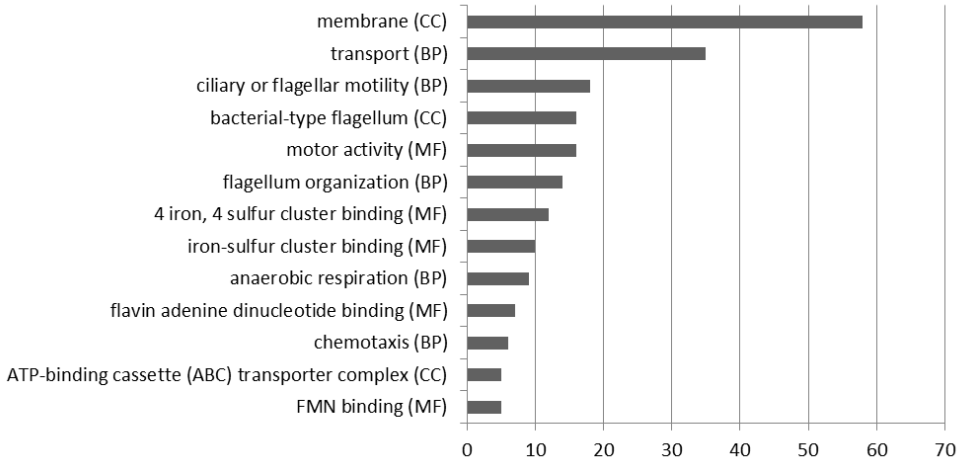
The results obtained with RNase R seemed paradoxical, as RNase R protein levels are known to increase in stationary phase (Andrade et al., 2006). However, further work demonstrated that this is due to a stabilization of the protein and not to an increase in the mRNA levels (Chen & Deutscher, 2010; Liang & Deutscher, 2012). To determine if the transcriptomics data is comparable with the protein levels of the different ribonucleases, both in exponential and stationary phase, proteomics studies should be performed to complement our RNA-seq data.

Exoribonucleases in exponential phase

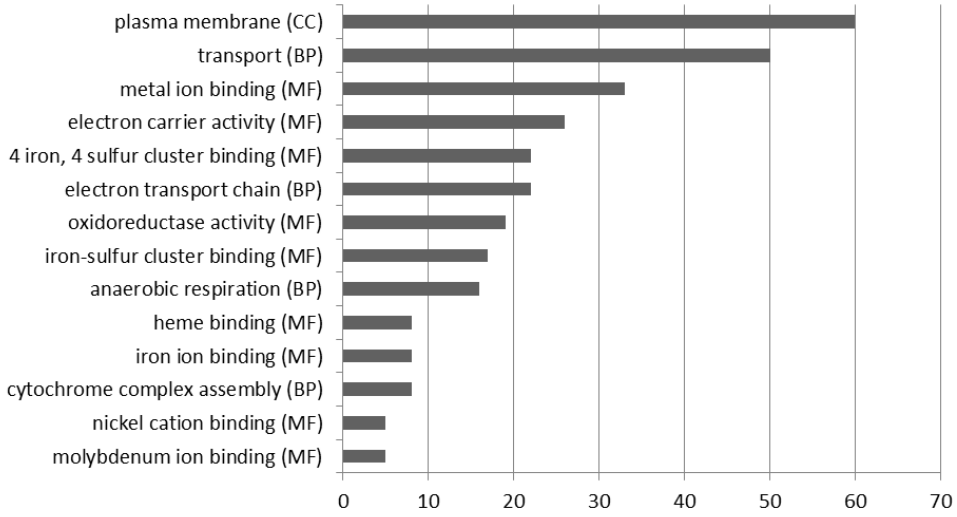
To determine the role of the three different exoribonucleases in exponential phase we used Cufflinks to compare the Δ*rnb*, Δ*rnr* and Δ*pnp* mutants with the wild-type. We then cluster the list of differentially expressed transcripts into different functional categories using GeneCodis (see materials and methods).

In the RNase II mutant, 187 transcripts are differentially expressed when compared with the wild-type (Table S2). Most of the transcripts that are affected by an RNase II deletion in exponential phase are related to flagellar assembly and motility (Figure 4A). Moreover, all the transcripts that are affected by the RNase II deletion and that belong to the Kegg pathway of flagellar assembly (Figure S1) are down-regulated (Table S2). Interestingly, the transcript that is most up-regulated in the Δ*rnb* mutant in exponential phase with a log2 fold change of 3.36 is Antigen-43 (*flu)* known to promote aggregation and inhibit bacterial motility (Ulett et al., 2006). Therefore, the RNase II deletion global effects on flagellar assembly can be an indirect effect due to the high levels of antigen-43 in the Δ*rnb* mutant.

A – RNase II mutant



B – RNase R mutant



C – PNPase mutant

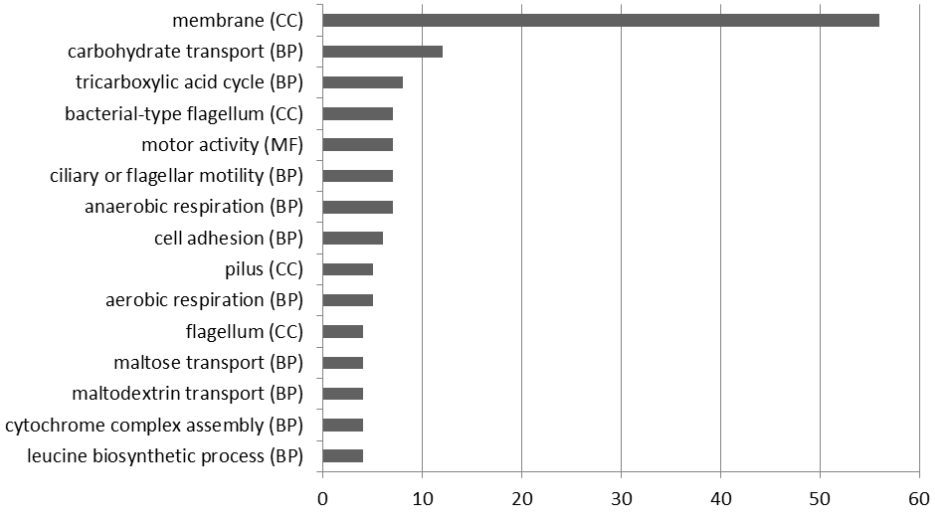


Figure 4 – Differentially expressed transcripts distribution in different functional categories. A) The differentially expressed transcripts between Δ*rnb* and wild-type in exponential phase. B) The differentially expressed transcripts between Δ*rnr* and wild-type in exponential phase. C) The differentially expressed transcripts between Δ*pnp* and wild-type in exponential phase. Transcripts were grouped into different functional categories (CC – cellular component, BP – Biological process and MF – metabolic function). Not all the transcripts are represented in this figure. These results were obtained using GeneCodis (Tabas-Madrid et al., 2012).

The deletion of RNase R affects the expression of 202 transcripts involved in many metabolic function and biological processes such as metal-ion binding, electron transport chain and oxidoreductase activity (Figure 4B). When analysing the transcripts with the highest fold-change we observed that most of these transcripts are from anaerobic respiration and all are up-regulated (Table S3). One of these transcripts is the small RNA FnrS (log2 fold change of 2.35). FnrS reprograms metabolism in response to anaerobiosis and its expression was observed only under anaerobic growth conditions (Durand & Storz, 2010). The fact that FnrS expression levels are increased in the Δ*rnr* mutant under normal aerobic conditions indicates that RNase R regulates this sRNA and might even have an important role in the respiration process of *E. coli*.

As for the PNPase mutant, there are 226 differentially expressed transcripts (Table S4). We can cluster these transcripts into functional categories like carbohydrate transport and ciliary or flagellar motility (Figure 4C). However, the number of transcripts grouped into the different functional categories is low, indicating that PNPase affects many different pathways in the cell but does not affect many transcripts of each pathway. Comparing the Δ*pnp*, the Δ*rnb* and Δ*rnr* differentially expressed transcripts in exponential phase we observed that there is an overlap in the functional categories of the three exoribonucleases (Figure 4). The deletion of any of the exoribonucleases appears to affect transcripts from the anaerobic respiration, although deletion of RNase R affects more transcripts involved in anaerobic respiration than deletion of RNase II or PNPase. Also in the Δ*rnb* and Δ*pnp* mutants the transcripts of the anaerobic respiration are down-regulated contrarily to what happens in the Δ*rnr* mutant (Tables S2, S3 and S4). Another functional category in which there is an overlap is the ciliary and flagellar motility. In both Δ*rnb* and Δ*pnp* mutants the transcripts are down-regulated, but deletion of PNPase seems to have a fewer impact than the deletion of RNase II (Table S2 and S4).

A striking difference between Δ*pnp* mutant and Δ*rnb* or Δ*rnr* mutants is the fact that many of the differentially expressed transcripts in Δ*pnp* mutant are tRNAs, rRNAs and sRNAs. Although in Δ*rnb* and Δ*rnr* these classes of RNAs were also present they were only a minority. The total number of tRNAs, rRNAs and sRNAs in Δ*rnb* is 11, in Δ*rnr* is 13 while in the Δ*pnp* there are 53. This result suggests that PNPase has a very important role in the regulation of these RNAs.

As mentioned before, there seems to be an overlap between these three exoribonucleases. To determine exactly how extensive is this overlapping we compared the differentially expressed transcripts of the three exoribonucleases to determine which were affected only by one of the exoribonucleases and those that were affected by more than one exoribonuclease (Figure 5). In exponential phase a total of 484 transcripts are being differentially expressed by the three exoribonucleases. Of these, 29 transcripts are common to the three exoribonucleases. RNase II and RNase R belong to the same family of enzymes and are very similar exoribonucleases, therefore it is interesting that PNPase shares more transcripts with RNase II (38 transcripts) and RNase R (23 transcripts) than RNase II shares with RNase R (only 12 transcripts). These results correlate with the results obtained for the functional categories clustering (Figure 4).

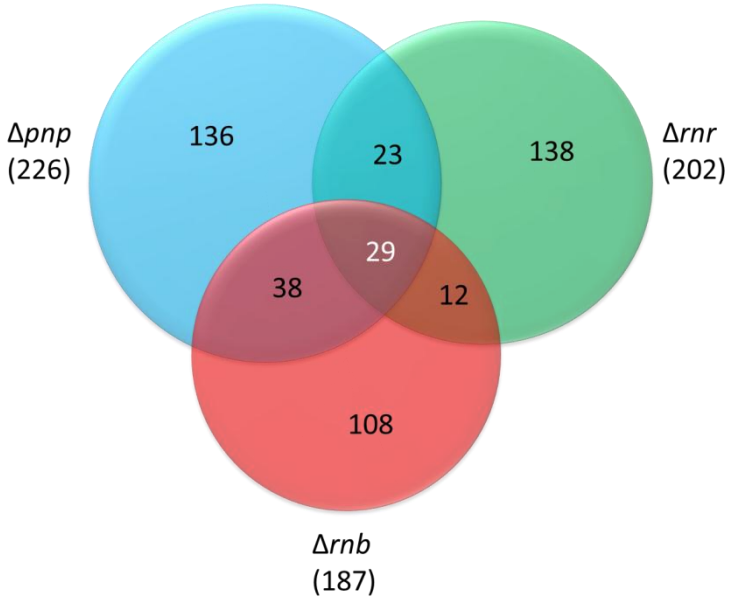


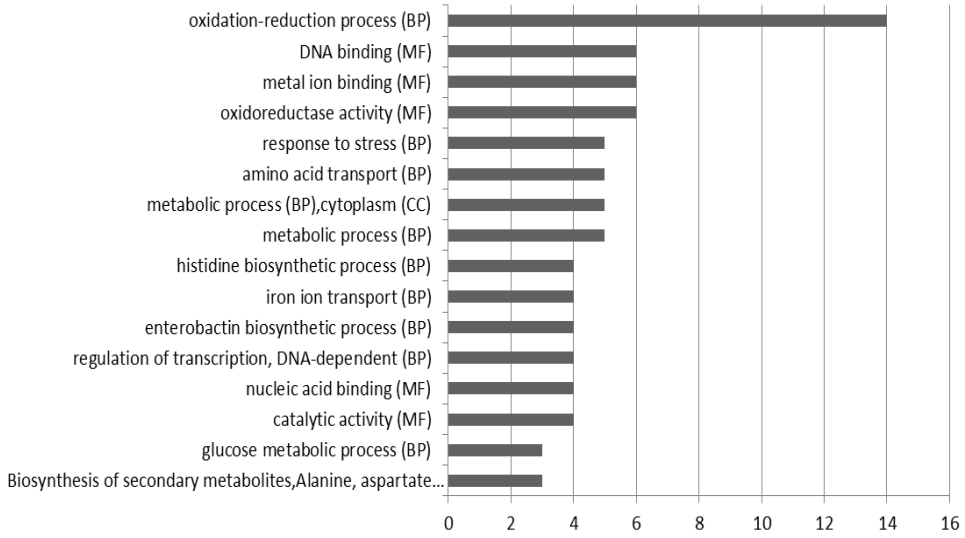
Figure 5 – Diagram comparing the number of transcripts that are differentially expressed in each of the three exoribonucleases in exponential phase.

Exoribonucleases in stationary phase

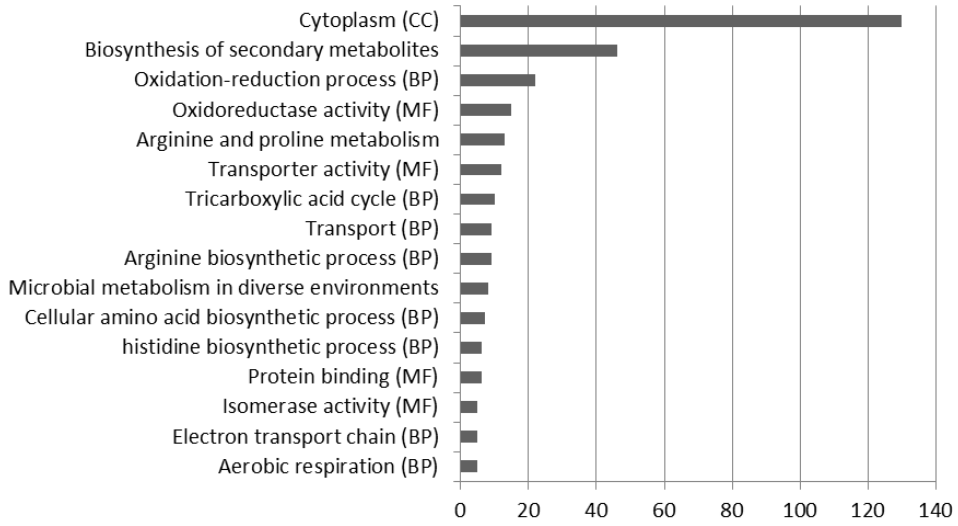
Using the same approach we clustered the differentially expressed transcripts for the three exoribonucleases in stationary phase.

There are 117 differentially expressed transcripts in the RNase II mutant (Table S5). In contrast, to what was observed in exponential phase, in stationary phase transcripts were mainly involved with oxidation-reduction processes and DNA and metal ion binding (Figure 6A). None of the RNase II differentially expressed transcripts in stationary phase is related with flagellar assembly or motility (Table S5).

A – RNase II



B – RNase R



C – PNPase

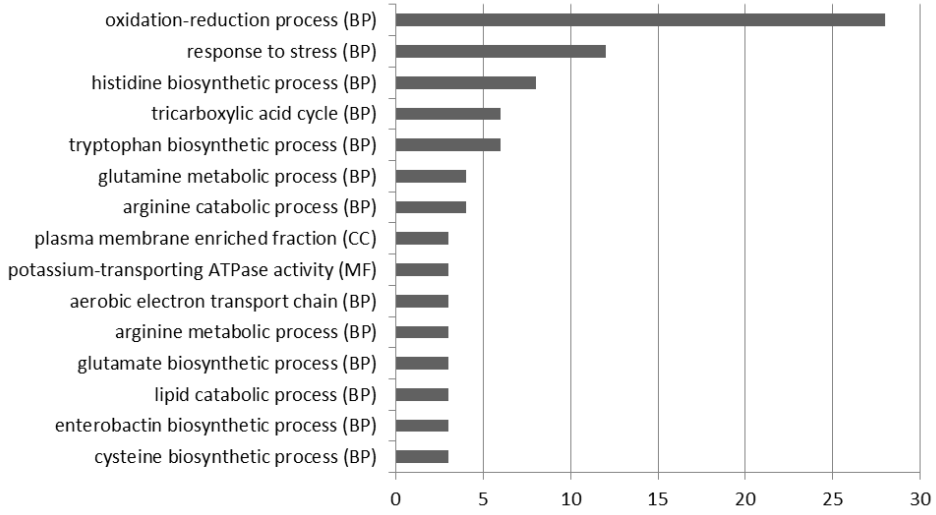


Figure 6 – Differentially expressed transcripts distribution in different functional categories. A) The differentially expressed transcripts between Δ*rnb* and wild-type in stationary phase. B) The differentially expressed transcripts between Δ*rnr* and wild-type in stationary phase. C) The differentially expressed transcripts between Δ*pnp* and wild-type in stationary phase. Transcripts were grouped into different functional categories (CC – cellular component, BP – Biological process and MF – metabolic function). Not all the transcripts are represented in this figure. These results were obtained using GeneCodis (Tabas-Madrid et al., 2012).

The number of transcripts that are differentially expressed in the Δ*rnr* mutant (694 transcripts) is much higher than for any other exoribonuclease (Table S6). Although RNase R deletion affects these many transcripts it does not seem to influence specific pathways. Instead we can cluster the transcripts in many different functional categories such as biosynthesis of secondary metabolites, oxidation-reduction processes, tricarboxylic acid cycle, protein binding and many more (Figure 6B). The difficulty in clustering all the transcripts that are differentially expressed in the Δ*rnr* mutant in stationary phase indicates how important this exoribonuclease is for the entire *E. coli* metabolism.

The number of transcripts that are differentially expressed in the Δ*pnp* mutant in stationary phase is practically the same as in exponential phase (228 and 226 respectively, Table S4 and Table S7). However, like RNase II and RNase R the pathways affected by the deletion of PNPase in exponential and stationary are different. For stationary phase we can cluster the transcripts into more biosynthetic and metabolic pathways like histidine and tryptophan biosynthetic processes and glutamine and arginine metabolic processes (Figure 6C). Again, exists an overlapping between the functional categories affected by of RNase II, RNase R and PNPase. The Δ*pnp* mutant affects functional categories like oxidation-reduction process and histidine biosynthetic process that are also affected in the RNase II and RNase R mutants. Transcripts related to response to stress are differentially expressed in both Δ*pnp* and Δ*rnb* mutants but not in the Δ*rnr* mutant. Contrarily, transcripts from the tricarboxylic acid cycle are differentially expressed in the Δ*pnp* and Δ*rnr* mutants but not in the Δ*rnb* mutant (Figure 6). Although the deletion of the different exoribonucleases affects the same functional categories the transcripts affected are different.

Comparing the three exoribonucleases in stationary phase we observed that from a total of 857 differentially expressed transcripts 694 transcripts are

regulated by RNase R (Figure 7). This clearly indicates that RNase R is the exoribonuclease with the most relevant role in stationary phase. From all of these transcripts only 26 are common to the three exoribonucleases. RNase R has more transcripts in common with PNPase (76 transcripts) than with RNase II (38 transcripts). In stationary phase PNPase only affects 16 transcripts that are also affected by RNase II but not by RNase R (Figure 7).

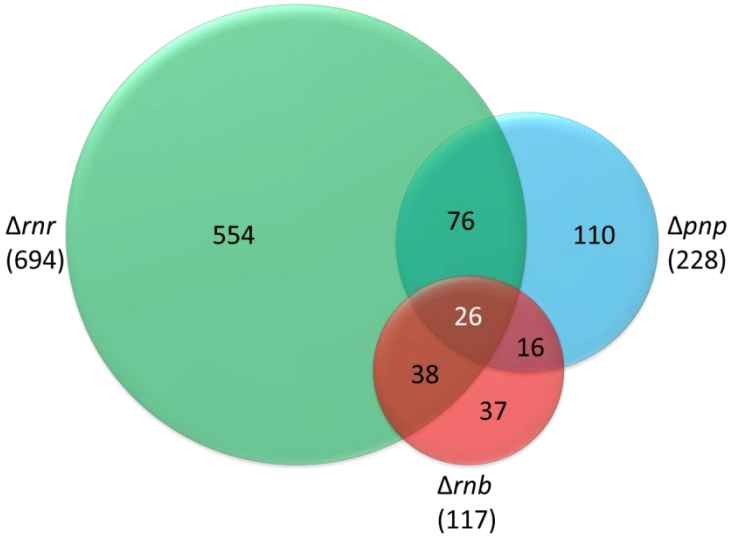


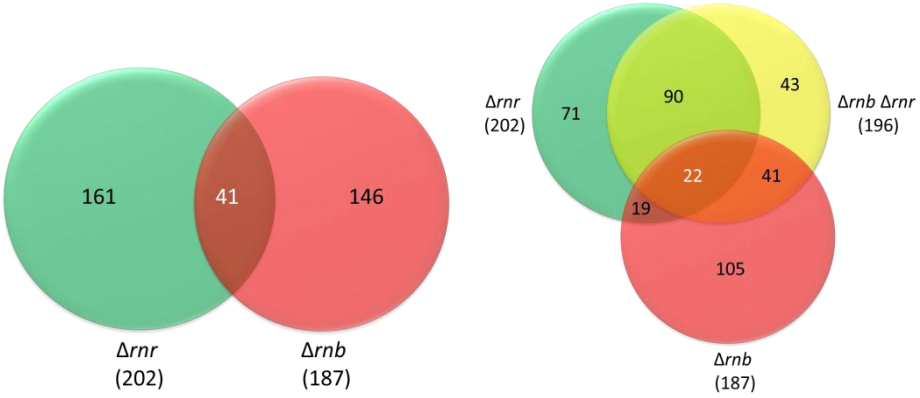
Figure 7 – Diagram comparing the number of transcripts that are differentially expressed in each of the three exoribonucleases in stationary phase.

The double mutant for both RNase II and RNase R behaves differently than the single mutants

Exoribonucleases were found to share some substrates and there is the possibility that these enzymes could compensate themselves by having overlapping functions in the cell. To decipher these pathways we intended to study multiple exoribonucleases mutants. Double mutants Δ*rnb* Δ*pnp* or Δ*rnr* Δ*pnp* are lethal (Donovan & Kushner, 1986; Deutscher, 1993; Cairrão et al., 2003), but we could delete both RNase II and RNase R (Δ*rnb* Δ*rnr* mutant) from the cell. This mutant is extremely important as the hydrolytic pathway of exonucleolytic RNA degradation is severely affected.

In the double mutant, 196 transcripts are differentially expressed in exponential phase (Table S8) while only 101 transcripts are significantly affected in stationary phase (Table S9). When compared with the number of transcripts that are differentially expressed in the single mutants we observed that the double mutant is not a simple addition of the Δ*rnb* and Δ*rnr* mutations (Figure 8). In spite of this, most of the transcripts that are differentially expressed in the double mutant are also differentially expressed in the Δ*rnb* or Δ*rnr* single mutants in both exponential and stationary phase (Figure 8). In exponential phase from the 41 transcripts that are common to Δ*rnb* and Δ*rnr* only 22 transcripts are also differentially expressed in the Δ*rnb* Δ*rnr* double mutant (Figure 8A). In stationary phase this difference is even higher; from the 64 transcripts common to Δ*rnb* and Δ*rnr* only 24 transcripts are also affected in the Δ*rnb* Δ*rnr* mutant (Figure 8B). The results also show that the double mutant has more transcripts common to the Δ*rnr* than to the Δ*rnb* mutant, especially in exponential phase (Figure 8).

A – Exponential phase



B – Stationary phase

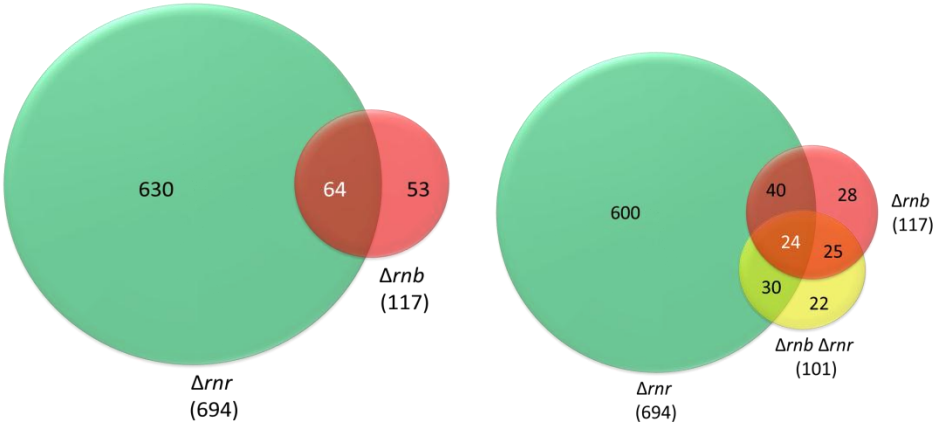
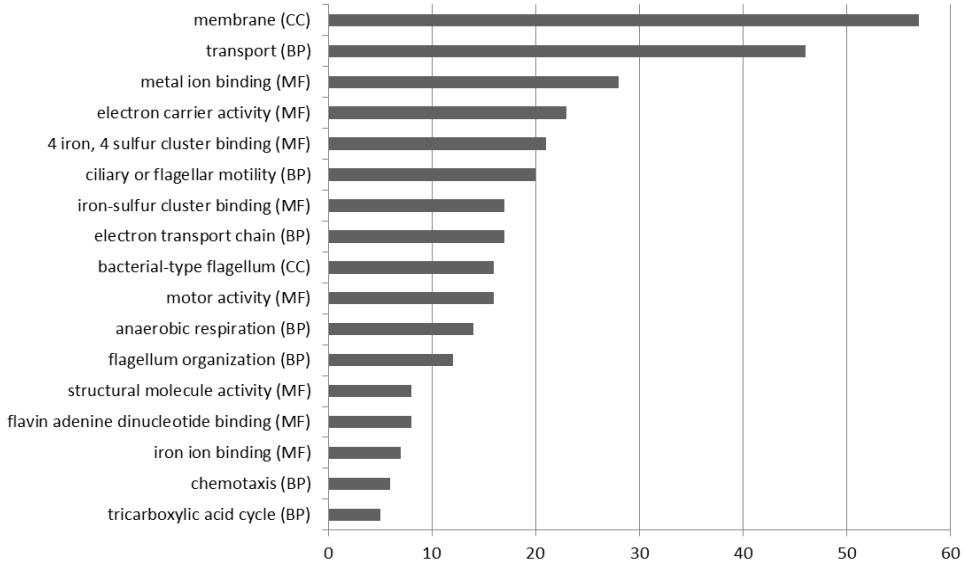


Figure 8 – Number of transcripts that are differentially expressed in the hydrolytic exoribonucleases mutants. A) Comparison of differentially expressed transcripts in the Δ*rnb* and Δ*rnr* single mutants and the Δ*rnb* Δ*rnr* double mutant in exponential phase.
B) Comparison of differentially expressed transcripts in the Δ*rnb* and Δ*rnr* single mutants and the Δ*rnb* Δ*rnr* double mutant in stationary phase.

Considering these results, is not so surprising that the clustering of transcripts results in the same functional categories than those existing for the single mutants (Figures 4, 6 and 9). For example in exponential phase we can identify transcripts that belong to metal ion binding and anaerobic respiration

that were common in the Δ*rnr* single mutant but we also detect transcripts from ciliary or flagellar motility and chemotaxis that were characteristic of the Δ*rnb* single mutant (Figure 4A and 4B). The same happens in stationary phase, as for example, the tricarboxylic acid cycle and arginine biosynthetic process are common to the Δ*rnr* mutant and the histidine biosynthetic and enterobactin biosynthetic processes are common to the Δ*rnb* mutant (Figure 6A and 6B).

A – Exponential phase



B – Stationary phase

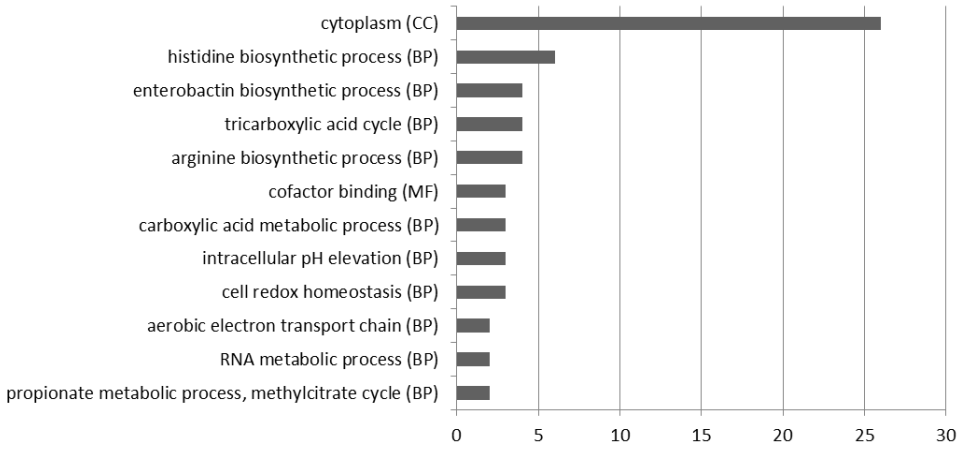


Figure 9 – Differentially expressed transcripts distribution into different functional categories. A) The differentially expressed transcripts between Δ*rnb* Δ*rnr* and wild-type in exponential phase. B) The differentially expressed transcripts between Δ*rnb* Δ*rnr* and wild-type in stationary phases. Transcripts were grouped into different functional categories (CC – cellular component, BP – Biological process and MF – metabolic function). Not all the transcripts are represented in this figure. These results were obtained using GeneCodis (Tabas-Madrid et al., 2012).

However, when comparing the lists of the Δ*rnb*, Δ*rnr* and the Δ*rnb* Δ*rnr* mutants we see that there are many transcripts that were differentially expressed in the single mutants, but not in the double mutant (Tables S2, S3, S5, S6, S8 and S9). This happens mainly in the stationary phase; for example *sib*B sRNA is up-regulated in the Δ*rnr* mutant however it does not appear in the list of the differentially expressed transcripts in the double mutant. Others can be up or down-regulated in the single mutant but then in the double this is inverted, one example of this is Sra (Stationary-phase-induced ribosome-associated protein) that is down-regulated in the Δ*rnr* single mutant (log2 fold change of -1.75) but in the double is up-regulated (log2 fold change of 1.86). The same is true for exponential phase and for Δ*rnb* differentially expressed transcripts. For example, *nrdI* is up-regulated in the Δ*rnb* single mutant (log2 fold change of 2.39) however it does not appear in the double mutant list of the differentially expressed

transcripts. These results indicate that the cell is able to somehow compensate the deletion of the two hydrolytic exoribonucleases and further studies are necessary to determine exactly how the cell adapts in the absence of RNase II and RNase R.

The role of exoribonucleases in small RNAs

Small RNAs can control many different targets and small changes in their levels can greatly affect its regulatory pathways. As shown previously (Table 2) most of the sRNAs are up-regulated in stationary phase and it is in this growth phase that sRNAs exert a major role (Waters & Storz, 2009). Therefore we will focus now only on the results for sRNAs in stationary phase. The effect of each exoribonuclease on the sRNA levels was calculated (Figure 10). RNase II is shown to have a minor impact in the degradation of sRNAs with more than 50% of the sRNAs not changing their levels in the Δ*rnb* mutant while 24% are down-regulated. Only 20% of sRNAs are up-regulated in the Δ*rnr* mutant. This suggests that RNase R is not significantly contributing for the degradation of the majority of sRNAs. On the other hand, PNPase deletion leads to an up-regulation of 41% of sRNAs. According to these results PNPase is the main exoribonucleases involved in sRNA degradation in stationary phase, in agreement with previous results from this lab (Andrade & Arraiano, 2008; Andrade et al., 2012).

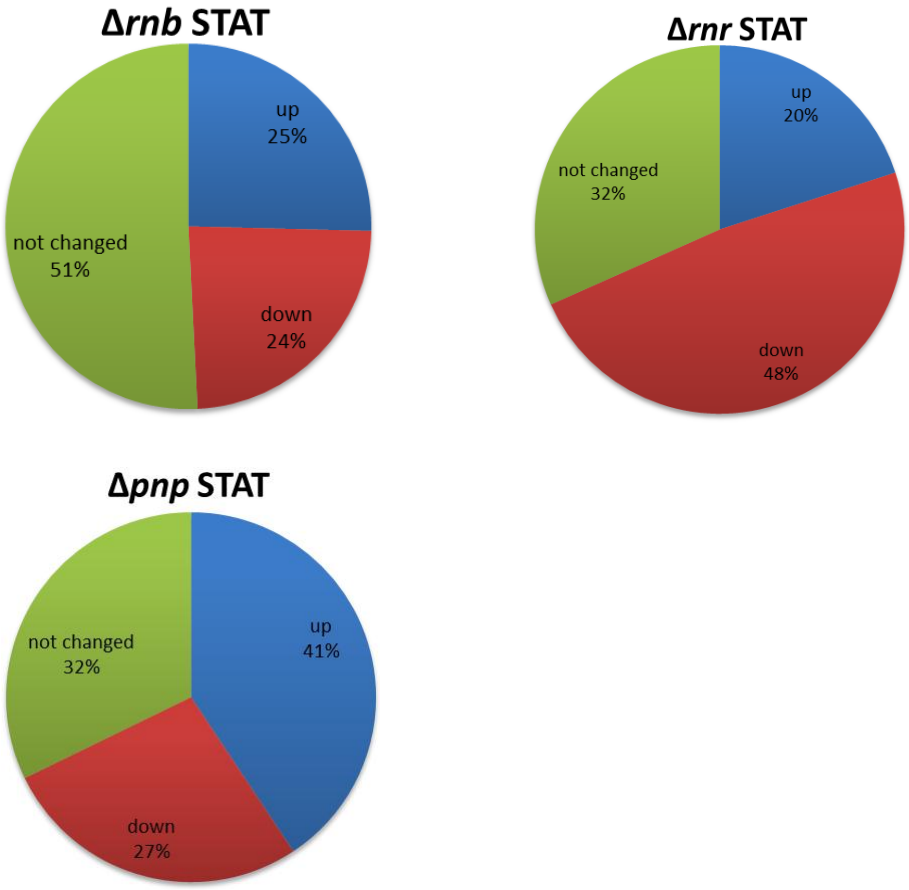


Figure 10 – Exoribonucleases role in sRNAs in stationary phase. All the annotated sRNAs were used to calculate the percentage of sRNAs that were up or down-regulated. The log2 fold-change for each sRNA was calculated and all the sRNAs with a log2 fold-change inferior to 0.5 was considered not significantly changed.

Discussion

The adaptation to stationary phase requires not only a rearrangement of the transcription, but also modulation of the RNA stability. Exoribonucleases are essential for RNA degradation and therefore here we try to determine their role in both exponential and stationary phase. RNA-seq was done to analyse the global transcriptome of mutants for the three main exoribonucleases in *E. coli* (RNase II, RNase R and PNPase). We found that the roles of RNase II, RNase R and PNPase are different depending on the growth phase. However, in both exponential and stationary phase PNPase overlaps with RNase II and RNase R. This was already expected because the double mutants (Δ*pnp* Δ*rnb* and Δ*pnp* Δ*rnr*) are not viable (Donovan & Kushner, 1986; Deutscher, 1993; Cairrão et al., 2003). On the other hand, the double mutant Δ*rnb* Δ*rnr* is viable indicating that essential RNase II and RNase R roles can be carried on by other ribonucleases.

In this work we studied the different roles of the three main *E. coli* exoribonucleases in exponential and stationary phases. Furthermore, we have shown what transcriptome rearrangements occur when *E. coli* goes from exponential to stationary phase. Approximately 85% of all transcripts were shown to be down-regulated in stationary phase when compared with exponential phase. Considering that in their natural habitat bacteria stays mostly in stationary phase due to limited nutrient availability (Kolter et al., 1993), therefore it is expected that the cells stop the transcription of most of the housekeeping genes as a defense mechanism to save energy. However, 14% of transcripts are up-regulated in stationary phase. Most of these are stress related transcripts that will confer *E. coli* cells resistance to the harsh conditions subsequent to stationary phase. It is well known that *E. coli* undergoes physical and morphological changes when entering stationary phase (Ishihama, 1997; Hengge-Aronis, 1999), therefore is not so surprisingly that most of the differentially expressed transcripts between

exponential and stationary phase are related to *E. coli* membrane and transport. One of the most important up-regulated transcript in stationary phase is the sigma factor *rpo*S. RpoS interacts with RNA polymerase and controls the expression of about 10% of the *E. coli* genome (Battesti et al., 2011). Our results show that *rpo*S RNA levels are 38% higher in stationary than in exponential phase (Table S1). This is in agreement with other reports stating that the protein levels of RpoS are 30% higher in stationary phase (Jishage & Ishihama, 1995). Another transcript essential for stationary phase adaptation is the small RNA *ssr*S (6S). The 6S RNA binds to both σ^{70} (*rpo*D, the σ subunit involved in the transcription of most genes during exponential growth) and to RNA polymerase supressing the σ^{70}dependent transcription during stationary phase (Wassarman & Storz, 2000). Here we showed that 6S is the most expressed small RNA in stationary phase even though it is not the one with the highest fold-change (Table 2). So far most of the studies carried on sRNAs were done in stationary phase as most sRNAs expression is higher in this condition. Interestingly, we have found 4 small RNAs (*tff*, *ryf*A, *psr*O and *rtt*R) up-regulated in exponential phase (Table 2). Not much is known about these sRNAs and further studies should be done to address the relevance of the increasing levels of these sRNAs in exponential phase.

The deletion of the exoribonucleases has different effects on the transcriptome depending on the growth phase. Interestingly, the deletion of exoribonucleases causes a down-regulation of a high percentage of transcripts. This is at first unexpected since the removal of an exoribonuclease should lead to the stabilization and consequently up-regulation of transcripts. Although it has been reported that in some cases an exoribonuclease can protect a transcript from degradation (Marujo et al., 2000; Mohanty & Kushner, 2003; De Lay & Gottesman, 2011) it is unlikely that all the transcripts down-regulated in the exoribonucleases mutants are result from such a protection effect. It is plausible that the down-regulated transcripts observed in the exoribonucleases mutants

can be due to indirect effect of the deletion of the RNase II, RNase R or PNPase. The same can happen with the up-regulated transcripts. Although many of the up-regulated transcripts can be substrates for the exoribonucleases it is possible that some of these transcripts are up-regulated because of an indirect effect of the exoribonucleases. Some transcription factors are differentially expressed in the exoribonucleases mutants when compared to the wild-type cells and therefore, so transcription can be a factor responsible for the indirect effects of the exoribonucleases in the transcriptome. Altogether it is important to consider these results as global effects of the exoribonucleases in the cell transcriptome, and not only as direct effects of these enzymes in the transcripts.

In exponential phase an RNase II mutation significantly affects 187 transcripts (Table S2). Most of these transcripts belong to the functional category of flagellar assembly (Figure 3A and Figure S1) and are down-regulated suggesting that this mutant may present motility deficiencies. Interestingly, the transcript which is found more up-regulated in the Δ*rnb* mutant with a log2 fold change of 3.36 is Antigen-43 (*flu*). Antigen-43 is an autotransporter protein that promotes aggregation and inhibits bacterial motility (Ulett et al., 2006). This suggests that RNase II deletion global effects on flagellar assembly might be a consequence of the high levels of antigen-43. Although RNase R is a member of the RNase II family, the two hydrolytic exoribonucleases are very different enzymes. The main difference is that RNase R is able to easily degrade structured RNAs while RNase II activity is blocked by secondary structures (Cannistraro & Kennell, 1999; Spickler & Mackie, 2000). The differences between these two enzymes are more evident when comparing the transcripts affected by the deletion of RNase II or RNase R (Table S2 and S3). In exponential phase the deletion of RNase R significantly affects the expression of 202 transcripts. Of all of these transcripts only 41 transcripts are also affected by RNase II. However, most of these are down-regulated in the Δ*rnb* mutant but up-regulated in the Δ*rnr* mutant. For example

*nir*B (Nitrite reductase [NAD(P)H] large subunit) is down-regulated in Δ*rnb* with a log2 fold-change of -1.46 while in the Δ*rnr* mutant nirB is up-regulated with a log2 fold-change of 3.19 (Table S2 and S3). This might indicate that RNase II and RNase R have very distinct roles in the cell. The up-regulated transcripts with highest fold-change in the Δ*rnr* mutant can be clustered into the anaerobic respiration functional category (Figure 3B and Table S3). One of the most important transcripts being up-regulated in the Δ*rnr* mutant is *fnr*S small RNA. FnrS reprograms metabolism in response to anaerobiosis and so far, *fnr*S was only shown to be expressed under anaerobic growth conditions (Durand & Storz, 2010). Considering that all cell cultures were grown at the same time and in the same conditions, the expression of *fnr*S and other anaerobic related transcripts in the Δ*rnr* mutant under normal aerobic conditions indicates that RNase R plays an essential role in the aerobic respiration process of *E. coli*. A small number of these anaerobic respiration transcripts are also differentially expressed in the Δ*rnb* and Δ*pnp* mutants. However, contrarily to what happens in the Δ*rnr* mutant the deletion of RNase II or PNPase leads to a down-regulation of these transcripts. Moreover, *fnr*S is not differentially expressed in either Δ*rnb* or Δ*pnp* mutants. This suggests that deletion of RNase II or PNPase can affect the cell respiratory processes but in a different mechanism than RNase R. Besides transcripts belonging to the anaerobic respiration functional categories, PNPase deletion also affected many other transcripts. In fact, PNPase was shown to be the exoribonuclease that affected more transcripts in exponential phase, in a total of 226 differentially expressed transcripts (Table S4). Interestingly, many of these transcripts (53 transcripts) are stable RNAs (rRNAs, tRNAs and sRNAs) indicating that PNPase has a very important role in the regulation of stable RNAs. This is in agreement with previous studies demonstrating that PNPase is involved in the processing and degradation of rRNAs and tRNAs (Cheng & Deutscher, 2003; Maes et al., 2012). It was also suggested that PNPase protects some sRNAs in

exponential phase (De Lay & Gottesman, 2011; Andrade et al., 2012). According to our results this happens with some small RNAs but is far from generalization, in fact only 4 of the differentially expressed sRNAs in the Δ*pnp* mutant are down-regulated. The other 8 differentially expressed sRNA are up-regulated meaning that PNPase is involved in their degradation. These results suggesting that PNPase role regarding small RNAs is still unclear and re-enforce the need for more studies to be carried out in exponential phase when analysing sRNAs.

In stationary phase, RNase R is the exoribonuclease with the more predominant role. The deletion of RNase R significantly affected 694 transcripts while deletion of RNase II or PNPase only affected 117 transcripts and 226 transcripts respectively. Also the number of overlapped transcripts of RNase R and the two other exoribonucleases is higher in stationary phase than in exponential phase (Figure 4 and Figure 5). Consequently we can observe a higher overlap of the functional categories affected by the three exoribonucleases (Figure 5). It was more difficult to cluster the differentially expressed transcripts for each of the exoribonucleases mutants in stationary phase than it was for exponential phase. This was mainly due to the fact that the transcripts could be clustered into many more functional categories and the number of transcripts in each category was smaller in stationary than in exponential phase. The potential roles previously presented for RNase II in flagellar assembly and RNase R in anaerobic respiration seems to be restricted to exponential phase. In stationary phase all the three exoribonucleases seem to have a broader role in RNA metabolism. Interestingly, at this stage of growth, all the three exoribonucleases affected oxidation-reduction processes (Figure 5). Considering that in stationary phase, there is an increase of oxidized proteins (Navarro Llorens et al., 2010) it makes sense that the cell maintains the transcripts involved in the response to oxidative stress under tight control. The deletion of any of the exoribonucleases will disturb the oxidation-reduction processes. For the same reasons is also not surprising that the

three exoribonucleases affect several metabolic and biosynthetic pathways (Figure 5).

RNase II and RNase R apparently affect several identical metabolic pathways in the cell. However, even though these two exoribonucleases affect the same pathway they do it in a distinct manner. In this work we also compared the Δ*rnb* and Δ*rnr* single mutants with the double mutant Δ*rnb* Δ*rnr* (Figure 7). Interestingly the Δ*rnb* Δ*rnr* is not a simple addition of the Δ*rnb* and Δ*rnr* mutations. The number of transcripts differentially expressed in the double mutant is lower than the sum of the transcripts affected by the single mutations in both exponential and stationary phase (Figure 7). These results indicate that the cell is able to compensate the deletion of both RNase II and RNase R. Still the vast majority of the transcripts that are differentially expressed in the Δ*rnb* Δ*rnr* mutant are also differentially expressed in the single mutants. Also when clustering the differentially expressed transcripts of the Δ*rnb* Δ*rnr* mutant we observed that there is a mixture between the functional categories affected by the Δ*rnb* and Δ*rnr* single mutations. So far there is no other study about the deletion of both RNase II and RNase R in the cell, so these results are very important to understand how the cell adapts when the two major exoribonucleases are absent. However, more studies are needed to clarify the collaboration of the different exoribonucleases to maintain the RNA levels in the cell.

Although our results raised some questions about small RNAs in exponential phase they corroborate the general view that small RNAs are mainly expressed in stationary phase. Our results confirm that PNPase is the main exoribonuclease involved in the decay of sRNAs in stationary phase. These results are in agreement with recent studies demonstrating that PNPase is extremely

important for small RNA degradation (Andrade & Arraiano, 2008; Andrade et al., 2012).

In conclusion, this work presents a wider overview on the role of exoribonucleases in exponential and stationary phase. All of these results have to be experimentally validated using other techniques like northern blot and RT-PCR. Still this work provides us a vast amount of information that aimed to expand our knowledge on RNA metabolism.

Materials and Methods

Growth conditions, strains and plasmids

Bacteria were grown at 37ºC, with shaking at 200 rpm in Luria-Bertani (LB) medium supplemented with thymine (50 μg ml^{-1}). When required, antibiotics were present at the following concentrations: kanamycin, 50 μg ml^{-1}; tetracycline, 20 μg ml^{-1}; streptomycin/spectinomycin 20 μg ml^{-1}. The *E. coli* strains used in this work are listed in Table 5.

Table 5- Bacterial strains used in this study

Strain	Relevant genotype	Reference
MG1693	*thyA715*	(Arraiano et al., 1988)
CMA201	*thyA715 ∆rnb*	(Andrade et al., 2006)
HM104	*thyA715 Δrnr*	(Andrade et al., 2006)
HM103	*thyA714 Δrnb Δrnr*	This work
SK10019	*thyA715 Δpnp*	(Mohanty & Kushner, 2003)

RNA extraction for high-throughput sequencing

Overnight cultures from isolated colonies were diluted in fresh medium to an initial OD_{600}~0.03 and grown to exponential (OD_{600}~0.3) or stationary-phase. RNA was isolated following cell lysis and phenol:chloroform extraction as described on Chapter 2. After precipitation step in ethanol and 300 mM sodium acetate, RNA was resuspended in MilliQ-water. The integrity of RNA samples was evaluated by agarose gel electrophoresis. Turbo DNase (Ambion) treatment was used to remove contaminant DNA following the instructions of the supplier. RNA samples (20 μg) were sent to Vertis Biotechnologie AG, Germany, for library preparation and sequencing. No biological replicates were sequenced.

Vertis Biotechnologies used the follow protocol for library preparation:

1- Depletion of rRNA

Ribosomal RNA molecules were depleted from the total RNA preparations using the MICROBExpress Bacterial mRNA Enrichment Kit (Ambion). Aliquots of the rRNA depleted samples were examined by capillary electrophoresis.

2- cDNA synthesis

The rRNA depleted RNAs were fragmented with Rnase III and the 5'PPP structures were removed using RNA 5' Polyphosphatase (Epicentre). Afterwards, the RNA fragments were poly(A)-tailed using poly(A) polymerase. Then a RNA adapter was ligated to the 5´-phosphate of the RNA fragments. Firststrand cDNA synthesis was performed using an oligo(dT)-adapter primer and M-MLV reversetranscriptase. The resulting cDNA was PCR-amplified to about 30 ng/µl using a high fidelity DNA polymerase. PCR cycles performed and barcode sequences, which are part of the 3' sequencing adaptor, are included in Table 2. The cDNA was purified using the Agencourt AMPure XP kit (Beckman Coulter Genomics) and analyzed by capillary electrophoresis.

3- Description of the cDNA

The cDNA is double stranded and has a size of about 200–500 bp. The primers used for PCR amplification were designed for TruSeq sequencing according to the instructions of Illumina. The following adapter sequences flank the cDNA insert:

TrueSeq_Sense_primer

5´- AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-3’

TrueSeq_Antisense_primer Barcode
5'-CAAGCAGAAGACGGCATACGAGAT-NNNNNN-
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC(dT25)-3'

The cDNA pool was sequenced on an Illumina HiSeq 2000 machine and the number of reads varied between 6.369.529 to 8.422.390.

High-throughput sequencing analysis

The Vertis Biotechnologie AG bioinformatics department did a preliminary analysis of the high-throughput sequencing results. We confirmed the raw data quality using FastQC tool (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). After the quality control, the reads were mapped against *E. coli* genome (NC_000913 downloaded from NCBI genome database) using BowTie alignment tool (Langmead et al., 2009). Approximately 94% of all reads were mapped against the genome, the reads that were not mapped were saved in a different fasta file and will be analysed posteriorly. The BowTie output is in SAM format that was then used to run HTSeq (Python framework to process and analyse high-throughput sequencing) to add gene annotations to the mapped reads. The HTSeq output is also a SAM file with gene names associated with the mapped reads. The FastQC, BowTie and HTSeq were run using the Linux versions.

The SAM data was uploaded into Galaxy, an open web-based platform for high-throughput sequencing analysis (Giardine et al., 2005; Blankenberg et al., 2010; Goecks et al., 2010). Galaxy was used to run Cufflinks (estimates the relative abundance of the transcripts) and after Cuffdiff to find significant changes in transcript expression when comparing two samples (Trapnell et al., 2010). Cufflinks uses the "average length method" to normalize the data (Trapnell et al., 2010). Although Galaxy can also be used for FastQC and BowTie the Linux

versions are much faster and allow you to choose the version of the genome and the annotations files to be used.

The R packages DESeq (Anders & Huber, 2010) and edgeR (Robinson et al., 2010) were also used to find significant changes in the transcripts expression. Although these two packages are very useful for the differential expression analysis of high-throughput data they presented several issues when analysing data without biological replicates. Therefore only Cuffdiff results were taken in consideration for these data analysis. The gene list resulted from Cuffdiff were then analysed using GeneCodis3, a web-based tool for the ontological analysis of large lists of genes (Tabas-Madrid et al., 2012).

References

Aiso T, Murata M, Gamou S. 2011. Transcription of an antisense RNA of a *gad*E mRNA is regulated by GadE, the central activator of the acid resistance system in *Escherichia coli*. *Genes to cells : devoted to molecular & cellular mechanisms 16*:670-680.

Anders S, Huber W. 2010. Differential expression analysis for sequence count data. *Genome biology 11*:R106.

Andrade JM, Arraiano CM. 2008. PNPase is a key player in the regulation of small RNAs that control the expression of outer membrane proteins. *RNA 14*:543-551.

Andrade JM, Cairrão F, Arraiano CM. 2006. RNase R affects gene expression in stationary phase: regulation of *ompA*. *Mol Microbiol 60*:219-228.

Andrade JM, Hajnsdorf E, Régnier P, Arraiano CM. 2009a. The poly(A)-dependent degradation pathway of *rps*O mRNA is primarily mediated by RNase R. *RNA 15*:316-326.

Andrade JM, Pobre V, Matos AM, Arraiano CM. 2012. The crucial role of PNPase in the degradation of small RNAs that are not associated with Hfq. *RNA 18*:844-855.

Andrade JM, Pobre V, Silva IJ, Domingues S, Arraiano CM. 2009b. The role of 3'-5' exoribonucleases in RNA degradation. *Prog Mol Biol Transl Sci 85*:187-229.

Arraiano CM, Andrade JM, Domingues S, Guinote IB, Malecki M, Matos RG, Moreira RN, Pobre V, Reis FP, Saramago M, Silva IJ, Viegas SC. 2010. The critical role of RNA processing and degradation in the control of gene expression. *FEMS Microbiol Rev 34*:883-923.

Arraiano CM, Yancey SD, Kushner SR. 1988. Stabilization of discrete mRNA breakdown products in *ams pnp rnb* multiple mutants of *Escherichia coli* K-12. *J Bacteriol 170*:4625-4633.

Awano N, Rajagopal V, Arbing M, Patel S, Hunt J, Inouye M, Phadtare S. 2010. *Escherichia coli* RNase R has dual activities, helicase and RNase. *Journal of bacteriology 192*:1344-1352.

Battesti A, Majdalani N, Gottesman S. 2011. The RpoS-mediated general stress response in *Escherichia coli*. *Annual review of microbiology 65*:189-213.

Blankenberg D, Von Kuster G, Coraor N, Ananda G, Lazarus R, Mangan M, Nekrutenko A, Taylor J. 2010. Galaxy: a web-based genome analysis tool for experimentalists. *Current protocols in molecular biology / edited by Frederick M Ausubel [et al] Chapter 19*:Unit 19 10 11-21.

Cairrão F, Cruz A, Mori H, Arraiano CM. 2003. Cold shock induction of RNase R and its role in the maturation of the quality control mediator SsrA/tmRNA. *Mol Microbiol 50*:1349-1360.

Cannistraro VJ, Kennell D. 1999. The reaction mechanism of ribonuclease II and its interaction with nucleic acid secondary structures. *Biochem et Biophys Acta 1433*:170-187.

Chen C, Deutscher MP. 2010. RNase R is a highly unstable protein regulated by growth phase and stress. *RNA 16*:667-672.

Cheng ZF, Deutscher MP. 2002. Purification and characterization of the *Escherichia coli* exoribonuclease RNase R. Comparison with RNase II. *J Biol Chem 277*:21624-21629.

Cheng ZF, Deutscher MP. 2003. Quality control of ribosomal RNA mediated by polynucleotide phosphorylase and RNase R. *Proc Natl Acad Sci USA 100*:6388-6393.

Coburn GA, Mackie GA. 1996. Overexpression, purification, and properties of *Escherichia coli* ribonuclease II. *J Biol Chem 271*:1048-1053.

De Lay N, Gottesman S. 2011. Role of polynucleotide phosphorylase in sRNA function in *Escherichia coli*. *RNA 17*:1172-1189.

Deutscher MP. 1993. Promiscuous exoribonucleases of *Escherichia coli*. *Journal of bacteriology 175*:4577-4583.

Donovan WP, Kushner SR. 1986. Polynucleotide phosphorylase and ribonuclease II are required for cell viability and mRNA turnover in *Escherichia coli* K-12. *Proc Natl Acad Sci USA 83*:120-124.

Durand S, Storz G. 2010. Reprogramming of anaerobic metabolism by the *FnrS* small RNA. *Mol Microbiol 75*:1215-1231.

Folichon M, Marujo PE, Arluison V, Le Derout J, Pellegrini O, Hajnsdorf E, Régnier P. 2005. Fate of mRNA extremities generated by intrinsic termination: detailed analysis of reactions catalyzed by ribonuclease II and poly(A) polymerase. *Biochimie 87*:819-826.

Gatewood ML, Jones GH. 2010. (p)ppGpp inhibits polynucleotide phosphorylase from streptomyces but not from *Escherichia coli* and increases the stability of bulk mRNA in *Streptomyces coelicolor*. *Journal of bacteriology 192*:4275-4280.

Geissen R, Steuten B, Polen T, Wagner R. 2010. *E. coli* 6S RNA: a universal transcriptional regulator within the centre of growth adaptation. *RNA Biol 7*:564-568.

Giardine B, Riemer C, Hardison RC, Burhans R, Elnitski L, Shah P, Zhang Y, Blankenberg D, Albert I, Taylor J, Miller W, Kent WJ, Nekrutenko A. 2005. Galaxy: a platform for interactive large-scale genome analysis. *Genome research 15*:1451-1455.

Goecks J, Nekrutenko A, Taylor J. 2010. Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome biology 11*:R86.

Guell M, Yus E, Lluch-Senar M, Serrano L. 2011. Bacterial transcriptomics: what is beyond the RNA horiz-ome? *Nature reviews Microbiology 9*:658-669.

Hajnsdorf E, Steier O, Coscoy L, Teysset L, Régnier P. 1994. Roles of RNase E, RNase II and PNPase in the degradation of the *rpsO* transcripts of *Escherichia coli*: stabilizing function of RNase II and evidence for efficient degradation in an *ams pnp rnb* mutant. *EMBO J 13*:3368-3377.

Hengge-Aronis R. 1999. Interplay of global regulators and cell physiology in the general stress response of *Escherichia coli*. *Curr Opin Microbiol 2*:148-152.

Ishihama A. 1997. Adaptation of gene expression in stationary phase bacteria. *Curr Opin Genet Dev 7*:582-588.

Jishage M, Ishihama A. 1995. Regulation of RNA polymerase sigma subunit synthesis in *Escherichia coli*: intracellular levels of sigma 70 and sigma 38. *Journal of bacteriology 177*:6832-6835.

Kolter R, Siegele DA, Tormo A. 1993. The stationary phase of the bacterial life cycle. *Annu Rev Microbiol 47*:855-874.

Langmead B, Trapnell C, Pop M, Salzberg SL. 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome biology 10*:R25.

Liang W, Deutscher MP. 2012. Post-translational modification of RNase R is regulated by stress-dependent reduction in the acetylating enzyme Pka (YfiQ). *RNA 18*:37-41.

Maes A, Gracia C, Hajnsdorf E, Régnier P. 2012. Search for poly(A) polymerase targets in *E. coli* reveals its implication in surveillance of Glu tRNA processing and degradation of stable RNAs. *Mol Microbiol 83*:436-451.

Marujo PE, Hajnsdorf E, Le Derout J, Andrade R, Arraiano CM, Régnier P. 2000. RNase II removes the oligo(A) tails that destabilize the *rpsO* mRNA of *Escherichia coli*. *RNA 6*:1185-1193.

Mohanty BK, Kushner SR. 2000. Polynucleotide phosphorylase, RNase II and RNase E play different roles in the *in vivo* modulation of polyadenylation in *Escherichia coli*. *Mol Microbiol 36*:982-994.

Mohanty BK, Kushner SR. 2000 Polynucleotide phosphorylase functions both as a 3' right-arrow 5' exonuclease and a poly(A) polymerase in *Escherichia coli*. *Proc Natl Acad Sci USA 97*:11966-11971.

Mohanty BK, Kushner SR. 2003. Genomic analysis in *Escherichia coli* demonstrates differential roles for polynucleotide phosphorylase and RNase II in mRNA abundance and decay. *Mol Microbiol 50*:645-658.

Mohanty BK, Kushner SR. 2006. The majority of *Escherichia coli* mRNAs undergo post-transcriptional modification in exponentially growing cells. *Nucleic acids research 34*:5695-5704.

Navarro Llorens JM, Tormo A, Martinez-Garcia E. 2010. Stationary phase in gram-negative bacteria. *FEMS Microbiol Rev 34*:476-495.

Pepe CM, Maslesa-Galic S, Simons RW. 1994. Decay of the IS10 antisense RNA by 3' exoribonucleases: evidence that RNase II stabilizes RNA-OUT against PNPase attack. *Mol Microbiol 13*:1133-1142.

Robinson MD, McCarthy DJ, Smyth GK. 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics 26*:139-140.

Slomovic S, Portnoy V, Yehudai-Resheff S, Bronshtein E, Schuster G. 2008. Polynucleotide phosphorylase and the archaeal exosome as poly(A)-polymerases. *Biochim Biophys Acta 1779*:247-255.

Spickler C, Mackie A. 2000. Action of RNases II and Polynucleotide Phosphorylase against RNAs containing stem-loops of defined structure. *J Bacteriol 182*:2422-2427.

Tabas-Madrid D, Nogales-Cadenas R, Pascual-Montano A. 2012. GeneCodis3: a non-redundant and modular enrichment analysis tool for functional genomics. *Nucleic Acids Res 40*:W478-483.

Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L. 2010. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nature biotechnology 28*:511-515.

Ulett GC, Webb RI, Schembri MA. 2006. Antigen-43-mediated autoaggregation impairs motility in *Escherichia coli*. *Microbiology 152*:2101-2110.

Vincent HA, Deutscher MP. 2006. Substrate recognition and catalysis by the exoribonuclease RNase R. *J Biol Chem 281*:29769-29775.

Wang Z, Gerstein M, Snyder M. 2009. RNA-Seq: a revolutionary tool for transcriptomics. *Nature reviews Genetics 10*:57-63.

Wassarman KM, Storz G. 2000. 6S RNA regulates *E. coli* RNA polymerase activity. *Cell 101*:613-623.

Waters LS, Storz G. 2009. Regulatory RNAs in bacteria. *Cell 136*:615-628.

Supplemental data

Figure S1 – Transcripts involved in the flagellar assembly.

Table S1 – Differential expressed transcript list for exponential versus stationary phase. Chapter 4\Supplementary Table 1.xlsx[1]

Table S2 – Differential expressed transcript list for Δ*rnb* mutant in exponential phase. Chapter 4\Supplementary Table 2.xlsx[1]

Table S3 – Differential expressed transcript list for Δ*rnr* mutant in exponential phase. Chapter 4\Supplementary Table 3.xlsx[1]

Table S4 – Differential expressed transcript list for Δ*pnp* mutant in exponential phase. Chapter 4\Supplementary Table 4.xlsx[1]

Table S5 – Differential expressed transcript list for Δ*rnb* mutant in stationary phase. Chapter 4\Supplementary Table 5.xlsx[1]

Table S6 – Differential expressed transcript list for Δ*rnr* mutant in stationary phase. Chapter 4\Supplementary Table 6.xlsx[1]

Table S7 – Differential expressed transcript list for Δ*pnp* mutant in stationary phase. Chapter 4\Supplementary Table 7.xlsx[1]

Table S8 – Differential expressed transcript list for Δ*rnb* Δ*rnr* mutant in exponential phase. Chapter 4\Supplementary Table 8.xlsx[1]

Table S9 – Differential expressed transcript list for Δ*rnb* Δ*rnr* mutant in stationary phase. Chapter 4\Supplementary Table 9.xlsx[1]

1 The Supplementary Excel Tables for this chapter were not printed and are made available in the accompanying digital format of this Dissertation.

Figure S1

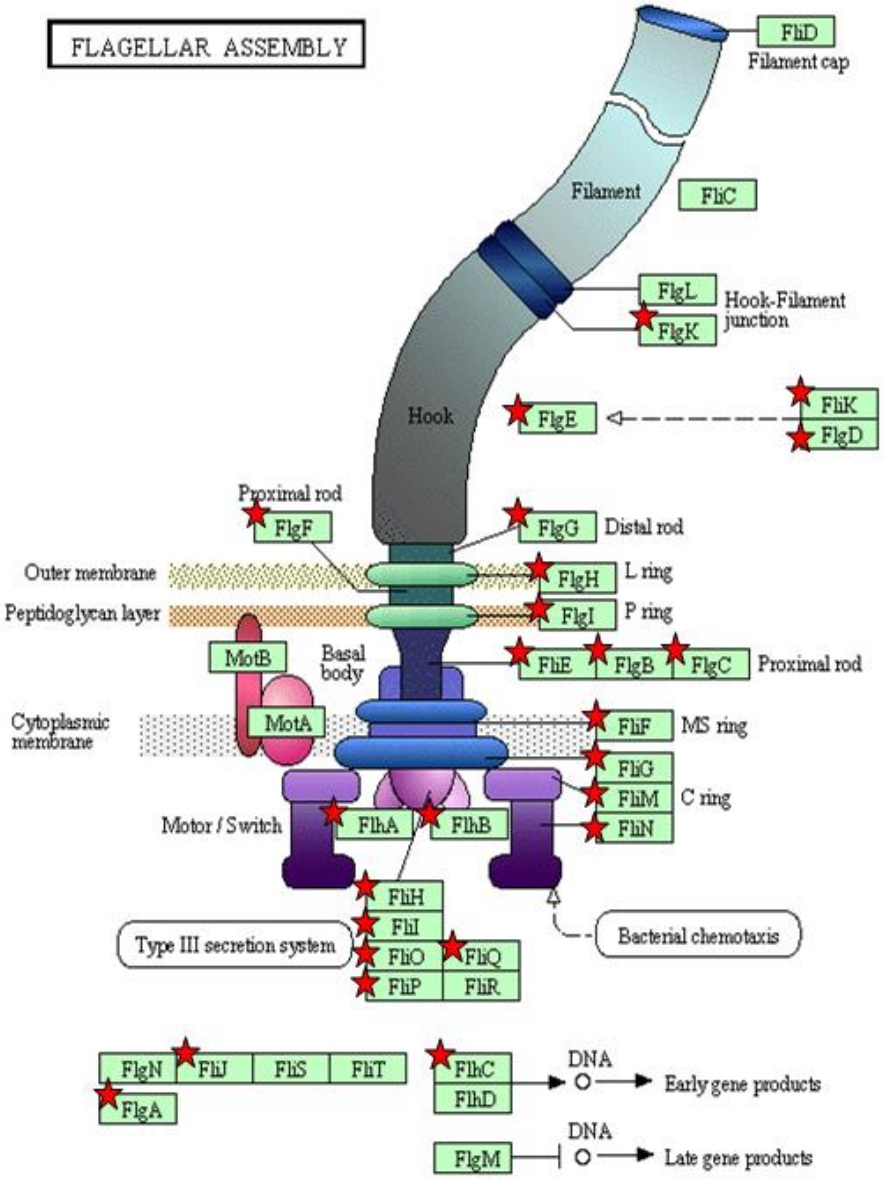


Figure S1 – Transcripts involved in the flagellar assembly. The transcripts involved in the flagellar assembly are in green boxes. All the transcripts with a red star are down-regulated in the Δ*rnb* mutant in exponential phase. Figure adapted from flagellar assembly Kegg pathway.

Chapter 5

General Discussion

Table of Contents

General Discussion

RNA degradation is a major factor involved in the control of the RNA levels and it has been the main focus of this Dissertation. In this work we studied RNA degradation mechanisms and investigated how exoribonucleases, Hfq and small RNAs can influence RNA levels in the cell and consequently affect gene expression.

PNPase degradation of small RNAs when not bound to Hfq

In this Doctoral work we wanted to study the degradation pathways of small RNAs. Small non-coding RNAs are highly structured regulatory molecules that control many genetic pathways. Usually sRNAs act by an antisense mechanism and bind to their mRNA targets with full (*cis*-encoded) or partial complementarity (*trans*-encoded) (Viegas & Arraiano, 2008). They typically bind to the ribosome binding site of the target mRNAs, blocking ribosome binding and promoting mRNA degradation (Urban & Vogel, 2007; Viegas & Arraiano, 2008). In *E. coli m*ost of the *trans*-encoded sRNAs studied so far interact with the RNA chaperone Hfq. Hfq mediates RNA-RNA interaction (Moller et al., 2002) and accelerates duplex formation between the sRNA and the mRNA target (Kawamoto et al., 2006). In some cases Hfq binds to the sRNA or to the mRNA refolding their structure and thus allowing binding (Geissmann & Touati, 2004). Hfq is known to stabilize sRNAs and in its absence most of sRNAs became very unstable (Massé et al., 2003; Andrade et al., 2012). In *E. coli* RNA degradation is carried out mainly by two endoribonucleases (RNase E and RNase III) and three exoribonucleases (RNase II, RNase R and PNPase) (Arraiano et al., 2010).

It was thought that Hfq would protect sRNAs mainly by binding to the same site as RNase E, preventing the RNase E cleavage of sRNAs (Massé et al., 2003; Moll et al., 2003). However our results showed that, the PNPase contribution is far greater than RNase E in the degradation of sRNAs, especially when they are not bound to Hfq (results from **Chapter 2**). We found that small RNAs in their Hfq-free state are rapidly degraded by PNPase, mainly in the stationary-phase of growth. In the absence of Hfq, PNPase inactivation resulted in increased levels of at least the MicA, SgrS, RyhB and GlmY sRNAs. This was shown to be consequence of the higher stability of these fragments (Andrade et al., 2012). Our results also demonstrated that the impact of RNase E on Hfq^{-} cells may not be as common as previously believed. Even though both RyhB and GlmY are RNase E substrates in cells lacking Hfq, RNase E depletion did not affect the levels of SgrS and MicA RNA. However, in the presence of Hfq MicA is a substrate for RNase E. This suggests that RNase E requires Hfq in order to degrade MicA. This had already been described for the degradation of OxyS by RNase E (Basineni et al., 2009). Nevertheless, RNase E can also affect the levels of some sRNAs independently of Hfq (Andrade et al., 2012).

Besides PNPase and RNase E, we also tested the impact of other ribonucleases on the small RNA levels. RNase II and RNase R were not found to be involved in the degradation of MicA either in the presence or absence of Hfq (Andrade & Arraiano, 2008; Andrade et al., 2012). Surprisingly, the absence of RNase R resulted in the reduction of MicA levels in cells without Hfq. The paradoxical protection of RNA by a ribonuclease has already been described for both RNase II and PNPase (Marujo et al., 2000; De Lay & Gottesman, 2011). RNase III inactivation in the absence of Hfq does not affect the sRNA levels. However, in the presence of Hfq and in the absence of RNase III MicA becomes extremely stabilized (results from **Chapter 2** and **Chapter 3**). In an *hfq* mutant the MicA levels strongly decrease and consequently the probability of MicA to base pair

with target mRNAs diminishes. Also Hfq is essential for MicA to bind to its targets. Our results from **Chapter 3** show that when the MicA binding to its mRNA targets is compromised there is an impairment in RNase III action on MicA, which is in agreement with *in vitro* studies (Viegas et al., 2011). Therefore the degradation of small RNAs can occur in two different pathways (Figure 1). When they are not associated with Hfq or the binding with the targets is compromised the degradation mainly occurs in a target-independent pathway, in which RNase III has a reduced impact. In this case PNPase is the main ribonuclease involved in the degradation of the small RNAs. In the other pathway if the sRNA is bound to its target mRNA forming a duplex then it can be initially cleaved by RNase III and then PNPase and possibly other exoribonucleases intervene in the degradation of both sRNA and mRNA.

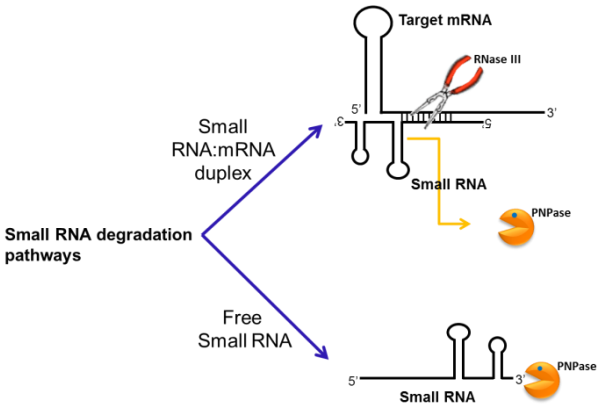


Figure 1 – Model for small RNA degradation pathways. In the cell there are two subsets of small RNA populations. One subset is the sRNA when bound to its mRNA targets while the other subset is the sRNA when not bound to its targets ("free" sRNA). Consequently there are two distinct pathways for the degradation of a small RNA. The target-dependent pathway where RNase III is the principal responsible for the degradation of the sRNA and the target-independent pathway where PNPase is the main ribonuclease involved in the sRNA decay.

Our results also showed that the lack of poly(A) polymerase I (PAP I) affected the levels of small RNAs in the absence of Hfq. Small RNAs that were highly affected by polyadenylation were also substrates for PNPase. However,

some sRNAs that were not affected by polyadenylation were also substrates for PNPase. Therefore the importance of PNPase on sRNAs levels in the absence of Hfq is independent of polyadenylation. On the other hand, it has been reported that PNPase biosynthetic activity is increased in the absence of Hfq (Mohanty et al., 2004; Slomovic et al., 2008). This fact may account for the impact of PNPase on the degradation of sRNAs in the absence of Hfq.

It has been recently demonstrated that Hfq binds U-rich sequences at the 3'-end of small RNAs (Otaka et al., 2011; Sauer & Weichenrieder, 2011; Pobre et al., 2012). Therefore another possibility for the great impact of PNPase on sRNA levels in the absence of Hfq is that Hfq protects the 3' end of the RNA against PNPase degradation. Although our results show that the 3' ends of the small RNAs are shortened in the absence of Hfq, PNPase does not seem to be the main responsible for the initial 3' end attack. Apparently Hfq does protect the 3' ends of the small RNAs because in the presence of Hfq the shorter sRNAs are barely detected.

Another factor influencing the degradation pathways of sRNAs is the growth phase. In the stationary-phase PNPase is shown to be the main enzyme in the degradation of small RNAs (Andrade & Arraiano, 2008; Andrade et al., 2012). On the other hand, recent studies proposed that in exponential phase PNPase can protect small RNAs from degradation by other ribonucleases, namely from RNase E (De Lay & Gottesman, 2011). In fact previous work that reported that RNase E was the main enzyme involved in the decay of sRNAs was also performed in exponential phase (Massé et al., 2003). Therefore growth phase can have a large impact on the choice of enzymes and degradation pathways.

RNA determinants involved in small RNA stability and target selectivity

Small RNAs are not "innocent" molecules waiting to be degraded. The sequence and structural features of the small RNAs influence their degradation. Most of the sRNAs are extremely structured RNAs with several stem loops that provide them some protection against exoribonucleolytic degradation and consequently sRNAs are usually very stable molecules. RNase II is a 3'-5' exoribonuclease that stalls before it reaches double stranded regions (Cannistraro & Kennell, 1999; Spickler & Mackie, 2000). So it is not so surprising that so far RNase II was not shown to be involved in the degradation of sRNAs. On the other hand, RNase R is the only 3'-5' exoribonuclease capable of degrading structured RNAs by itself (Cheng & Deutscher, 2002, 2003; Awano et al., 2010). However RNase R also does not seem to have any relevant role in the degradation of sRNAs. In contrast, PNPase seems to have very important roles in the degradation of sRNAs (Andrade & Arraiano, 2008; De Lay & Gottesman, 2011; Andrade et al., 2012). Like PNPase, the endoribonucleases RNase E and RNase III are also involved in the degradation of sRNAs. However RNase III-mediated decay pathway is apparently dependent on the binding to the target (Viegas et al., 2011; Andrade et al., 2012; Pobre et al., 2012).

Most of the studies on sRNA degradation are focused in the role of the exoribonucleases or in the interaction between sRNA-mRNA. So we focused on the intrinsic characteristics of a sRNA. For this study we used as a model the small RNA MicA. MicA was initially identified as a repressor of several major outer membrane proteins (OMPs) (Rasmussen et al., 2005; Udekwu et al., 2005; Bossi & Figueroa-Bossi, 2007). Recently MicA target mRNAs list was expanded through the use of microarray studies (Gogol et al., 2011). MicA is a 72bp RNA molecule with a 5' linear region, a predicted Hfq-binding A/U-rich region that is between two stem

loops and finally a U-rich linear stretch in the 3' end (Udekwu et al., 2005; Pobre et al., 2012). Other small RNAs, like RybB and SgrS have a similar modular organization than MicA (Balbontín et al., 2010; Papenfort et al., 2010; Otaka et al., 2011; Rice & Vanderpool, 2011). By introducing mutations in the different MicA regions (modules) we constructed several different MicA mutated forms to study the MicA determinants involved in the sRNA stability and target binding. Our **Chapter 3** results demonstrate that distinct MicA modules play distinct roles in protecting MicA from degradation. The 5' end of MicA was suggested to be the principal target recognition domain (Papenfort & Vogel, 2009). Mutations in the 5' end domain resulted in the stabilisation of this sRNA. This is probably a consequence of the disruption of the base pairing between the sRNA and the mRNA targets that will impair the RNase III activity against MicA. And as previously mentioned the degradation pathway for a sRNA bound to the target is distinct from the degradation pathway of a free sRNA (Figure 1). The stem-loops protect the sRNA against degradation by acting as physical barriers against 3'-5' exoribonucleolytic degradation or by sequestering sequences susceptible to RNase E cleavage (Arraiano et al., 2010). The two stem-loops present in MicA play distinct roles. Thermodynamically, STEM1 is weaker than STEM2 and the extensive disruption of STEM1 did not significantly affect the stability of MicA. In contrast STEM2 disruption greatly affected the stability of MicA, suggesting that this stem-loop is extremely important for the protection of the sRNA against degradation.

We confirmed that Hfq binds to MicA at two distinct sites. Not only Hfq binds to the internal A/U-rich region that was its predicted binding site, but Hfq also binds to the 3' U-rich tail after the transcriptional terminator (**Chapter 3** results). As shown in **Chapter 2** Hfq can protect sRNAs from degradation. Therefore is not surprising that mutations disrupting the Hfq-binding sites lead to more unstable MicA's, probably because these MicA variants become more

vulnerable to the action of RNases. The mutations introduced in the 3' U-rich region resulted in more destabilized MicA than mutations introduced in the internal A/U-rich. However in both cases PNPase was shown to be the main enzyme involved in the degradation of these MicA variants.

Our work also contributed to a better understanding on how small RNAs control different targets. Although the 5' end of MicA is critical for repression of some targets (such as *omp*A expression) the 3' end of MicA may be more relevant for regulation of other targets (such as *tsx* mRNA). The two stem-loops present in MicA were found to affect the MicA targets in different ways. MicA STEM1 was shown to strongly affect *tsx* mRNA while barely affect *omp*A and *ecn*B mRNAs. On the other hand, MicA STEM2 was shown to affect *omp*A and *ecn*B mRNAs but hardly affected *tsx* mRNA. There are other sRNAs that interact with their mRNA targets through the stem-loops. OxyS RNA forms "kissing" complexes with its mRNA target *fhlA*. This interaction occurs between the two stem loops of OxyS RNA and two stem loops of *fhlA* mRNA (Argaman & Altuvia, 2000). Hfq is essential not only for the stability of the sRNA (Andrade et al., 2012) but also for the binding to the target (Moller et al., 2002; Kawamoto et al., 2006). Our results showed that the two Hfq binding sequences play distinct roles in the regulation of the different targets. Mutations in the A/U-rich region affected more strongly *omp*A mRNA levels than the other targets. Disruption of the 3' poly(U) tail of MicA had a more generalized effect on the mRNA targets tested. These results suggest that interaction of Hfq with the sRNA seems to greatly depend on the target itself or might require the interaction with additional factors. These results can most probably be extended to other regulatory RNAs and can be useful to design synthetic regulatory RNAs to program gene expression networks.

The role of 3'-5' exoribonucleases investigated by RNA-Seq

The *E. coli* exoribonucleases roles have been extensively studied (Andrade et al., 2009). However most studies are directed to their roles in the degradation of specific targets, but what are their global roles in the cell? How do exoribonucleases choose their targets? Are there specific metabolic pathways affected by a specific exoribonuclease? These are some of the questions that are still unanswered. There are not many studies carried out from a global perspective in *E. coli*. However there are two studies that addressed the role of ribonucleases in RNA degradation using DNA microarrays (Mohanty & Kushner, 2003; Bernstein et al., 2004). In both of these studies a PNPase mutant was analysed, in one study from a degradosome point of view (Bernstein et al., 2004) and in the other study the role of PNPase was compared with the role of RNase II in the decay of *E. coli* mRNAs (Mohanty & Kushner, 2003). PNPase role was shown to be independent of the degradosome assembly (Bernstein et al., 2004). PNPase deletion affected the steady-state levels of more mRNAs than inactivation of RNase II (Mohanty & Kushner, 2003). Also it was shown that a large number of *E. coli* mRNAs are decreased in the absence of RNase II, suggesting that this exoribonuclease can protect specific mRNAs from the activity of other ribonucleases (Marujo et al., 2000; Mohanty & Kushner, 2003). Both of these studies were done in exponential phase and provided valuable information about the role of ribonucleases in the mRNA decay. However DNA microarrays information is limited and nowadays there are other techniques much more advanced. RNA-Seq is the most advanced high-throughput technique. It uses deep-sequencing technologies and provides reliable information about the entire RNA content in the cell (Wang et al., 2009). Because is still a recent technique the monetary costs are high. For this reason our RNA-Seq results are based in only one sample for each condition and consequently all the results still need to be experimentally validated. Moreover there is still no straightforward bioinformatics

pipeline to analyse RNA-Seq data, meaning that the analysis of the data takes a long time and an extensive knowledge of bioinformatics.

The exoribonuclease levels in the cell are affected by many factors like stress, metabolites and growth phases (Cairrão et al., 2001; Cairrão et al., 2003; Chen & Deutscher, 2010; Gatewood & Jones, 2010). The majority of studies on the role of exoribonucleases are done in exponential phase, however in the last years this tendency has been reverted mainly because small RNAs are known to be up-regulated in stationary phase. For this reason we analysed the role of exoribonucleases on both exponential and stationary phase. The transition from exponential to stationary phase requires extensive gene expression rearrangements (Ishihama, 1997; Hengge-Aronis, 1999). Our results shown that, approximately 85% of all transcripts are down-regulated in stationary phase. Contrarily only 14% of the transcripts are up-regulated (**Chapter 4**). In stationary phase cells have limited nutrient availability (Kolter et al., 1993), therefore it is expected that the cells stop the transcription of most of the genes as a survival mechanism. However, because cells in stationary phase have to deal with several stresses some genes should be up-regulated (Navarro Llorens et al., 2010). In stationary phase the sigma factor *rpo*S interacts with RNA polymerase and controls the expression of about 10% of the *E. coli* genome (Battesti et al., 2011). We showed that RpoS levels are 38% higher in stationary phase. This values are in agreement with another report showing that the protein levels of RpoS are 30% higher in stationary phase (Jishage & Ishihama, 1995). Besides rpoS there are other transcripts that are important in stationary phase adaptation. For example, during stationary phase the 6S small RNA is up-regulated and supresses the σ^{70} dependent transcription (σ subunit involved in the transcription of most genes during exponential growth) (Wassarman & Storz, 2000). In spite the fact that our results are based on only one sample several transcripts levels are in agreement

with other published results. This allows us to have some confidence in our results.

Our results indicate that the PNPase roles overlap with RNase II and RNase R roles, but their roles differ from exponential to stationary phase. In exponential phase an RNase II mutation significantly affects 187 transcripts, the deletion of RNase R significantly affects the expression of 202 transcripts, while PNPase deletion affected 226 transcripts. This results are comparable with the DNA microarrays results (Mohanty & Kushner, 2003). Interestingly, most of the transcripts affected by RNase II deletion are down-regulated and belong to the functional category of flagellar assembly suggesting that this mutant may present motility deficiencies. This might be a consequence derived by the fact that in the absence of RNase II the Antigen-43 (promotes aggregation and inhibits bacterial motility (Ulett et al., 2006)) is up-regulated. Contrarily to RNase II, RNase R affected transcripts related mainly to anaerobic respiration suggesting that RNase R might play an essential role in the aerobic respiration process of *E. coli*. Curiously 41 transcripts that are affected by the RNase R deletion are also affected by RNase II deletion. However, most of these transcripts are down-regulated in the Δrnb mutant but up-regulated in the Δrnr mutant. This seems to indicate that RNase II have opposing roles to RNase R in the cell. On the other hand, PNPase shares some roles with both RNase II and RNase R exoribonucleases. However, PNPase apparently has a specific role in the regulation of stable RNAs (rRNAs, tRNAs and sRNAs). This is in accordance with other reports that highlighted the PNPase role in the degradation of small RNAs (Andrade & Arraiano, 2008; De Lay & Gottesman, 2011; Andrade et al., 2012; Pobre et al., 2012).

In stationary phase, like in exponential phase, there is an overlap between the roles of PNPase, RNase II and RNase R. However, in stationary phase RNase R

is clearly the most important exoribonuclease. The deletion of RNase R significantly affected 694 transcripts while deletion of RNase II affected 117 transcripts and deletion of PNPase affected 226 transcripts. Moreover the number of overlapped transcripts is higher in stationary phase than in exponential phase and consequently there is a higher overlap in the functional categories affected by the three exoribonucleases. In stationary phase it is more difficult to cluster the differentially expressed transcripts than it was for exponential phase. This suggests that in stationary phase the exoribonucleases have broader roles in the RNA metabolism than in exponential phase. Most of the functional categories affected by the exoribonucleases can be related to stress resistance (oxidative stress, nutrient depletion and so on). A possible explanation resides in the fact that in stationary phase, cells need to have a tighter control of gene expression to deal with all the stresses inherent to stationary phase (Navarro Llorens et al., 2010).

Our results show that RNase II and RNase R have different roles in the cell and in fact PNPase has a higher overlap with RNase II and RNase R than RNase II has with RNase R. This might explain why the double mutant Δrnb Δrnr is viable while the double mutants Δrnb Δpnp and Δrnr Δpnp are not (Cairrão et al., 2003). Although the most transcripts differentially expressed in the double mutant Δrnb Δrnr are the same as the transcripts differentially expressed in the single mutants, the double mutant is not an “addition” of both mutants. Our results might suggest that the cell can somehow compensate the deletion of the two hydrolytic exoribonucleases, RNase II and RNase R.

A vast majority of small RNAs are up-regulated in stationary phase and consequently most of the sRNA studies are done in this growth phase. Our results suggest that PNPase is the main exoribonuclease involved in the decay of sRNAs in

stationary phase. This is in agreement to the previous results that we had during this Doctoral work (**Chapter 2** and **Chapter 3**).

The work described in this Dissertation expanded our knowledge on RNA degradation pathways, highlighting the roles of exoribonucleases, Hfq and small RNA sequence and structures on the RNA decay mechanisms. Our work not only provided several important answers regarding RNA degradation but also opened new perspectives. The modulations of sRNAs to control different targets and the study of the unexpected roles of RNase II in motility and RNase R in anaerobic respiration are only a small part of the future work subsequent to this Doctoral thesis.

References

Andrade JM, Arraiano CM. 2008. PNPase is a key player in the regulation of small RNAs that control the expression of outer membrane proteins. *RNA 14*:543-551.

Andrade JM, Pobre V, Matos AM, Arraiano CM. 2012. The crucial role of PNPase in the degradation of small RNAs that are not associated with Hfq. *RNA 18*:844-855.

Andrade JM, Pobre V, Silva IJ, Domingues S, Arraiano CM. 2009. The role of 3'-5' exoribonucleases in RNA degradation. *Prog Mol Biol Transl Sci 85*:187-229.

Argaman L, Altuvia S. 2000. *fhl*A repression by *OxyS* RNA: kissing complex formation at two sites results in a stable antisense-target RNA complex. *J Mol Biol 300*:1101-1112.

Arraiano CM, Andrade JM, Domingues S, Guinote IB, Malecki M, Matos RG, Moreira RN, Pobre V, Reis FP, Saramago M, Silva IJ, Viegas SC. 2010. The critical role of RNA processing and degradation in the control of gene expression. *FEMS Microbiol Rev 34*:883-923.

Awano N, Rajagopal V, Arbing M, Patel S, Hunt J, Inouye M, Phadtare S. 2010. *Escherichia coli* RNase R has dual activities, helicase and RNase. *Journal of bacteriology 192*:1344-1352.

Balbontín R, Fiorini F, Figueroa-Bossi N, Casadesus J, Bossi L. 2010. Recognition of heptameric seed sequence underlies multi-target regulation by RybB small RNA in *Salmonella enterica*. *Mol Microbiol 78*:380-394.

Basineni SR, Madhugiri R, Kolmsee T, Hengge R, Klug G. 2009. The influence of Hfq and ribonucleases on the stability of the small non-coding RNA *OxyS* and its target *rpoS* in *E. coli* is growth phase dependent. *RNA Biol 6*:584-594.

Battesti A, Majdalani N, Gottesman S. 2011. The RpoS-mediated general stress response in *Escherichia coli*. *Annual review of microbiology 65*:189-213.

Bernstein JA, Lin PH, Cohen SN, Lin-Chao S. 2004. Global analysis of *Escherichia coli* RNA degradosome function using DNA microarrays. *Proc Natl Acad Sci U S A 101*:2758-2763.

Bossi L, Figueroa-Bossi N. 2007. A small RNA downregulates LamB maltoporin in *Salmonella*. *Mol Microbiol 65*:799-810.

Cairrão F, Chora A, Zilhão R, Carpousis AJ, Arraiano CM. 2001. RNase II levels change according to the growth conditions: characterization of *gmr*, a new *Escherichia coli* gene involved in the modulation of RNase II. *Mol Microbiol 39*:1550-1561.

Cairrão F, Cruz A, Mori H, Arraiano CM. 2003. Cold shock induction of RNase R and its role in the maturation of the quality control mediator SsrA/tmRNA. *Mol Microbiol 50*:1349-1360.

Cannistraro VJ, Kennell D. 1999. The reaction mechanism of ribonuclease II and its interaction with nucleic acid secondary structures. *Biochem et Biophys Acta 1433*:170-187.

Chen C, Deutscher MP. 2010. RNase R is a highly unstable protein regulated by growth phase and stress. *RNA 16*:667-672.

Cheng ZF, Deutscher MP. 2002. Purification and characterization of the *Escherichia coli* exoribonuclease RNase R. Comparison with RNase II. *J Biol Chem 277*:21624-21629.

Cheng ZF, Deutscher MP. 2003. Quality control of ribosomal RNA mediated by polynucleotide phosphorylase and RNase R. *Proc Natl Acad Sci USA 100*:6388-6393.

De Lay N, Gottesman S. 2011. Role of polynucleotide phosphorylase in sRNA function in *Escherichia coli*. *RNA 17*:1172-1189.

Gatewood ML, Jones GH. 2010. (p)ppGpp inhibits polynucleotide phosphorylase from streptomyces but not from *Escherichia coli* and increases the stability of bulk mRNA in *Streptomyces coelicolor*. *Journal of bacteriology 192*:4275-4280.

Geissmann TA, Touati D. 2004. Hfq, a new chaperoning role: binding to messenger RNA determines access for small RNA regulator. *EMBO J 23*:396-405.

Gogol EB, Rhodius VA, Papenfort K, Vogel J, Gross CA. 2011. Small RNAs endow a transcriptional activator with essential repressor functions for single-tier control of a global stress regulon. *Proc Natl Acad Sci U S A 108*:12875-12880.

Hengge-Aronis R. 1999. Interplay of global regulators and cell physiology in the general stress response of *Escherichia coli*. *Curr Opin Microbiol 2*:148-152.

Ishihama A. 1997. Adaptation of gene expression in stationary phase bacteria. *Curr Opin Genet Dev 7*:582-588.

Jishage M, Ishihama A. 1995. Regulation of RNA polymerase sigma subunit synthesis in *Escherichia coli*: intracellular levels of sigma 70 and sigma 38. *Journal of bacteriology 177*:6832-6835.

Kawamoto H, Koide Y, Morita T, Aiba H. 2006. Base-pairing requirement for RNA silencing by a bacterial small RNA and acceleration of duplex formation by Hfq. *Mol Microbiol 61*:1013-1022.

Kolter R, Siegele DA, Tormo A. 1993. The stationary phase of the bacterial life cycle. *Annu Rev Microbiol 47*:855-874.

Marujo PE, Hajnsdorf E, Le Derout J, Andrade R, Arraiano CM, Régnier P. 2000. RNase II removes the oligo(A) tails that destabilize the *rpsO* mRNA of *Escherichia coli*. *RNA 6*:1185-1193.

Massé E, Escorcia FE, Gottesman S. 2003. Coupled degradation of a small regulatory RNA and its mRNA targets in *Escherichia coli*. *Genes Dev 17*:2374-2383.

Mohanty BK, Kushner SR. 2003. Genomic analysis in *Escherichia coli* demonstrates differential roles for polynucleotide phosphorylase and RNase II in mRNA abundance and decay. *Mol Microbiol 50*:645-658.

Mohanty BK, Maples VF, Kushner SR. 2004. The Sm-like protein Hfq regulates polyadenylation dependent mRNA decay in *Escherichia coli*. *Mol Microbiol 54*:905-920.

Moll I, Afonyushkin T, Vytvytska O, Kaberdin VR, Blasi U. 2003. Coincident Hfq binding and RNase E cleavage sites on mRNA and small regulatory RNAs. *RNA 9*:1308-1314.

Moller T, Franch T, Hojrup P, Keene DR, Bachinger HP, Brennan RG, Valentin-Hansen P. 2002. Hfq: a bacterial Sm-like protein that mediates RNA-RNA interaction. *Mol Cell 9*:23-30.

Navarro Llorens JM, Tormo A, Martinez-Garcia E. 2010. Stationary phase in gram-negative bacteria. *FEMS Microbiol Rev 34*:476-495.

Otaka H, Ishikawa H, Morita T, Aiba H. 2011. PolyU tail of *rho*-independent terminator of bacterial small RNAs is essential for Hfq action. *Proc Natl Acad Sci U S A 108*:13059-13064.

Papenfort K, Bouvier M, Mika F, Sharma CM, Vogel J. 2010. Evidence for an autonomous 5' target recognition domain in an Hfq-associated small RNA. *Proc Natl Acad Sci U S A 107*:20435-20440.

Papenfort K, Vogel J. 2009. Multiple target regulation by small noncoding RNAs rewires gene expression at the post-transcriptional level. *Research in microbiology 160*:278-287.

Pobre V, Andrade JM, Arraiano CM. 2012. Small RNA modules confer different stabilities and selectively choose among different targets. *PLoS One Accepted with modification.*

Rasmussen AA, Eriksen M, Gilany K, Udesen C, Franch T, Petersen C, Valentin-Hansen P. 2005. Regulation of *omp*A mRNA stability: the role of a small regulatory RNA in growth phase-dependent control. *Mol Microbiol 58*:1421-1429.

Rice JB, Vanderpool CK. 2011. The small RNA *SgrS* controls sugar-phosphate accumulation by regulating multiple PTS genes. *Nucleic Acids Res 39*:3806-3819.

Sauer E, Weichenrieder O. 2011. Structural basis for RNA 3'-end recognition by Hfq. *Proc Natl Acad Sci U S A*.

Slomovic S, Portnoy V, Yehudai-Resheff S, Bronshtein E, Schuster G. 2008. Polynucleotide phosphorylase and the archaeal exosome as poly(A)-polymerases. *Biochim Biophys Acta 1779*:247-255.

Spickler C, Mackie A. 2000. Action of RNases II and Polynucleotide Phosphorylase against RNAs containing stem-loops of defined structure. *J Bacteriol 182*:2422-2427.

Udekwu KI, Darfeuille F, Vogel J, Reimegard J, Holmqvist E, Wagner EG. 2005. Hfq-dependent regulation of *Omp*A synthesis is mediated by an antisense RNA. *Genes Dev 19*:2355-2366.

Ulett GC, Webb RI, Schembri MA. 2006. Antigen-43-mediated autoaggregation impairs motility in *Escherichia coli*. *Microbiology 152*:2101-2110.

Urban JH, Vogel J. 2007. Translational control and target recognition by *Escherichia coli* small RNAs *in vivo*. *Nucleic Acids Res 35*:1018-1037.

Viegas SC, Arraiano CM. 2008. Regulating the regulators: How ribonucleases dictate the rules in the control of small non-coding RNAs. *RNA Biol 5*:230-243.

Viegas SC, Silva IJ, Saramago M, Domingues S, Arraiano CM. 2011. Regulation of the small regulatory RNA MicA by ribonuclease III: a target-dependent pathway. *Nucleic Acids Res 39*:2918-2930.

Wang Z, Gerstein M, Snyder M. 2009. RNA-Seq: a revolutionary tool for transcriptomics. *Nature reviews Genetics 10*:57-63.

Wassarman KM, Storz G. 2000. 6S RNA regulates *E. coli* RNA polymerase activity. *Cell 101*:613-623.

Appendix

Publications

REVIEW ARTICLE

The critical role of RNA processing and degradation in the control of gene expression

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RNases; RNA; post-transcriptional control of gene expression.

Abstract

The continuous degradation and synthesis of prokaryotic mRNAs not only give rise to the metabolic changes that are required as cells grow and divide but also rapid adaptation to new environmental conditions. In bacteria, RNAs can be degraded by mechanisms that act independently, but in parallel, and that target different sites with different efficiencies. The accessibility of sites for degradation depends on several factors, including RNA higher-order structure, protection by translating ribosomes and polyadenylation status. Furthermore, RNA degradation mechanisms have shown to be determinant for the post-transcriptional control of gene expression. RNases mediate the processing, decay and quality control of RNA. RNases can be divided into endonucleases that cleave the RNA internally or exonucleases that cleave the RNA from one of the extremities. Just in *Escherichia coli* there are $>$ 20 different RNases. RNase E is a single-strand-specific endonuclease critical for mRNA decay in *E. coli*. The enzyme interacts with the exonuclease polynucleotide phosphorylase (PNPase), enolase and RNA helicase B (RhlB) to form the degradosome. However, in *Bacillus subtilis*, this enzyme is absent, but it has other main endonucleases such as RNase J1 and RNase III. RNase III cleaves double-stranded RNA and family members are involved in RNA interference in eukaryotes. RNase II family members are ubiquitous exonucleases, and in eukaryotes, they can act as the catalytic subunit of the exosome. RNases act in different pathways to execute the maturation of rRNAs and tRNAs, and intervene in the decay of many different mRNAs and small noncoding RNAs. In general, RNases act as a global regulatory network extremely important for the regulation of RNA levels.

Introduction

General outline

Many cellular mechanisms cannot be fully understood without a profound knowledge of the RNA metabolism. Protein production depends not only on the levels of mRNAs but also on other RNA species. The translation of mRNAs is mediated by tRNAs and rRNAs and functional RNAs also intervene in the regulation of gene expression. Synergies between the structure and function of RNAs contribute towards orchestrating their fundamental role in cell viability.

Bacterial mRNAs are rapidly degraded and this allows the microorganisms to rapidly adapt to changing environments. Even though transcription is quite important to determine steady-state levels, increasingly it is being established that the role of post-transcriptional control is critical in the regulation of gene expression. Analyzing RNA degradation in prokaryotes has been particularly difficult not only due to the coupling of transcription, translation and mRNA degradation but also because most mRNAs undergo a rapid exponential decay with an average of 1.3 min at 37 °C. The rRNAs and tRNAs are usually more stable, but in order to be functionally active, they have to be processed to the mature form. It has been shown that the levels of small noncoding RNAs (sRNAs) are also highly dependent on post-transcriptional events. The knowledge collected makes it clear how far our understanding of RNA degradation has come in the last few years and how much remains to be discovered about this important genetic regulatory process. Applications of this knowledge in medicine and biotechnology are underway.

FEMS MICROBIOLOGY REVIEWS

RNases are the enzymes that intervene in the processing, degradation and quality control of all types of RNAs. A limited number of RNases can exert a determinant level of control acting as a global regulatory network, monitoring and adapting the RNA levels to the cell needs. Many of them are essential, but others exhibit a functional overlap and are interchangeable. RNases can act alone or they can cooperate in RNA degradation complexes. During RNA degradation, they do not only act as 'molecular killers' eliminating RNA species. RNases act according to the requirements of growth in adaptation to the environment; they play an extremely important role in contributing to the recycling of ribonucleotides, and also carry out surveillance, destroying aberrant RNAs that would produce detrimental proteins.

Individual RNA species differ widely with respect to their stability. The rate of turnover has no relation to the length of the gene, the segments that decay more rapidly can be anywhere in the mRNA and the stability of the gene transcripts seems to be regulated by determinants localized to specific mRNA segments. Secondary structure features can also influence the degradation by RNases.

Several factors can intervene in the decay mechanism: the sequence/structure of RNAs can act as stabilizer or destabilizer elements to specific RNases; the presence of ribosomes during active translation can hide some RNA loci that are vulnerable to RNases; poly(A) stretches are the preferred substrate for several RNases – therefore, the addition of poly(A) tails can modulate the stability of full-length transcripts and degradation intermediates and accelerate the decay of defective stable RNAs; *trans*-acting factors can bind to the RNAs and expose or hide RNA sites that are preferential targets for RNases – for instance, the host factor Hfq is known to bind sRNAs and affect their turnover; and other factors such as helicases can act in *trans* and contribute to RNA degradation because they unwind RNA structures and can change their accessibility to RNases.

In this review, we will focus on RNA processing and degradation in *Escherichia coli*, but we will also provide comparative examples from many other microorganisms. Namely, we will include the description of enzymes that exist in *Bacillus subtilis* and are absent in *E. coli*, we will provide examples from archaea and we will also include a section that makes a parallel to what happens in yeast.

We will start by describing most of the known RNases, characterizing their structure and function and the regulation of their expression. They will be divided into endonucleases, which cleave the RNA internally, and exonucleases, which cleave the RNA from one of the extremities. After the characterization of RNases, we will focus on their protein complexes involved in decay mechanisms. Then we will focus on the 'RNases in action'. Examples will be provided regarding the processing and degradation of RNAs. We will describe the maturation of rRNAs and tRNAs, and characterize the decay of many different mRNAs and sRNAs. Finally, we will compare with what is known in eukaryotic microorganisms, namely yeast. A small degree of overlap is unavoidable between sections on related topics. This allows for each section to be read and understood as an independent unit.

This review is intended to be an exhaustive and updated overview of what is known on RNAs, RNases and the post-transcriptional control of gene expression in microorganisms. It is expected that it can be used as a reference to put in perspective the critical role of RNA processing and degradation as a major global regulatory network.

Endonucleases

RNase E

RNase E, encoded by the *rne* gene, was first identified by a temperature-sensitive mutation (*rne-3071*) (Apirion & Lassar, 1978) and was initially described as an activity required for the processing of the *E. coli* 9S rRNA gene (Ghora & Apirion, 1978). The *ams* (altered mRNA stability) locus was also identified by a temperature-sensitive mutation (*ams-1*) (Ono & Kuwano, 1980) and was shown to play an important role in *E. coli* RNA turnover (Ono & Kuwano, 1979). The combination of the Ams and RNase II ts-alleles plus deficiency in polynucleotide phosphorylase (PNPase) was shown to substantially increase the half-life of bulk mRNA, and specific messengers were highly stabilized in the *ams-1 rnb-500 pnp-7* mutant (Arraiano *et al.*, 1988). Later, it was shown that these two previously identified genes, *rne* and *ams*, were actually different mutant alleles of the same gene encoding RNase E (Mudd *et al.*, 1990; Babitzke & Kushner, 1991; Melefors & von Gabain, 1991; Taraseviciene *et al.*, 1991). This important endonuclease is essential for cell growth, and the inactivation of temperature-sensitive mutants impedes processing and prolongs the lifetime of bulk mRNA (Apirion & Lassar, 1978; Ono & Kuwano, 1979; Arraiano *et al.*, 1988; Mudd *et al.*, 1990; Babitzke & Kushner, 1991; Melefors & von Gabain, 1991; Taraseviciene *et al.*, 1991). It has been reported that RNase E plays a central role in the processing of precursors of the 5S rRNA gene (Apirion & Lassar, 1978; Misra & Apirion, 1979), the 16S rRNA gene (Li *et al.*, 1999b), tRNAs (Ow & Kushner, 2002), transfer mRNA (tmRNA) (Lin-Chao *et al.*, 1999) and the M1 RNA component of the RNase P ribozyme (Lundberg & Altman, 1995; Ko *et al.*, 2008). Homologues of RNase E have been identified in > 50 bacteria, archaea and plants (Lee & Cohen, 2003).

Escherichia coli RNase E is a 1061-residue enzyme composed of two distinct functional regions (Fig. 1a). The amino-terminal half forms the catalytic domain (residues 1–529) and is relatively conserved among prokaryotes (Marcaida *et al.*, 2006). The carboxy-terminal half of RNase

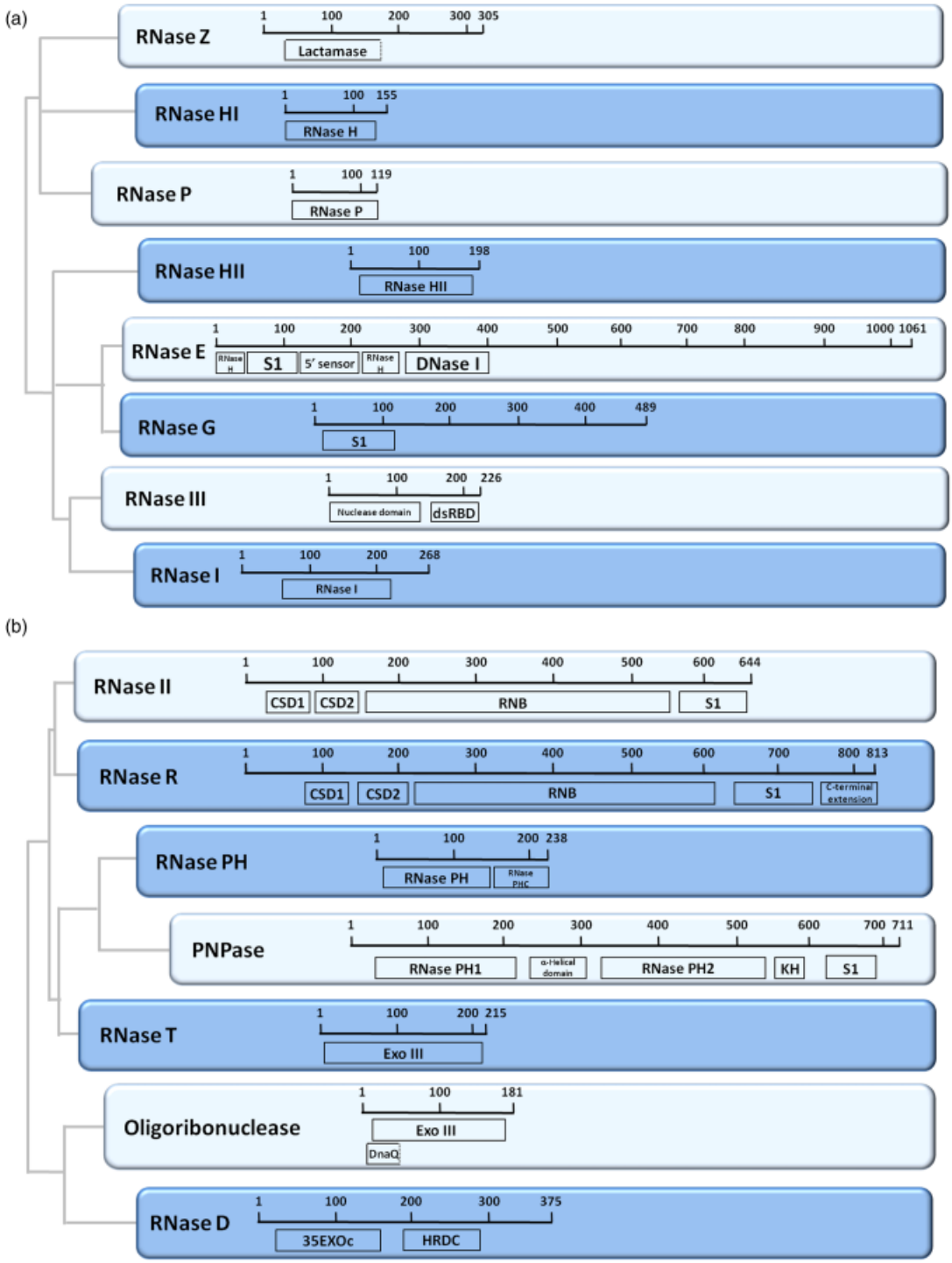

Fig. 1. Representative dendrograms of the endoribonucleases (a) and exoribonucleases (b) of *Escherichia coli*. This representation was based on the amino acid sequence of each enzyme, after a multiple alignment using the CLUSTAL program (Thompson *et al*., 1997). Near each enzyme is the length (number of amino acids) and architecture, emphasizing the domains of each enzyme. This representation was made based on the CDART program (Geer *et al*., 2002). These dendrograms were adapted from Barbas *et al*. (2006).

E (residues 530–1061) is a noncatalytic region, largely unstructured and poorly conserved (Callaghan *et al*., 2004). Segment A is located between residues 565 and 582 and is responsible for binding of RNase E to the inner cytoplasmic membrane (Khemici *et al*., 2008). Residues 601–700 form an arginine-rich segment that binds RNA *in vitro* and that is

believed to enhance the activity of RNase E in mRNA degradation *in vivo* (Lopez *et al.*, 1999; Ow *et al.*, 2000). Residues 701–1061 form a scaffold for interactions between RNase E and the other major components of the degradosome, a protein complex involved in mRNA decay (see Complexes of RNases) (Kaberdin *et al.*, 1998; Vanzo *et al.*, 1998).

The first crystal structure for a member of the RNase E family has been determined at 2.9 Å, and it reveals that the catalytic domain of RNase E forms a homotetramer with a molecular mass of roughly 260 kDa, organized as a dimer of dimers (Callaghan *et al.*, 2005a). Each protomer is composed of two globular portions: the 'large' and 'small' domains. The 'large' domain can be divided into four subdomains that closely resemble established folds. One is related to the RNase H endoribonuclease family, but is inactive. In this subdomain an S1 domain is embedded and has a fold that participates in the recognition of the 5′ terminus of RNA (5′-sensor). The rest of the large domain is similar to the repetitive structural element within the endodeoxyribonuclease DNase I. In isolation, each protomer appears elongated, with a large domain comprising the subdomains (S1, 5′-sensor, RNase H and DNase I), an elongated linker region (Zn-link) and then the small domain. The dimer–dimer interface is formed by the small domains. At the junction point, there is a zinc-binding site (Callaghan *et al.*, 2005a, b). The arrangement of the domains within each dimer resembles the blades and handles of an open pair of scissors.

Escherichia coli RNase E is a single-stranded, nonspecific endonuclease with a preference for cleaving A/U-rich sequences (Mackie, 1992; McDowall *et al.*, 1995). *In vitro* experiments have shown that purified *E. coli* RNase E prefers to cleave RNAs that are monophosphorylated at the 5′ end (Mackie, 1998). Recently, it was shown that RNA pyrophosphohydrolase (RppH) converts the 5′ terminus of primary transcripts from a triphosphate to a monophosphate (Celesnik *et al.*, 2007; Deana *et al.*, 2008). However, some structured substrates can be cleaved independent of its state of phosphorylation by RNase E even if the 5′ end forms a secondary structure (Baker & Mackie, 2003; Hankins *et al.*, 2007). This indicates that while the 5′-monophosphate-dependent pathway makes a significant contribution to mRNA degradation (Mackie, 1998, 2000), there is another pathway of initial substrate recognition by RNase E termed 'bypass' or 'internal entry' (Baker & Mackie, 2003; Kime *et al.*, 2009).

The crystal structure explains some features of the protein and suggests a mechanism of RNA recognition and cleavage. A pocket is formed between the 5′-sensor and the RNase H subdomains and can bind a monophosphate group at a 5′ end (Callaghan *et al.*, 2005a). The catalytic site is physically separated from the 5′-sensing site. It contains conserved residues on the surface of the DNase I subdomain of RNase E and coordinate a magnesium ion implicated in catalysis. A 'mouse-trap' model for communication between the 5′-sensing pocket and the site of catalysis has been suggested: S1- and 5′-sensing domains move together as one body to clamp down the substrate (Koslover *et al.*, 2008). This conformational change suggests a mechanism of RNA recognition and catalysis that explains the enzyme's preference for substrates with a 5′-monophosphate over a 5′-triphosphate and 5′-hydroxyl RNA. Substantial flexibility was also observed at one of the dimer–dimer interfaces, a deformation that may be essential to accommodate structured RNA for processing by internal entry.

The cellular level and activity of RNase E are subject to complex regulation. First, the enzyme concentration in the cell is regulated by a feedback loop in which RNase E modulates the decay of its own mRNA, maintaining the level of the enzyme within a narrow range (Mudd & Higgins, 1993; Jain & Belasco, 1995; Diwa *et al.*, 2000; Sousa *et al.*, 2001; Ow *et al.*, 2002). Second, the efficiency of RNase E cleavage depends on the structure of the substrates and the accessibility of putative cleavage sites. A 5′-monophosphate in substrate RNAs serves as an allosteric activator of RNase E activity (Mackie, 1998; Jiang & Belasco, 2004). Third, interactions of mRNA targets with Hfq and sRNAs play an important role in the cleavage of certain mRNAs by RNase E (Wagner *et al.*, 2002). Fourth, the activity of RNase E is globally affected by protein inhibitors, namely the L4 ribosomal protein, RraA and RraB (the regulator of RNase activity A and B, respectively) that interact with RNase E and inhibit RNase E endonucleolytic cleavages of a selective group of transcripts (Lee *et al.*, 2003; Gao *et al.*, 2006). Fifth, the membrane localization of RNase E and its association with the bacterial cytoskeleton may affect its function through various mechanisms (Liou *et al.*, 2001; Khemici *et al.*, 2008; Taghbalout & Rothfield, 2008).

Some variants of RNase E can be found in *Alphaproteobacteria*, *Synechocystis* spp. and in the high G+C Gram-positive bacteria (Condon & Putzer, 2002). In *Rhodobacter capsulatus*, RNase E is the enzyme responsible for the majority of the endonucleolytic cleavages. *Rhodobacter capsulatus* RNase E (118 kDa) has a conserved N-terminal region (Jäger *et al.*, 2001) and a C-terminal portion, probably involved in the scaffold of degradosome assembly. It was purified in two different complexes: one where it is associated with a helicase and an unidentified protein and the other, which was coupled with a helicase, Rho and an unidentified protein (Jäger *et al.*, 2001). Moreover, in *R. capsulatus*, this enzyme is involved in the endonucleolytic processing and stabilization of *cspA* mRNA (Jäger *et al.*, 2004). Similar to *R. capsulatus*, *Pseudomonas syringae*, a psychrophilic bacterium, also has an RNase E that is associated with RNase R and the DEAD-box helicase RhlE in a degradosome (see Complexes of RNases) (Purusharth *et al.*, 2005).

RNase G

Escherichia coli RNase G was initially identified by its role in chromosome segregation and cell division (Okada *et al.*, 1994). Overproduction of this protein led to morphological changes in which the bacteria formed anucleated chained cells containing long axial filaments, justifying its former name, *cafA* (cytoplasmic axial filament) (Okada *et al.*, 1994). RNase G was subsequently shown to exhibit endonuclease activity both *in vivo* (Li *et al.*, 1999b; Wachi *et al.*, 1999; Umitsuki *et al.*, 2001) and *in vitro* (Jiang *et al.*, 2000; Tock *et al.*, 2000). RNase G is a paralogue of RNase E (McDowall *et al.*, 1993), belonging to the RNase E/G family, and is also involved in the degradation and processing of RNA (Carpousis *et al.*, 2009).

A strong resemblance has been identified between RNase G and the amino-terminal portion of *E. coli* RNase E, sharing a high level of sequence identity (35%) and similarity (50%) (McDowall *et al.*, 1993) (Fig. 1a). Purified RNase G has *in vitro* properties similar to RNase E and both enzymes are required for a two-step sequential reaction of 5′ maturation of the 16S rRNA gene (Li *et al.*, 1999b; Wachi *et al.*, 1999). Their activity is 5′ end dependent and both RNases attack substrates in A+U-rich regions (Jiang *et al.*, 2000; Tock *et al.*, 2000). Moreover, residues of RNase E that can contact a 5′-monophosphorylated end and coordinate the catalytic magnesium ion are conserved in RNase G (McDowall *et al.*, 1993; Callaghan *et al.*, 2005a). RNase G seems to have a higher preference for 5′-monophosphorylated substrates than RNase E (Tock *et al.*, 2000) and the precise cleavage sites of RNase E and RNase G are not strictly conserved (Li *et al.*, 1999b; Tock *et al.*, 2000). The 5′-monophosphate end, which stimulates RNase G, is generated by RppH (Deana *et al.*, 2008) or by other endonucleases (Lee *et al.*, 2002).

Whereas cells lacking RNase E are normally nonviable (Apirion & Lassar, 1978; Ono & Kuwano, 1979), RNase G is dispensable for viability (Li *et al.*, 1999b; Wachi *et al.*, 1999) and is present in lower abundance (Lee *et al.*, 2002). Some functional homology between RNase G and RNase E was suggested by the observations that RNase G expression can confer viability to the *rne* deletion mutant strain (Lee *et al.*, 2002). However, at intracellular physiological levels, RNase G cannot complement RNase E mutations (Lee *et al.*, 2002; Ow *et al.*, 2003). Recently, single amino acid changes in the predicted RNase H domain of RNase G led to complementation of RNase E deletion mutants, suggesting that this region of the two proteins may help distinguish their *in vivo* biological activities (Chung *et al.*, 2010). However, these RNase G mutant proteins do not fully substitute RNase E in mRNA decay and tRNA processing (Chung *et al.*, 2010).

Microarray data showed that RNase G controls the level of transcripts associated with sugar metabolism centered on glycolysis (*adhE*, *pgi*, *glk*, *nagB*, *acs*, *eno*, *tpiA*) (Lee *et al.*, 2002), and it has been shown that strains defective in RNase G produce increased levels of pyruvic acid (Sakai *et al.*, 2007). These results suggest that RNase G is involved in the regulation of central metabolism.

RNase III

RNase III was originally identified by Robertson *et al.* (1968) in extracts of *E. coli* as the first specific double-stranded RNA (dsRNAs) endoribonuclease. Members of the RNase III family are widely distributed among prokaryotic and eukaryotic organisms, sharing structural and functional features (Lamontagne *et al.*, 2001) (Fig. 1a). However, until now, homologues of RNase III have not been found in the genomes of archaea (Condon & Putzer, 2002). All enzymes of this family are hydrolytic and have a specificity for dsRNAs, generating 5′-monophosphate and 3′-hydroxyl termini with a two-base overhang at the 3′ end (Meng & Nicholson, 2008). The RNase III family comprises four classes, according to their polypeptide structure. The class I is the simplest, containing an endonuclease domain (NucD), characterized in several bacteria by the presence of a highly conserved amino acid stretch NERLEFLGDS, and a dsRNA-binding domain (dsRBD) (Blaszczyk *et al.*, 2001). The class II is exemplified by the *Drosophila melanogaster* Drosha protein, which contains a long N-terminal extension, followed by two NucD and a single dsRBD. The class III is represented by Dicer, which has an N-terminal helicase/ATPase domain, followed by a domain of unknown function (DUF283), a centrally positioned Piwi Argonaute Zwille (PAZ) domain and a C-terminal configuration like Drosha, consisting of two NucD and one dsRBD (Drider & Condon, 2004; MacRae & Doudna, 2007). Finally, the class IV is only represented, to date, by the Mini-RNase III of *B. subtilis*, which is constituted by a single NucD domain (Redko *et al.*, 2008).

The class I members of the RNase III family are ubiquitously found in bacteria, bacteriophages and some fungi (MacRae & Doudna, 2007). *Escherichia coli* RNase III has served as the prototypical member of the family. In this model microorganism, RNase III is encoded by the *rnc* gene, and is active as a 52 kDa homodimer (Li & Nicholson, 1996). Each monomer contains a C-terminal dsRBD, located in the last 74 amino acids, which is responsible for substrate recognition and adopts a tertiary fold with the characteristic α_1-β_1-β_2-β_3-α_2 structure that is conserved throughout the RNase III family (Blaszczyk *et al.*, 2001). Additionally, each monomer contains an N-terminal NucD. When the two monomers are combined (RNase III homodimer), they form a single processing center in the subunit interface, in which each monomer contributes to the hydrolysis of one RNA strand of the duplex substrate. Ji and

colleagues (Blaszczyk *et al.*, 2004; Gan *et al.*, 2006) resolved the structure of the hyperthermophilic bacteria *Aquifex aeolicus* RNase III and the data have revealed two functional forms of dsRNA binding by RNase III: a catalytic form, functioning as a dsRNA-processing enzyme, cleaving both natural and synthetic dsRNA, and a noncatalytic form, in which RNase III plays the role of a dsRNA-binding protein (without cleaving). The latter activity is in agreement with previous studies in which this enzyme binds certain substrates in order to influence gene expression, affecting RNA structures (Court, 1993; Oppenheim *et al.*, 1993; Dasgupta *et al.*, 1998; Calin-Jageman & Nicholson, 2003). Furthermore, magnesium (Mg^{2+}) is the preferred cofactor. Recent data are indicative that each active site contains two divalent cations during substrate hydrolysis (Meng & Nicholson, 2008).

The RNase III substrate selection consists of a combination of structural determinants and sequence elements referred to as reactivity epitopes, such as the helix length, the strength of base-pairing or the occurrence of specific nucleotide pairs (termed proximal and distal boxes) located at defined positions related to the cleavage site. In addition, there are also two classes of double-helical elements that can function as negative determinants, which can either inhibit the recognition of this endoribonuclease or suppress the cleavage (without affecting recognition) (Zhang & Nicholson, 1997; Pertzev & Nicholson, 2006b).

RNase III in *E. coli* is not essential; however, it was observed that mutants for this endoribonuclease have a slow-growth phenotype (Nicholson, 1999). This enzyme was initially identified due to its role in the maturation of tRNA precursors and rRNA. Regarding the maturation of rRNA, RNase III is involved in the processing of 16S and 23S from a 30S rRNA gene precursor (Babitzke *et al.*, 1993). In *Salmonella* and other members of *Alphaproteobacteria*, RNase III is also responsible for the cleavage of the intervening sequences (IVS) found in their 23S rRNA gene (Evguenieva-Hackenberg & Klug, 2000), and is also involved in the decay of several mRNA species (Condon & Putzer, 2002; Calin-Jageman & Nicholson, 2003). For example, in *E. coli*, this enzyme participates in the first step of the decay of *pnp* mRNA (Régnier & Portier, 1986), the gene encoding PNPase, downregulating its synthesis (Régnier & Grunberg-Manago, 1990; Robert-Le Meur & Portier, 1992; Jarrige *et al.*, 2001). Interestingly, this endoribonuclease also has the ability to regulate its own synthesis with a specific cleavage near the 5′ end of its own mRNA that removes a stem loop, which acts as a degradation barrier (Bardwell *et al.*, 1989; Matsunaga *et al.*, 1996).

RNase III participates as a stress response modulator, controlling the steady-state levels of genes involved in cellular adaptation to stress (Santos *et al.*, 1997; Freire *et al.*, 2006; Sim *et al.*, 2010). It was seen in *Salmonella typhimurium* that RNase III regulates the levels of the sRNA MicA (Viegas *et al.*, 2007), a main regulator of the abundant outer membrane protein OmpA that plays an important structural role in the cell and is involved in pathogenesis (Guillier *et al.*, 2006). The enzyme is also involved in the decay of sRNA/mRNA complexes upon translational silencing (Vogel *et al.*, 2004; Afonyushkin *et al.*, 2005; Huntzinger *et al.*, 2005; Kaberdin & Blasi, 2006). In this way, cleavage by RNase III within the sRNA/mRNA duplex and the resulting subsequent decay of the mRNA intermediate by the *E. coli* RNA decay machinery could resemble the RNA interference (RNAi) in the eukaryotic cells (Agrawal *et al.*, 2003). RNAi is an evolutionarily conserved phenomenon that functions as a safeguard for the maintenance of genomic integrity. This phenomenon allows the selective post-transcriptional downregulation of target genes in the cells, in which RNase III-like enzymes dictate the degradation of dsRNA molecules (Jagannath & Wood, 2007; Ma *et al.*, 2007; Jinek & Doudna, 2009). Accordingly, the RNase III family has been associated with gene expression regulation, potential antivirus agents and tumor suppressors (Lamontagne *et al.*, 2001).

Bs-RNase III is a homologue of *E. coli* RNase III in *B. subtilis*. It is a 28-kDa protein (Mitra & Bechhofer, 1994), encoded by the *rncS* gene (Mitra & Bechhofer, 1994; Herskovitz & Bechhofer, 2000). In contrast to *E. coli* and *Staphylococcus aureus*, where the RNase III gene can be deleted without loss of viability, in *B. subtilis* and in the yeast, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, this enzyme is essential (Huntzinger *et al.*, 2005). Although the local environment of the site of Bs-RNase III cleavage appears to be very similar to that of *E. coli* RNase III, there are important differences in their substrate specificity (Mitra & Bechhofer, 1994; Wang & Bechhofer, 1997). Some of the substrates for this enzyme are the 30S ribosomal precursor RNA (Wang & Bechhofer, 1997) and the small cytoplasmic RNA (scRNA) (Oguro *et al.*, 1998; Yao *et al.*, 2007). More recently, another RNase III-like protein was identified in *B. subtilis* called Mini-III, reported to be involved in 23S rRNA gene maturation (Redko *et al.*, 2008). Interestingly, like Bs-RNaseIII, Mini-III does not seem to have endogenous mRNA substrates (Bechhofer, 2009). In *Lactococcus lactis*, RNase III is encoded by the *rnc* gene and plays a determinant role in the control of *citQRP* mRNA stability (Drider *et al.*, 1998, 1999). Complementation assays performed in *E. coli* showed that *L. lactis* RNase III can process *E. coli* rRNAs and regulate the levels of PNPase mRNA, substituting the endogenous RNase III (Amblar *et al.*, 2004).

Taken together, the functional and evolutionary conservation of the RNase III family in bacteria and higher organisms is indicative of their biological relevance in RNA maturation and degradation. Despite the fact that RNase E is

considered the major RNase that catalyzes the initial rate-determining cleavage of several transcripts, the RNase III family of enzymes has emerged as one of the most important groups of endoribonucleases in the control of RNA stability (Jaskiewicz & Filipowicz, 2008).

RNase H

Both RNase III and RNase H are representatives of components of the RNAi machinery and both are Mg^{2+}-dependent hydrolytic endoribonucleases. The analysis of the crystal structure of *E. coli* RNase H (Yang *et al.*, 1990) revealed the stepwise participation of two magnesium atoms in the enzyme mechanism (Nowotny & Yang, 2006).

RNases H are enzymes that cleave the RNA of RNA/DNA hybrids that are formed during replication and repair, preventing aberrant chromosome replication (for a review, see Condon & Putzer, 2002; Worrall & Luisi, 2007; Tadokoro & Kanaya, 2009). It is a ubiquitous enzyme distributed among all domains of life, and three different RNase H enzymes have been identified (HI, HII and HIII) (Ohtani *et al.*, 1999). In *E. coli*, 95% of RNase H activity is provided by RNase HI (widely distributed in *Proteobacteria*) and the remainder by RNase HII (Fig. 1a). In *B. subtilis*, RNase H activity is mostly provided by RNase HII and HIII. RNase H activity is essential to both bacteria. Thus, the inactivation of one of the *rnh* genes, but not both, is tolerated in these two organisms (Itaya *et al.*, 1999; Ohtani *et al.*, 1999).

RNase HII is widely distributed in bacteria and archaea, while RNase HIII is only present in a limited number of bacteria (Ohtani *et al.*, 1999). Proteins similar to HI and HII (named H1 and H2, respectively) can also be found in eukaryotes, but are larger and more complex than their prokaryotic counterparts (see Cerritelli & Crouch, 2009 for a review). The RNase H domain was also described in retroviruses (RNase HI), where it is associated with a reverse transcriptase (Davies *et al.*, 1991; Mian, 1997).

The PIWI domain of the eukaryotic Argonaute proteins, involved in RNA silencing, is structurally similar to the RNase H domain and conserves the residues necessary for RNase H endonucleolytic activity (Song *et al.*, 2004; Kitamura *et al.*, 2010). The eukaryotic Ago proteins showing endonuclease activity (slicer) can digest one RNA strand of the RNA/RNA hybrid. In contrast, the few prokaryotic Ago proteins known show a higher affinity for RNA/DNA hybrids. Very recently, it was reported for the first time that *Pyrococcus furiosus* RNase HII (*pf*-RNase HII) can digest an RNA/RNA hybrid in the presence of Mn^{2+} (Kitamura *et al.*, 2010).

RNase P

RNase P is a ribozyme considered to be a vestige from the 'RNA world'. It was discovered by Sidney Altman, almost 40 years ago (Robertson *et al.*, 1972), and for this, he received the Nobel Prize in Chemistry in 1989. This ancestral protein is a quasi-universal endoribonuclease found in all three domains of life: Bacteria, Eukarya (and eukaryotic organelles) and Archaea. RNase P is best known for universally catalyzing the endonucleolytic cleavage of the extra nucleotides in the 5′ end of the pre-tRNAs to generate the mature tRNAs (for a recent review by Sidney Altman, see Liu & Altman, 2009).

This ribozyme appears to have adapted to modern cellular life by adding protein to the RNA catalytic core. The bacterial version is the most simple, with a single RNA [350–400 nucleotide (nt), encoded by the *rpnB* gene] and a single small protein subunit (approximately 15 kDa, encoded by the *rpnA* gene) (Fig. 1a), both essential for cell viability (Shiraishi & Shimura, 1986; Kirsebom *et al.*, 1988; Baer *et al.*, 1989). In archaea and eukaryotes, the RNA subunit is bound by multiple proteins (at least four and nine proteins, respectively) with no relationship with their bacterial counterpart (Hall & Brown, 2002).

Five distinct structural classes of RNase P RNAs have been defined, based on the RNA secondary structure. In bacteria, two distinct types predominate: the A type (for ancestral), represented by *E. coli* RNase P RNA, and the B type (for *Bacillus*), confined to the low G+C Gram-positive bacteria (Chen *et al.*, 1998; Massire *et al.*, 1998; Smith *et al.*, 2007). Although evolution retained the catalysis function associated with the RNA subunit, the protein(s) play vital supporting roles. The higher protein : RNA mass ratio in the archaeal and eukaryal holoenzymes reflects a recruitment of protein cofactors during evolution, broadening the substrate spectrum in the more complex cellular environments (Liu & Altman, 1994).

In the bacterium *A. aeolicus*, candidate genes for *rpnA* and *rpnB* could not be identified (Willkomm *et al.*, 2002; Lombo & Kaberdin, 2008). However, recent work has demonstrated the existence of an RNase P-like activity in this hyperthermophilic bacterium (Marszalkowski *et al.*, 2008). The universality of RNase P is also challenged in the archaeon *Nanoarchaeum equitans* in which tRNAs are transcribed as primary 5′ mature tRNAs, and therefore, RNase P activity has been dispensed (Randau *et al.*, 2008). In eukaryotes, a different exception occurs. Human mitochondria and higher plant chloroplasts possess a protein-only version of the enzyme, known as 'Proteinaceous RNase P', which lacks the RNA subunit (Holzmann *et al.*, 2008; Gobert *et al.*, 2010). In this case, RNase P enzymes seem to have lost the RNA component during evolution.

Despite less efficiently than with tRNAs, RNase P has been shown to cleave other substrates, both *in vivo* and *in vitro*. Namely, the *E. coli* enzyme processes two other important stable RNA substrates involved in protein synthesis: the tmRNA (Gimple & Schon, 2001) and 4.5S RNA (Bothwell

et al., 1976; Peck-Miller & Altman, 1991). Other substrates include phage-induced regulatory RNAs (Hartmann *et al.*, 1995), sRNA duplex substrates and snoRNAs (Ko & Altman, 2007; Yang & Altman, 2007), riboswitches (Altman *et al.*, 2005; Seif & Altman, 2008) and intergenic regions of polycistronic operon mRNAs (Alifano *et al.*, 1994; Li & Altman, 2003).

Catalysis by RNase P RNA is hydrolytic and absolutely dependent on divalent metal ions (Mg^{2+} or Mn^{2+}) (Smith *et al.*, 1992; Kirsebom & Trobro, 2009). Its turnover rate is slow compared with other enzymes, what may reflect a specialization for cleavage-site selectivity and recognition of several different substrates rather than for rapid catalysis. This would explain the complex nature of this ancient ribozyme.

RNase Z

RNase Z is a conserved endonuclease that belongs to the β-lactamase superfamily of metal-dependent hydrolases (Fig. 1a). Genes encoding RNase Z homologues were identified in all three domains of life (Minagawa *et al.*, 2004; de la Sierra-Gallay *et al.*, 2005). The enzyme is mainly responsible for the 3′ end maturation of tRNAs.

Mature tRNAs all bear a CCA sequence at the end of the acceptor stem that is essential for aminoacylation and interaction with the ribosome. Two main modes for 3′ tRNA processing have been described: (1) a one-step maturation involving direct endonucleolytic cleavage by RNase Z at the 3′ end (CCA less tRNAs). The cleavage occurs after the discriminator base (the unpaired nucleotide immediately upstream the CCA motif) (Nashimoto, 1997; Pellegrini *et al.*, 2003) and provides the substrate for subsequent CCA addition by tRNA nucleotidyltransferase to generate the mature tRNA (Deutscher, 1990; Nashimoto, 1997; Schiffer *et al.*, 2002); and (2) multistep maturation involving endo- and exonucleases (e.g. in *E. coli* where all genes have the CCA encoded). Hence, the presence or not of the universal 3′-terminal CCA sequence in the tRNA primary transcript is the key determinant for the 3′-tRNA processing pathway (Deutscher, 1990; Schiffer *et al.*, 2002). In organisms such as *B. subtilis*, both types of 3′-tRNA processing may occur (see the section below on processing).

While the RNase Z gene is essential in *B. subtilis* for cell viability (Schilling *et al.*, 2004), in *E. coli*, mutants lacking RNase Z have no obvious growth phenotype (Schilling *et al.*, 2004). The *E. coli* RNase Z, also known as the ElaC protein, was initially identified as a zinc phosphodiesterase, ZiPD (Vogel *et al.*, 2002; Schilling *et al.*, 2004). It had been identified several years before as RNase BN, initially thought to be a cobalt-activated RNase with exonuclease activity (Asha *et al.*, 1983). The enzyme was required for the maturation of tRNA precursors encoded by phage T4. However, the gene encoding RNase BN (*rbn*) was originally misidentified, and was only recently shown to be the *elaC* gene, known to encode RNase Z (Ezraty *et al.*, 2005). Therefore, the *E. coli* enzyme is still called RNase BN occasionally. Other denominations include tRNase Z, 3′-tRNase and 3′-pre-tRNase.

The enzyme is a zinc-dependent metallo-hydrolase, and like RNase P, recognizes the tRNA structure in precursor molecules (Pellegrini *et al.*, 2003). RNase Z crystal structures have revealed that the enzyme forms a dimer of metallo-β-lactamase domains and has a characteristic domain, named a flexible arm or an exosite, which protrudes from the metallo-β-lactamase core and is involved in tRNA binding (de la Sierra-Gallay *et al.*, 2005). In the case of *Thermotoga maritima*, the structure of the flexible arm of the enzyme is different from those of homologue enzymes and may explain why, in this bacterium, tRNase Z exceptionally cleaves precisely after the CCA sequence (at 3′) and not after the discriminator base (Ishii *et al.*, 2005).

The intriguing presence of an RNase Z homologue in some members of the *Gammaproteobacteria*, such as *E. coli* and *Salmonella* spp., even though its action is not needed for tRNA maturation, has led to a search for other potential substrates for RNase Z. Surprising results were obtained when the *rnz* mutation was combined with a mutation in RNase E. The lack of both enzymes resulted in a drastic increase in the half-live compared with the absence of either enzyme alone (Perwez & Kushner, 2006a). These authors also observed that *E. coli* RNase Z was able to cleave *rpsT* mRNA *in vitro* at locations distinct from those obtained with RNase E. The enzyme is also capable of cleaving unstructured RNA substrates (Shibata *et al.*, 2006).

Deutscher and coworkers proposed that the *E. coli* enzyme (RNase BN) may differ in certain respects from the RNase Z homologues in other organisms; namely, it can have a dual exo- and an endoribonuclease activity (Dutta & Deutscher, 2009, 2010). This dual activity was also seen in RNase J from *B. subtilis*, another member of the zinc-dependent metallo-β-lactamases family (see the section on Other endonucleases) (Mathy *et al.*, 2007).

Other endonucleases

Several other endonucleases have been described not only in *E. coli* but also in other microorganisms. Below, we will briefly mention some of their main characteristics.

RNase I is a broad-specificity endoribonuclease, very active, present in the periplasmic space of *E. coli*. The enzyme belongs to the T2 superfamily of RNases, whose members are widely distributed throughout nature (Irie, 1997; Condon & Putzer, 2002) (Fig. 1a). Although RNase I activity is easily detected, its function in cell metabolism has never been clarified, because RNase I-deficient mutants are viable and do not affect global mRNA degradation (Zhu

et al., 1990). The enzyme can cleave RNA between every residue to yield mononucleotides and its activity is not inhibited in the presence of EDTA. It was proposed to be implicated in the scavenging of ribonucleotides from the extracellular environment (Condon & Putzer, 2002).

There are reports of other broad-specificity endoribonucleases that are RNase I related, namely, RNase I* (Cannistraro & Kennell, 1991) and RNase M (Cannistraro & Kennell, 1989). However, their existence was never confirmed and seems to consist merely of different manifestations of RNase I (Subbarayan & Deutscher, 2001).

Escherichia coli RNase LS is an RNase that, despite playing a minor role in noninfected bacteria (reviewed in Uzan, 2009), seems to constitute an important cellular defense mechanism against bacteriophage invasion (Otsuka & Yonesaki, 2005). Namely, bacteriophage T4 uses a combination of host- and phage-encoded enzymes to degrade its mRNAs in a stage-dependent manner. Phage T4 encodes RegB, a sequence-specific endoribonuclease (Sanson & Uzan, 1995; Uzan, 2001) that inactivates T4 early transcripts shortly after infection. The middle and late T4 mRNAs are protected from degradation by the viral factor Dmd. In T4-phages defective for the *dmd* gene, RNase LS (for late-gene silencing in T4) cleaves these T4 mRNAs, inhibiting phage multiplication. Therefore, this endonuclease acts as an antagonist of T4 phage replication and Dmd is required for overcoming the host's RNase LS defense role.

Escherichia coli also encodes for a large number of suicide or toxin genes. Their expression is toxic to their host cells, causing growth arrest and eventual cell death. For example, *E. coli* RelE and MazF are two different families of bacterial toxins that inhibit translation by specific endonucleolytic mRNA cleavage (Pedersen *et al*., 2003; Neubauer *et al*., 2009; Yamaguchi & Inouye, 2009).

In *B. subtilis*, it was shown that the majority of the ribonucleolytic activity is phosphorolytic. However, several studies showed that PNPase is not responsible for the initial step in RNA decay in *B. subtilis*, but is a secondary enzyme that acts after the decay has been initiated by other RNases (Bechhofer, 2009). Recently, two proteins (RNase J1 and RNase J2) with cleavage activity equivalent to *E. coli* RNase E were purified in this organism (Even *et al*., 2005). Moreover, these enzymes share many other characteristics with RNase E, which may be related to their similar endonucleolytic activities (Bechhofer, 2009). RNase J1 and J2 are around 61 kDa and have both endonucleolytic and 5′–3′ exonucleolytic activity, which is sensitive to the 5′ phosphorylation state of the substrate. These enzymes were the first described 5′–3′ exonucleases in bacteria (Mathy *et al*., 2007), the J1 activity being twofold higher than J2 (Mathy *et al*., 2010) (see also under the topic Exonucleases the section on RNase J1/J2). Furthermore, RNase J1 is essential, while RNase J2 is not (Even *et al*., 2005).

RNase J1 plays a major role in RNA stability (Mader *et al*., 2008) and maturation. It functions as a 5′–3′ exoribonuclease in the maturation of 16S rRNA gene and in regulating the mRNA stability of the stationary-phase insecticidal protein transcript *cryIIIA* (Mathy *et al*., 2007; Deikus *et al*., 2008). RNase J1 is also responsible for increasing the stability of the downstream fragments that result from the endonucleolytic cleavage of *thrS* and *thrZ* mRNAs (Even *et al*., 2005). A recent study using a bacterial two-hybrid system showed that PNPase, RNase J1 and two glycolytic enzymes can interact with RNase Y and potentially form a degradosome-like complex (Commichau *et al*., 2009) (see Complexes of RNases). Moreover, it was shown recently that RNase J1 and J2 in wild-type cells are mostly in a complex. While the individual enzymes have similar endonucleolytic cleavage activities and specificities, as a complex, they behave synergistically to alter cleavage site preference and to increase cleavage efficiency at specific sites (Mathy *et al*., 2010).

RNase J1 homologues are widely distributed in several other bacteria and archaea (Even *et al*., 2005). The enzyme is a member of the β-CASP subfamily of zinc-dependent metallo-β-lactamases. The enzyme is composed of three domains: an N-terminal β-lactamase domain, a β-CASP and a C-terminal domain necessary for the enzyme activity. A binding pocket coordinating the phosphate and base moieties of the nucleotide in the surrounding area of the catalytic center provides a basis for the 5′-monophosphate-dependent 5′–3′ exoribonuclease activity (de la Sierra-Gallay *et al*., 2008). The endonucleolytic activity of the enzyme is not dependent of 5′-monophosphate. For the initiation of endonuclease cleavage, RNase J1 either binds to the 5′ end or directly to the internal site of the mRNA. The upstream product is rapidly degraded by the 3′–5′ exonuclease activity of PNPase. The downstream RNA fragment with the 5′-monophosphate end can be a target of new RNase J1 endonuclease cleavage or processive 5′–3′ exonucleolytic decay from the 5′ end (Bechhofer, 2009). It was also shown that RNase J1 requires a single-stranded 5′ end with AU-rich regions to allow the exoribonucleolytic activity (Mathy *et al*., 2007). This was observed in *infC* leader RNA (Choonee *et al*., 2007), *trp* leader RNA (Deikus *et al*., 2008) and the RNA species called scRNA (Yao *et al*., 2007).

Similar to what happens with *B. subtilis*, we can find RNase J1 and J2 also in *Streptococcus pyogenes*. While in *B. subtilis* only RNase J1 is an essential protein, in *S. pyogenes*, both proteins are essential for growth. In this bacterium, RNases J1 and J2 were also seen to affect the decay of several mRNAs (Bugrysheva & Scott, 2009).

Another endonuclease sensitive to the 5′ end phosphorylation state of the substrate was discovered recently. RNase Y is involved in the initiation of turnover of *B. subtilis S*-adenosylmethionine-dependent riboswitches (Shahbabian

et al., 2009), which controls the expression of 11 transcriptional units (Winkler & Breaker, 2005; Henkin, 2008). The enzyme has a major function in the initiation of mRNA degradation in this organism, affecting mRNA stability > 30% in an RNase J1/J2 double-mutant strain. RNase Y orthologues are present in about 40% of the eubacteria; however, this enzyme is absent from archaea and eukaryotic organisms, with the exception of *Drosophila willistoni* (Shahbabian *et al.*, 2009).

Other endonucleases are described in *B. subtilis* such as RNase M5, RNase Z (see the above section on RNase Z), RNase Bsn and RNase P (see the above section on RNase P). However, neither RNase M5 nor RNase Z appears to have mRNA targets in *B. subtilis* (Condon *et al.*, 2002). RNase M5's major role is the maturation of the 5S rRNA gene (Sogin & Pace, 1974) and can only be found in low G+C Gram-positive bacteria (Condon *et al.*, 2001). Bsn is an extracellular nuclease, apparently with no sequence specificity. It can cleave RNA endonucleolytically to yield 5′-phosphorylated oligonucleotides. The enzyme is found in some members of low G+C Gram-positive bacteria (Nakamura *et al.*, 1992).

Barnase is a guanyl-specific extracellular RNase. Although it is found in many of the *Bacilli*, it is not present in *B. subtilis*. Orthologues of *Bacillus amyloliquefaciens* Barnase and its inhibitor Barstar are also found in *Clostridium acetobutylicum* and the Gram-positive *Yersinia pestis*. It appears that some organisms have lost their copy of the Barnase gene because it was no longer required for a selective advantage. Alternatively, they acquired the resistance gene because other organisms sharing the same niche produced Barnase (Belitsky *et al.*, 1997).

Besides the well-known endonucleases, there are some DNA-binding proteins in archaea with RNase endonucleolytic activity; however, the physiological relevance of these proteins with respect to RNA metabolism is not clear (Evguenieva-Hackenberg & Klug, 2009). The attempts to purify novel RNase activities from archaea resulted in the isolation of very different proteins. Two proteins with RNase activity were purified from *Sulfolobus solfataricus* (called p1 and p2). It was shown that divalent cations are not required for their activity, and they were capable of cleaving yeast tRNA (Fusi *et al.*, 1993; Shehi *et al.*, 2001). Another 9-kDa protein, called SaRD, whose RNase activity is not affected in the presence of different divalent cations, was purified from *Sulfolobus acidocaldarius* (Kulms *et al.*, 1995). Furthermore, two different dehydrogenases were identified in the same organism, with RNase III-like properties and cleavage patterns dependent on $MgCl_2$: an aspartate-semialdehyde dehydrogenase and acyl-CoA dehydrogenase (Evguenieva-Hackenberg *et al.*, 2002). A homologue of the eukaryotic initiation factor 5A (eIF-5A) called archaeal initiation factor 5A (aIF-5A), from *Halobacterium salinarum*, was also described as an RNase with activity in low salt concentrations without addition of $MgCl_2$ (Wagner & Klug, 2007). It was shown that aIF5A efficiently binds structured RNA containing certain motifs and that the interaction is hypusine dependent (Xu *et al.*, 2004).

Exonucleases

PNPase

PNPase belongs to the PDX family of exoribonucleases, which also includes RNase PH from bacteria, and the core of the exosome in archaea and eukaryotes (Mian, 1997; Zuo & Deutscher, 2001; Pruijn, 2005) (Fig. 1b). In 1959, Severo Ochoa received the Nobel Prize for his studies on the polymerase activity of this enzyme, being the first to synthesize RNA outside the cell. This was a major contribution towards deciphering the genetic code. PNPase is also involved in global mRNA decay, being widely conserved from bacteria to plants and metazoans (Zuo & Deutscher, 2001; Bermúdez-Cruz *et al.*, 2005).

PNPase is encoded by the *pnp* gene and is transcribed from two promoters (Portier & Regnier, 1984). *pnp* expression is negatively autoregulated at the post-transcriptional level by the concerted action of PNPase and RNase III (Portier *et al.*, 1987; Robert-Le Meur & Portier, 1992, 1994; Jarrige *et al.*, 2001; Carzaniga *et al.*, 2009). This autoregulation can be disrupted by ribosomal protein S1, which binds to the *pnp* mRNA 5′-UTR (Briani *et al.*, 2008). In an RNase III-deficient strain, there is a 10-fold increase in the PNPase levels (Portier *et al.*, 1987). PNPase levels are also affected by polyadenylation. It is likely that polyadenylated transcripts titrate out the amount of PNPase available to carry out normal autoregulation (Mohanty & Kushner, 2002). PNPase and RNase II are cross-regulated (Zilhão *et al.*, 1996a). In the absence of RNase II, PNPase levels are increased and PNPase overexpression leads to a decrease in RNase II activity (Zilhão *et al.*, 1996a).

PNPase does not seem to be indispensable to *E. coli* at optimal temperature, unless either RNase II or RNase R is also missing (Donovan & Kushner, 1986; Cheng *et al.*, 1998). However, PNPase is essential for *E. coli* growth at low temperatures (Luttinger *et al.*, 1996; Piazza *et al.*, 1996; Zangrossi *et al.*, 2000) and certain mutations of the RNA-binding domains have been shown to confer a cold-sensitive phenotype (García-Mena *et al.*, 1999; Briani *et al.*, 2007; Matus-Ortega *et al.*, 2007). Higher levels of RNase II allow lower levels of PNPase, and in fact, overexpression of RNase II could complement the cold-shock function of PNPase (Zilhão *et al.*, 1996a; Awano *et al.*, 2008). PNPase was also shown to be involved in the long-term survival of *Campylobacter jejuni* at temperatures > 10 °C (Haddad *et al.*, 2009). In *E. coli*, cold-temperature induction of *pnp* expression occurs at post-transcriptional levels including the reversal of

pnp autoregulation (Zangrossi *et al.*, 2000; Beran & Simons, 2001; Mathy *et al.*, 2001).

PNPase processively catalyzes the 3′–5′ phosphorolytic degradation of RNA, releasing nucleoside 5′-diphosphates. Although the degrading activity of *E. coli* PNPase is known to be blocked by dsRNA structures (Spickler & Mackie, 2000), PNPase can form complexes with other proteins, allowing it to degrade through extensive structured RNA. The main multiprotein complex that integrates PNPase is the degradosome (see the Complexes of RNases). To degrade certain dsRNAs, PNPase can form a complex ($\alpha_3\beta_2$) with RNA helicase B (RhlB) (Liou *et al.*, 2002; Lin & Lin-Chao, 2005). PNPase also forms complexes with Hfq and PAP I (Mohanty *et al.*, 2004). The enzyme was reported to degrade a stem-loop without the assistance of RhlB, but this could be related to the low thermodynamic stability of the stem-loop (Mohanty & Kushner, 2010). In the Gram-negative bacteria *Thermus thermophilus*, the PNPase homologue (Tth PNPase) was shown to have phosphorolytic activity at the optimal temperature of 65 °C. Surprisingly, it is able to completely degrade RNAs with very stable intramolecular secondary structures (Falaleeva *et al.*, 2008).

A minimal 3′ overhang of 7–10 unpaired ribonucleotides is required for an RNA molecule to be bound by PNPase (Py *et al.*, 1996; Cheng & Deutscher, 2005), and the action of the enzyme on folded RNAs is known to be stimulated by 3′ polyadenylation (Xu & Cohen, 1995; Py *et al.*, 1996; Carpousis *et al.*, 1999; Spickler & Mackie, 2000). PNPase is also able to catalyze the polymerization of RNA from nucleoside diphosphates at a low inorganic phosphate (Pi) concentration (Godefroy, 1970; Littauer & Soreq, 1982; Sulewski *et al.*, 1989). *In vivo*, PNPase is essentially devoted to the processive degradation of RNA, but is also responsible for adding the heteropolymeric tails observed in *E. coli* mutants devoid of the main polyadenylating enzyme PAP I (Mohanty & Kushner, 2000b; Slomovic *et al.*, 2008). In exponentially growing *E. coli*, $>$ 90% of the transcripts are polyadenylated and Rho-dependent transcription terminators were suggested to be modified by the polymerase activity of PNPase (Mohanty & Kushner, 2006). In spinach chloroplasts, *Cyanobacteria* and *Streptomyces coelicolor*, PNPase seems to be the main tail polymerizing enzyme (Yehudai-Resheff *et al.*, 2001; Rott *et al.*, 2003; Sohlberg *et al.*, 2003). PNPase-dependent RNA tailing and degradation are believed to occur mainly at low ATP concentrations, because ATP has been shown to inhibit both activities (Del Favero *et al.*, 2008). Recently, it was shown that *B. subtilis* PNPase, in the presence of Mn^{2+} and low levels of Pi, is also able to degrade ssDNA, while in the presence of Mg^{2+} and higher amounts of Pi, it degrades RNA. This suggests that PNPase degradation of RNA and ssDNA occurs by mutually exclusive mechanisms (Cardenas *et al.*, 2009). Because of the ability of PNPase to carry out several distinct activities, the enzyme can be considered as a multifunctional protein. It is a pleiotropic regulator, involved in a number of different pathways of RNA degradation. Indeed, it is the only exoribonuclease in *Streptomyces* and is an essential enzyme in these organisms (Bralley & Jones, 2003; Bralley *et al.*, 2006). In *E. coli*, PNPase is now believed to play a greater role in mRNA degradation than previously thought and its inactivation increases the steady-state levels of many transcripts (Deutscher & Reuven, 1991; Mohanty & Kushner, 2003). The enzyme was also reported to play an important role in protecting *E. coli* cells under oxidative stress (Wu *et al.*, 2009). In *B. subtilis*, the RNA decay is primarily phosphorolytic and this major activity is attributed to the PNPase, which is the principal 3′–5′ exoribonuclease in this organism. The deletion of PNPase in *B. subtilis* causes a number of phenotypes such as competence deficiency, cold and tetracycline sensitivity, and filamentous growth (Hahn *et al.*, 1996; Luttinger *et al.*, 1996; Wang & Bechhofer, 1996).

X-ray crystal structures of *E. coli* and *Streptomyces antibioticus* PNPase reveal a homotrimeric subunit organization with a ring-like architecture (Symmons *et al.*, 2000; Shi *et al.*, 2008; Nurmohamed *et al.*, 2009). Each monomer exhibits a five-domain arrangement: at the N-terminus, two RNase PH domains (PH1 and PH2) are linked by an α-helical domain; two RNA-binding domains, KH and S1, are found in the C-terminal end. In the quaternary structure, the KH and S1 domains are found together in one face of the trimer, while the active site is found in the opposite side.

PNPase mutants lacking either the S1 or the KH domain retain phosphorolytic activity (Jarrige *et al.*, 2002; Stickney *et al.*, 2005; Matus-Ortega *et al.*, 2007). However, the presence of both KH and S1 domains is required for a proper binding (Matus-Ortega *et al.*, 2007), and their absence was proposed to affect product release and enzyme cycling, leading to a decreased turnover number (Stickney *et al.*, 2005). The crystal structure of a KH/S1 deletion mutant, along with biochemical and biophysical data, strongly suggests that these domains are involved not only in RNA binding but also contribute to the formation of a more stable trimeric structure (Shi *et al.*, 2008). Indeed, a previous study has shown that the S1 domain from PNPase was able to induce trimerization of a chimeric RNase II containing PNPase S1 (Amblar *et al.*, 2007).

The association of the three subunits encloses a central channel. A properly constricted channel and the conserved basic residues located in the neck region have been shown to play critical roles in trapping RNA for processive degradation (Shi *et al.*, 2008). Two constricted points have been identified in the channel, and the structure of PNPase in complex with RNA clearly indicates that the pathway followed by the RNA molecule is along the central pore in the direction of the active site (Symmons *et al.*, 2000; Shi *et al.*, 2008; Nurmohamed *et al.*, 2009). The ability of the

aperture at the central channel and its neighboring regions to undergo conformational changes is likely to be a key aspect of the dynamic translocation of RNA by PNPase (Nurmohamed *et al.*, 2009).

The catalytic site of PNPase is composed of structural elements of both PH1 and PH2 core domains, and several mutations introduced into the PNPase core abolish or drastically decrease all catalytic activities of the enzyme (Jarrige *et al.*, 2002; Briani *et al.*, 2007). However, other mutations in the core region were analyzed that do not affect phosphorolytic or polymerase activities, but rather RNA binding is severely impaired (Regonesi *et al.*, 2004). *Streptomyces antibioticus* PNPase catalytic center has been identified using tungstate (a phosphate analogue), which is coordinated by T462 and S463 (Symmons *et al.*, 2000). *Escherichia coli* PNPase crystals obtained in the presence of Mn^{2+} (which can substitute for Mg^{2+} to support catalysis) showed that the metal is coordinated by the conserved residues D486, D492 and K494 (Nurmohamed *et al.*, 2009). Indeed, the substitution of D492 abolished both phosphorolysis and polymerization activities (Jarrige *et al.*, 2002).

PNPase has been described to play a role in the establishment of virulence in several pathogens. In *Salmonella*, PNPase activity decreases the expression of genes from the pathogenicity islands SPI 1 (containing genes for invasion) and SPI 2 (containing genes for intracellular growth) (Clements *et al.*, 2002). Similarly, in *Dichelobacter nodosus*, PNPase acts as a virulence repressor in benign strains by decreasing twitching motility (Palanisamy *et al.*, 2009). In contrast, in *Yersinia*, PNPase modulates the type three secretion system (TTSS) by affecting the steady-state levels of TTSS transcripts and controlling the secretion rate (Rosenzweig *et al.*, 2005, 2007). This is probably the reason why the *pnp* deletion results in a less virulent strain in a mouse model (Rosenzweig *et al.*, 2007). In *C. jejuni* PNPase is involved in motility (Haddad *et al.*, 2009). Finally, in *S. pyogenes*, PNPase activity is rate-limiting for the decay of *sagA* and *sda*, which code for the important virulence factors streptolysin S and streptodornase (a DNase), respectively (Barnett *et al.*, 2007).

RNase II

Escherichia coli RNase II is the prototype of the RNase II family of enzymes (Mian, 1997; Mitchell *et al.*, 1997; Zuo & Deutscher, 2001; Frazão *et al.*, 2006; Grossman & van Hoof, 2006) (Fig. 1b). RNase II-like proteins are widespread among the three domains of life, and in eukaryotes, they are the catalytic component of the exosome (Liu *et al.*, 2006b; Dziembowski *et al.*, 2007).

RNase II is encoded by the *rnb* gene that can be transcribed from two promoters P1 and P2 and terminates in a Rho-independent terminator 10 nucleotides downstream of the *rnb* stop codon (Zilhão *et al.*, 1993, 1995a, 1996b). PNPase regulates RNase II expression by degrading the *rnb* mRNA (Zilhão *et al.*, 1996a). RNase III and RNase E endonucleases are also involved in the control of RNase II expression at the post-transcriptional level. RNase III does not affect *rnb* mRNA directly, but affects PNPase levels, and RNase E is directly involved in the *rnb* mRNA degradation (Zilhão *et al.*, 1995b).

The protein stability of RNase II is known to be post-translationally regulated and its levels are adjusted according to the growth conditions. *gmr* (gene modulating RNase II) is located downstream of *rnb* and the related protein is involved in the modulation of the stability of RNase II (Cairrão *et al.*, 2001). Gmr has a PAS domain that can act as an environmental sensor detecting changes in growth conditions.

Escherichia coli RNase II is a sequence-independent hydrolytic exoribonuclease that processively degrades RNA in the 3′–5′ direction, yielding 5′-nucleoside monophosphates. However, the processive degradation of an RNA molecule by RNase II is easily blocked by secondary structures, and the enzyme is known to stall around seven nucleotides before it reaches a double-stranded region (Cannistraro & Kennell, 1999; Spickler & Mackie, 2000). In *E. coli*, RNase II is the major hydrolytic enzyme and participates in the terminal stages of mRNA degradation (Deutscher & Reuven, 1991). However, the enzyme is not essential for *E. coli* growth unless PNPase is also missing (Donovan & Kushner, 1986; Zilhão *et al.*, 1996a). Although RNase II-degrading activity is sequence independent, the most reactive substrate is the homopolymer poly(A). Because the presence of a poly(A) tail is often needed for the RNA degradative process, the rapid degradation of polyadenylated stretches by RNase II can paradoxically protect some RNAs by impairing the access of other exoribonucleases (Hajnsdorf *et al.*, 1994; Pepe *et al.*, 1994; Coburn & Mackie, 1996a; Marujo *et al.*, 2000; Mohanty & Kushner, 2000a; Folichon *et al.*, 2005). Indeed, in the absence of RNase II, a large number (31%) of *E. coli* mRNAs are decreased, especially ribosomal protein genes, suggesting a major function for this enzyme in the protection of specific mRNAs through poly(A) tail removal (Mohanty & Kushner, 2003).

The structure of *E. coli* RNase II and its RNA-bound complex was determined (Frazão *et al.*, 2006) (Fig. 2a). This was the first structure of an exoribonuclease from the RNase II family that has been resolved (Frazão *et al.*, 2006). The overall X-ray crystallographic structure of the wild-type enzyme (Frazão *et al.*, 2006; Zuo *et al.*, 2006) revealed four domains, as predicted previously by Amblar *et al.* (2006) (see Figs 1b and 2a). Three RNA-binding domains have been identified: two cold-shock domains (CSD1 and CSD2) in the N-terminal region and an S1 RNA-binding domain at

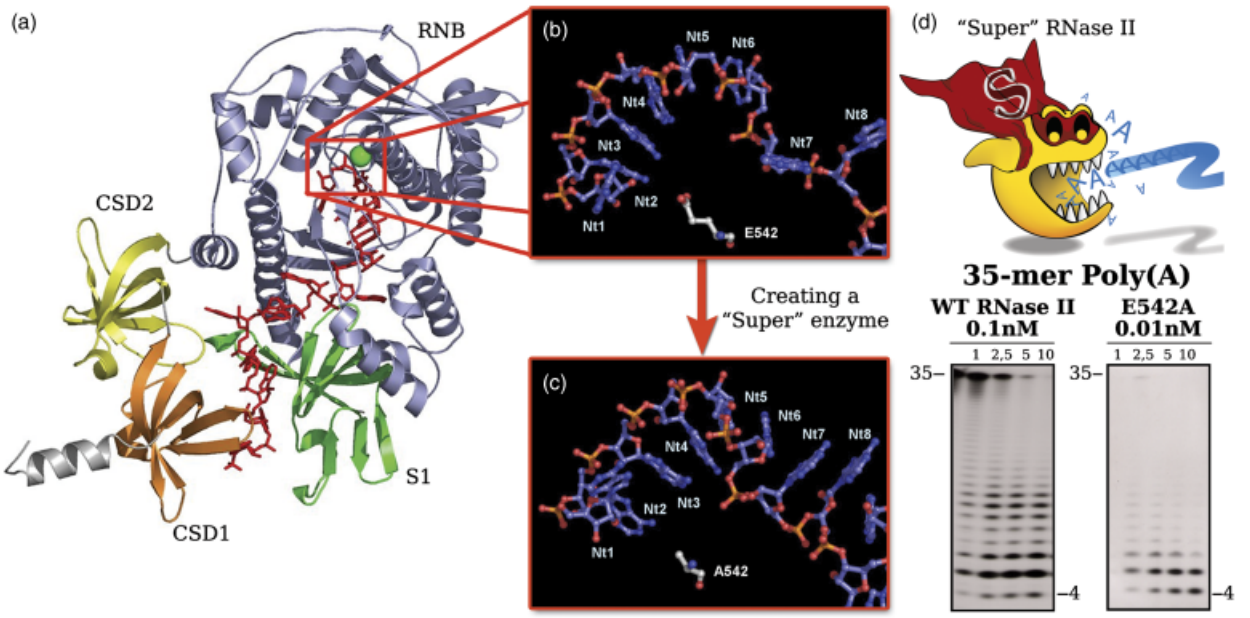

Fig. 2. The making of a 'super-enzyme'. (a) RNase II is composed of two N-terminal cold shock domains (CSD1 in orange and CSD2 in yellow), a central catalytic domain (RNB in gray), a C-terminal S1 domain (in green). (b) Zooming the catalytic cavity of RNase II. (c) Modelling the E542A mutant with the Poly(A) RNA strand in the RNB domain. Substitution in position 542 of the negatively charged glutamic side-chain for the smaller neutral methyl group of alanine could reduce significantly both electrostatic and steric surfaces in the RNA-binding interface. (d) Exoribonuclease activity with the Poly(A) substrate: comparison of wild-type and E542A proteins. It is possible to see that we need to use higher concentrations of RNase II when compared with the E542A mutant, which is 110-fold more active when compared with the wild type (Barbas *et al.*, 2009. ©The American Society for Biochemistry and Molecular Biology).

the C-terminus. The catalytic site resides in the central RNB domain, whose structure has shown an unprecedented fold characteristic of this family. This domain contains four highly conserved sequence motifs (I–IV) with several invariant carboxylate residues (Mian, 1997). The RNA-binding domains (CSD1, CSD2 and S1) are grouped together on one side of the structure, while the active site is on the other side of the molecule (Frazão *et al.*, 2006).

Elimination of the N-terminal CSD1 resulted in an increase in the RNA-binding affinity of the enzyme for poly(A), suggesting that this domain may play a role in controlling the movement of the enzyme on the poly(A) chain (Amblar *et al.*, 2006; Arraiano *et al.*, 2008). Interestingly, without all the RNA-binding domains, the enzyme is still able to degrade RNA, although with much less efficiency than the wild-type enzyme (Matos *et al.*, 2009; Vincent & Deutscher, 2009).

The structure of the RNA-bound enzyme revealed that the RNA fragment interacts with the protein at two non-contiguous regions: the 'anchor' and catalytic regions (Cannistraro & Kennell, 1994; Frazão *et al.*, 2006) (Fig. 2a). Nucleotides 1–5, at the 5′ end of the 13-mer RNA fragment, are located in the 'anchor' region in a deep cleft between the two CSDs and the S1 domain. The final nucleotides 9–13 are located in a cavity deep within the RNB domain, stacked and 'clamped' between the conserved residues Phe358 and Tyr253. A 10-nucleotide fragment is the shortest RNA able to retain contacts with both the anchor and the catalytic regions. This explains why RNase II is processive on long RNA molecules, but becomes distributive on substrates shorter than 10–15 nucleotides. When the RNA molecule is shorter than five nucleotides, the required packing of the bases can no longer occur, preventing the translocation of the RNA, and a final end product of four nucleotides is released (Frazão *et al.*, 2006). Tyr-253 has been identified as the residue responsible for setting the RNase II end product, and its substitution was shown to alter the smallest end product of degradation from 4 to 10 nucleotides (Barbas *et al.*, 2008). This mutation has been proposed to cause loosening of the RNA substrate at the catalytic site and, as a consequence, binding at the anchor region would be essential to keep the RNA attached to the protein and allow cleavage. Molecules shorter than 10 nucleotides are too small to be simultaneously bound at both sites, meaning that they would have to be degraded in a distributive manner (Barbas *et al.*, 2008).

The access to the catalytic pocket is restricted to single-stranded RNA by steric hindrance, which explains the inability of RNase II to degrade dsRNA. DNA is not a substrate because there is a specific interaction between the protein and the ribose rings of nucleotides that directly contact the enzyme (Frazão *et al.*, 2006). Residues Tyr-313

and Glu-390 have been demonstrated to be responsible for the discrimination of the cleavage of RNA vs. DNA (Barbas *et al.*, 2009).

Several residues in the catalytic region are important for catalysis (Amblar & Arraiano, 2005; Frazão *et al.*, 2006). Asp-201 and Asp-210 substitution led to a significant loss of RNase II activity, and Arg-500 has also been shown to be crucial for RNA cleavage (Frazão *et al.*, 2006; Barbas *et al.*, 2008, 2009). However, Asp-209 is the only essential residue for RNA degradation (Barbas *et al.*, 2008). The conserved residue Glu-542 has been proposed to facilitate the elimination of the exiting nucleotide upon phosphodiester cleavage (Frazão *et al.*, 2006). Interestingly, its substitution by alanine rendered the mutant RNase II much more active than the wild type and significantly increased the RNA-binding ability (Fig. 2b–d). Three-dimensional modelling of the mutant enzyme indicated that the substitution induced a subtle conformational change in the RNB domain. This resulted in a reorganization of the RNA-binding interface that transformed the RNase II into the so-called 'super-enzyme', an enzyme with extraordinary catalysis and binding abilities. When compared with the wild-type RNase II, the 'super-enzyme' exhibits >100-fold increase in the exoribonucleolytic activity (Fig. 2d) and about a 20-fold increase in the RNA-binding affinity (Barbas *et al.*, 2009).

RNase R

RNase R encoded by the *rnr* gene (previously *vacB*) is a 3′–5′ hydrolytic exoribonuclease from the RNase II family of exoribonucleases (Cheng & Deutscher, 2002; Vincent & Deutscher, 2006). The *rnr* gene is second in an operon, together with *nsrR* (a transcriptional regulator), *rlm*B (rRNA methyltransferase) and *yjfI* (unknown function). Transcription is driven from a putative σ^{70} promoter upstream of *nsrR* (Cheng *et al.*, 1998; Cairrão *et al.*, 2003). *rnr* mRNAs are post-transcriptionally regulated by RNase E, although RNase G may also participate (Cairrão & Arraiano, 2006). RNase R is a processive and sequence-independent enzyme, with a wide impact on RNA metabolism (Cairrão *et al.*, 2003; Cheng & Deutscher, 2005; Oussenko *et al.*, 2005; Andrade *et al.*, 2006, 2009a; Purusharth *et al.*, 2007). It is unique among the RNA-degradative exonucleases present in *E. coli* as it can easily degrade highly structured RNAs (Cheng & Deutscher, 2002, 2003; Awano *et al.*, 2010). RNase R is able to degrade an RNA duplex, provided there is a single-stranded 3′ overhang (Cheng & Deutscher, 2002; Vincent & Deutscher, 2006). In fact, RNase R was shown to be a key enzyme involved in the degradation of polyadenylated RNA (Andrade *et al.*, 2009a).

RNase R shows a modular organization of RNA-binding domains (CSD1 and CSD2 located at the N-terminus and a C-terminal S1 domain) flanking the central catalytic RNB domain, typically found on RNase II-family members (Fig. 1b). A three-dimensional model of RNase R has been proposed based on the structure of its paralogue RNase II (Barbas *et al.*, 2008). Mutational analysis identified important residues located in the active center: D272, D278 and D280 (Matos *et al.*, 2009). A D280N mutant showed no exonucleolytic activity, similarly to what was reported with the D209N mutant in RNase II (Amblar & Arraiano, 2005; Matos *et al.*, 2009; Awano *et al.*, 2010). RNase R degradation is processive, and unlike RNase II, the final end product of digestion is a dinucleotide. Tyrosine Y324 was found to be responsible for setting the final end product of RNase R (Matos *et al.*, 2009).

RNase R was shown to bind RNA more tightly within its catalytic channel than does RNAase II (Matos *et al.*, 2009; Vincent & Deutscher, 2009). Surprisingly, a mutant expressing only the nuclease domain (RNB) is able to degrade a perfect dsRNA (Matos *et al.*, 2009). Paradoxically, the presence of the RNA-binding domains (CDS1, CDS2 and S1) requires the presence of a short tail in order to degrade dsRNA (Matos *et al.*, 2009). The RNA-binding domains 'block' the entrance of dsRNA into the catalytic channel. Accordingly, it was proposed that RNA-binding domains actually discriminate the substrates that can be processed by RNase R, favoring the selection of RNA molecules harboring a 3′ linear tail. It has been suggested that RNase R can function both as an exoribonuclease as well as an RNA 'helicase' (Awano *et al.*, 2010). RNase R intrinsic 'helicase' unwinding activity is dependent on RNA-binding regions (S1, CDS1, and most importantly, CDS2). The dsRNA must have a 3′ linear overhang in order to become a suitable substrate for RNase R helicase activity. Altogether, RNA-binding domains of RNase R seem to be responsible for the selection of RNA substrates harboring a 3′ linear region, which can be provided by polyadenylation (Andrade *et al.*, 2009a; Matos *et al.*, 2009). Clearly, only the resolution of the RNase R structure will allow a full understanding of its remarkable modes of action.

RNase R is critical in RNA quality control, namely in the degradation of defective tRNAs (Vincent & Deutscher, 2006; Awano *et al.*, 2010) and rRNA (Cheng & Deutscher, 2003). Together with PNPase, RNase R eliminates aberrant fragments of the 16S and 23S rRNA genes, whose accumulation potentially affects ribosome maturation and assembly. Furthermore, the importance of RNase R in the accuracy of gene expression is broadened with its role in protein quality control. In the absence of RNase R, the small stable SsrA/tmRNA is not processed properly, leading to defects in *trans*-translation and significant errors in protein tagging for proteolysis (Cairrão *et al.*, 2003). RNase R has also emerged as an important novel contributor to mRNA degradation. The absence of both RNase R and PNPase results in the strong accumulation of REP-containing mRNA sequences (Cheng & Deutscher, 2005). However, the presence of only

one of these exoribonucleases is sufficient to remove such transcripts, revealing again a functional overlap between these two enzymes. Remarkably, RNase R was also shown to degrade the *ompA* transcript in a growth-phase-specific manner (Andrade *et al.*, 2006). In the stationary phase of growth, the single inactivation of RNase R results in the accumulation of *ompA* mRNA and this correlated with increasing intracellular levels of OmpA protein. This work revealed a role for RNase R in the control of gene expression that could not be replaced by any of the other exoribonucleases (Andrade *et al.*, 2006).

The activity of RNase R is modulated according to the growth conditions of the cell and responds to environmental stimuli. RNase R seems to be a general stress-induced protein, whose levels are increased under several stresses, namely in cold shock, and the stationary phase of growth (Cairrão *et al.*, 2003; Andrade *et al.*, 2006). RNase R-like enzymes are widespread in most sequenced genomes. Although most of the knowledge on this protein came from work in *E. coli*, many RNase R from other bacterial species have been identified. Notably, RNase R has also been implicated in the establishment of virulence in a growing number of pathogens.

In *Shigella flexneri*, RNase R was shown to be required for the expression of the invasion factors IpaB, IpaC, IpaD and VirG (Tobe *et al.*, 1992). The disruption of the VacB gene in other *Shigella* spp. and enteroinvasive *E. coli* resulted in the reduced expression of virulence phenotypes (Tobe *et al.*, 1992). In *Legionella pneumophila* RNase R is the only hydrolytic exoribonuclease present. This protein is not essential for growth at optimal temperature; however, it is important for growth and viability at low temperatures and induces the competence (Charpentier *et al.*, 2008). To date, only one exoribonuclease, RNase R (MgR), was identified in *Mycoplasma genitalium*, where it is an essential protein (Hutchison *et al.*, 1999). MgR shares some properties of both *E. coli* RNase R and RNase II and can carry out a broad range of RNA processing and degradative functions (Lalonde *et al.*, 2007). Similar to what happens in *E. coli*, RNase R from *Aeromonas hydrophila* is also a cold-shock protein essential for viability at lower temperatures and its absence leads to a reduction in *A. hydrophila* motility (Erova *et al.*, 2008). The infection of mouse cells with Δ*rnr* strains shows that the virulence is attenuated, confirming the role of this enzyme in the pathogenesis of this organism (Erova *et al.*, 2008). In *Streptococcus pneumoniae*, there is a unique homologue of the RNase II family of enzymes that was shown to be a RNase R-like protein (Domingues *et al.*, 2009). RNase R from *Salmonella* showed a reduction in its activity and the ability to bind to RNA when compared with *E. coli* RNase R (Domingues *et al.*, 2009). Proteins isolated from different strains regarding their virulence ability (virulent vs. nonvirulent) are different regarding their activity and RNA affinity (Domingues *et al.*, 2009). Further studies are still necessary to confirm whether the differences observed in RNase R protein are responsible for the virulence of these strains.

In *P. syringae*, RNase R is the exoribonuclease present in the degradosome as opposed to most other systems, where PNPase is part of such complexes (Purusharth *et al.*, 2005) (see Complexes of RNases). Like in *E. coli*, RNase R is also particularly important at low temperatures, because inactivation of the *rnr* gene inhibits the growth of both *Pseudomonas putida* (Reva *et al.*, 2006) and *P. syringae* (Purusharth *et al.*, 2007) at 4 °C. In *P. syringae*, RNase R is involved in 3′ end maturation of the 16S and 5S rRNA genes and in tmRNA turnover (Purusharth *et al.*, 2007). Genomic studies revealed that *P. putida* RNase R plays an important role in mRNA turnover because its absence led to the accumulation of several mRNAs (Fonseca *et al.*, 2008). On the other hand, RNase R (previously YvaJ) from *B. subtilis* was suggested not to play a critical role in RNA degradation; however, it may play a role in mRNA turnover when polyadenylation at the 3′ end occurs (Oussenko *et al.*, 2005). Moreover, *B. subtilis* RNase R was shown to be important for the quality control of tRNAs (Campos-Guillen *et al.*, 2010).

Overall, RNase R-deficient bacteria have been shown to be less virulent than the wild-type parental strains. However, how this is achieved is still not completely clear. This is probably related to critical RNA degradation pathways. The fact that RNase R was found to be key in the degradation of sRNAs, namely the virulence regulator SsrA/tmRNA, paves the way to broaden its role in pathogenesis. It has also been suggested that RNase R may control the export of proteins involved in virulence mechanisms. Altogether, the available data suggest that bacterial RNase R may be attractive as a potential therapeutic agent, but clearly more studies are required.

Oligoribonuclease

The end products resulting from the degradation of previously described RNases constitute a severe problem to the cell viability, because these enzymes release RNA fragments of 2–5 nucleotides in length whose accumulation may be deleterious (Ghosh & Deutscher, 1999). Oligoribonuclease is the enzyme that degrades these short oligoribonucleotides (Stevens & Niyogi, 1967; Niyogi & Datta, 1975). From the known exoribonuclease genes in *E. coli* the oligoribonuclease gene, *orn*, is the only one required for cell viability (Ghosh & Deutscher, 1999).

Oligoribonuclease belongs to the DEDD family of exoribonucleases (Zuo & Deutscher, 2001), and is a homodimeric ($\alpha 2$) enzyme (Zhang *et al.*, 1998) that produces mononucleotides and requires the presence of divalent cations (Mn^{2+}) (Niyogi & Datta, 1975) (Fig. 1b). The hydrolysis is

processive in the 3′–5′ direction; this enzyme has a higher affinity to 5-mer oligoribonucleotides and the reaction rate decreases with increasing chain length (Datta & Niyogi, 1975). This enzyme requires a free 3′-OH end and is not sensitive to the 5′-phosphorylation state of the RNA (Datta & Niyogi, 1975). Only the preliminary X-ray characterization of the *E. coli* oligoribonuclease structure has been reported (Fiedler *et al.*, 2004). It was shown recently that Orn can degrade short DNA oligos, like its human homologue Sfn, but this degradation requires higher enzyme concentrations than the RNA-directed activity (Mechold *et al.*, 2006).

Bacillus subtilis does not have an oligoribonuclease (Orn) homologue. However, a functional analogue of Orn was identified in this organism that was named YtqI (NrnA). Surprisingly, this protein *in vitro* can degrade not only short oligonucleotides (with a preference for 3-mer) but also 3′-phosphoadenosine 5′-phosphate (pAp). This suggests the existence of a closer link between sulfur and RNA metabolism in *B. subtilis* (Mechold *et al.*, 2007). More recently, a second nanoRNase was discovered and named YngD (NrnB). This protein is a member of the DHH/DHHA1 protein family of phosphoesterases, and degrades nanoRNA 5-mers *in vitro* similar to oligoribonuclease from *E. coli* (Fang *et al.*, 2009).

In *Streptomyces griseus* and *S. coelicolor*, the gene *ornA* encodes the oligoribonuclease protein. It is transcribed from two promoters: one that is developmentally regulated and the other that is a constitutive promoter (Ohnishi *et al.*, 2000). Unlike *E. coli*, in which oligoribonuclease is an essential enzyme, if the *ornA* gene is deleted, the cells are viable, but not able to form aerial hyphae (Ohnishi *et al.*, 2000). It was also shown that the degradation of RNA oligomers by oligoribonuclease is critical for the completion of the life cycle (Sello & Buttner, 2008).

In RNA metabolism, oligoribonuclease acts as the 'finishing enzyme' to degrade oligoribonucleotides of two to five nucleotides in length to mononucleotides in a wide range of organisms.

RNase J1/J2

Recently, the discovery of RNase J1 and J2 shed new light on the mechanism of RNA degradation in *B. subtilis*. These enzymes were the first to be demonstrated to have bacterial 5′–3′ exoribonucleolytic activity (Mathy *et al.*, 2007). Moreover, two different activities can be observed for these enzymes, because they can act both as endo- and as exoribonucleases (Even *et al.*, 2005). RNases J1 and J2 had already been described under endoribonucleases (see the above section on Other endonucleases). RNase J1 is an essential protein (Even *et al.*, 2005) and its exoribonucleolytic activity depends on the phosphorylation state at the 5′ end, with a preference for monophosphate substrates (Mathy *et al.*, 2007). It was also shown that RNase J1 requires a single-stranded 5′ end to allow the exoribonucleolytic activity (Mathy *et al.*, 2007). It also functions as a 5′–3′ exoribonuclease in the maturation of the 16S rRNA gene and in regulating the mRNA stability of the *Bacillus thuringiensis* stationary-phase insecticidal protein transcript *cryIIIA* and the *trp* leader sequence (Mathy *et al.*, 2007; Deikus *et al.*, 2008). There are indications that RNase J1 plays an important role both in the maturation or degradation of specific RNAs and in governing global mRNA stability (Mader *et al.*, 2008). Interestingly, RNase J homologues are not present in *Gammaproteobacteria* such as *E. coli*, but are widely distributed in other bacteria and in archaea (Even *et al.*, 2005; Mathy *et al.*, 2007).

Other 3′–5′ exonucleases

In *E. coli*, besides the exoribonucleases mentioned above, three others are present in the cell: RNase PH, RNase D and RNase T.

RNase PH belongs to the same family of PNPase, the PDX family of exoribonucleases (see Fig. 1b). It is encoded by the *rph* gene and cotranscribed with pyrE, a gene necessary for pyrimidine synthesis that is located upstream of *rph* (Ost & Deutscher, 1991). However, while PNPase has an important function in mRNA degradation, RNase PH is involved in tRNA metabolism, namely in the processing of tRNA precursors (Deutscher *et al.*, 1988; Kelly *et al.*, 1992). RNase PH can act as a phosphorolytic RNase by removing nucleotides following the CCA terminus of tRNA and also as a nucleotidyltransferase by adding nucleotides to the ends of RNA molecules (Jensen *et al.*, 1992; Kelly & Deutscher, 1992). RNase PH can also cleave off the 3′ end of other sRNAs, including M1, 6S and 4.5S RNA (Li *et al.*, 1998). Deletion of the *rph* gene has no effect on the growth or the viability of the cells. However, the combination of this deletion with RNase T or PNPase deletions affects growth. These data suggest that RNase PH has overlapping functions *in vivo* with both RNase T and PNPase (Kelly *et al.*, 1992). In *B. subtilis*, there are two pathways for tRNA maturation and RNase PH seems to be the most important for the maturation of tRNA precursors with CCA motifs, while RNase Z is responsible for the processing of CCA-less tRNA precursors (Wen *et al.*, 2005). The crystal structure of *B. subtilis* RNase PH has been determined with a medium resolution and it can be superimposed to the second core domain structure of PNPase. Similar to what happens with RNase PH from *A. aeolicus* and *Pseudomonas aeruginosa*, the protein crystallizes as a hexamer arranged as a trimer of dimers and the substrate interacts with the dimer (Ishii *et al.*, 2003; Choi *et al.*, 2004; Harlow *et al.*, 2004). However, the hexameric ring formation is essential for the binding of precursor tRNA and also for exoribonucleolytic activity (Choi *et al.*,

2004). In *Streptomyces*, an RNase PH-like enzyme encoded by the *SCO2904* gene was identified. Similar to PNPase, this can polyadenylate the 3′ end of RNA *in vitro*; however, *in vivo* studies showed that RNase PH may not be involved in the synthesis or the maintenance of poly(A) tails in *S. coelicolor* (Bralley *et al.*, 2006). In *Streptomyces*, all essential tRNA genes must encode the CCA end and the RNase PH must be required to induce maturation of the 3′ end of these tRNAs (Bralley *et al.*, 2006) (see also below the section on processing).

RNase D is a 3′–5′ hydrolytic exoribonuclease from the DEDD superfamily, which contains both DNA and RNA exonucleases (Zuo & Deutscher, 2001) (Fig. 1b). As a member of this family, it has three conserved motifs. In motif III, the presence of a tyrosine or histidine led to the division of this family into two subgroups, DEDDy and DEDDh, with RNase D belonging to the first one (Zuo & Deutscher, 2001). RNase D requires divalent metal ions for its activity and has a high degree of substrate specificity; its substrates include denatured and damaged tRNAs, as well as tRNA precursors with extra 3′ residues following the CCA sequence, but not ssRNA (Cudny & Deutscher, 1980; Cudny *et al.*, 1981; Zhang & Deutscher, 1988b) (see also below the section on processing). RNase D overexpression seems to be deleterious for the cell (Zhang & Deutscher, 1988a). The chromosomal gene uses UUG as the initiation codon and has an abnormally high level of rare codons, which could limit the levels of endogenous protein (Kane, 1995). Moreover, it was shown that RNase D expression is negatively regulated at the translational level by the initiation codon (Zhang & Deutscher, 1989). The crystal structure of RNase D shows that this protein has one DEDD catalytic domain and two HRDC domains with a funnel-shaped ring architecture that could be important to define the exoribonucleolytic activity of RNase D, which may be processive (Zuo *et al.*, 2005). RNase D homologues have been found in many organisms, except archaea, and, in some genomes, it is possible to find more than one homologue (Zuo & Deutscher, 2001).

RNase T is a 3′–5′ exoribonuclease that belongs to the DEDD superfamily of RNases and to the DEDDh subgroup (Zuo & Deutscher, 2001) (Fig. 1b). It is a single-strand-specific exonuclease and the activity is dependent on the presence of divalent metal ions, such as Mg^{2+} or Mn^{2+} (Deutscher & Marlor, 1985; Zuo & Deutscher, 2002). Besides the ability to cleave RNA molecules, RNase T also has DNA exonuclease activity (Viswanathan *et al.*, 1998). RNase T has a distributive activity and an unusual base specificity, discriminating against pyrimidines and, particularly, C residues (Zuo & Deutscher, 2002). This sequence specificity is largely determined by the last four nucleotides at the 3′ end (Zuo & Deutscher, 2002). It is involved in the final step of maturation of many stable RNAs and seems to be the most important RNase with that function (Li & Deutscher, 1995, 1996; Li *et al.*, 1998). In fact, it was shown that RNase T is essential for the maturation of the 3′ ends of 5S and 23S rRNA genes (Li & Deutscher, 1995; Li *et al.*, 1999a), and it is also involved in the end turnover of tRNAs (Deutscher *et al.*, 1985). The crystal structures of RNase T from both *E. coli* and *P. aeruginosa* show that the protein adopts an oligoribonuclease-like homodimer architecture, which was shown to be required for its activity (Li *et al.*, 1996; Zuo *et al.*, 2007). The two monomers are facing opposite ends, which means that the active site of one monomer is facing the binding site of the other. This arrangement allows the binding of the RNA molecule from one monomer to be close to the active site of the other one (Zuo *et al.*, 2007). Despite its critical role in RNA metabolism, RNase T orthologues are just found in a small group of bacteria, the *Gamma* division of *Proteobacteria* (Zuo & Deutscher, 2001).

Both *E. coli* and *Salmonella* belong to the *Enterobacteriaceae* family. A recent work showed that the two hydrolytic enzymes present in *E. coli*, RNase II and RNase R, are also found in *Salmonella* and behave quite similarly in terms of their the ability to degrade structured substrates and the final product that is released. However, the proteins from *Salmonella* showed a reduction in their activity and an ability to bind to RNA when compared with the *E. coli* enzymes (Domingues *et al.*, 2009).

In *B. subtilis*, besides the proteins mentioned above, we can find other RNase, YhaM. This protein has been implicated in DNA replication (is able to degrade ssDNA), and *in vitro* studies showed that is also able to cleave RNA into the 3′–5′ direction in a Mn^{2+}-dependent manner. However, the *in vivo* function of YhaM in RNA metabolism remains to be determined (Noirot-Gros *et al.*, 2002; Oussenko *et al.*, 2002). Sequence homologues of YhaM were found only in Gram-positive bacteria (Oussenko *et al.*, 2002).

Cyanobacteria are prokaryotes organisms that may be related to the ancestor of chloroplasts. In the genome of *Synechocystis*, it is possible to find genes that have a high homology to RNase E, PNPase, RNase II/R and PAP, the most important proteins involved in mRNA degradation and polyadenylation (Rott *et al.*, 2003). However, the product of the putative PAP gene has nucleotidyltransferase and not PAP activity, and the reaction of polyadenylation in *Synechocystis* is performed by PNPase, which originates heterogeneous poly(A)-rich tails, like it occurs in chloroplasts. These tails are found in the amino acid coding region, the 5′ and 3′ untranslated regions of mRNAs, in rRNA and the single intron located at the tRNAfmet (Rott *et al.*, 2003). PNPase is an essential protein for this organism because the deletion of this gene causes lethality. The same is observed when the gene for RNase II/R is disrupted (Rott *et al.*, 2003). There is no degradosome complex in cyanobacteria (see Complexes of RNases).

Complexes of RNases

RNA-degrading machines

The degradosome is a large multiprotein complex involved in RNA degradation. It is believed to act as a general RNA decay machine in which the components of the degradosome cooperate during the decay of many RNAs. The complex formation contributes to the coordination of the endoribonucleolytic cleavage with the exoribonucleolytic degradation (Py *et al.*, 1994, 1996; Miczak *et al.*, 1996; Vanzo *et al.*, 1998).

In *E. coli*, this multiprotein complex is formed by RNA degradation enzymes RNase E and the exonuclease PNPase, as well as the ATP-dependent RhlB and the glycolytic enzyme enolase (Py *et al.*, 1994; Miczak *et al.*, 1996; Vanzo *et al.*, 1998). RNase E provides the organizing scaffold for the degradosome, through its carboxy-terminal half. In the carboxy-terminal half, four segments were found to show a tendency to form a secondary structure (Callaghan *et al.*, 2004), namely A, B, C and D. Segment A localizes the degradosome to the inner cytoplasmic membrane (Khemici *et al.*, 2008). RhlB binds a 69-residue conserved segment downstream of segment B, a coiled coil that may engage RNA (Chandran *et al.*, 2007; Worrall *et al.*, 2008b). Segment C is the enolase-binding site (Chandran & Luisi, 2006), and segment D interacts with PNPase (Callaghan *et al.*, 2004).

Under normal growth conditions, crystallographic and biophysical measurements indicate that one enolase dimer and one helicase protomer interact with one RNase E monomer (Chandran & Luisi, 2006; Chandran *et al.*, 2007; Worrall *et al.*, 2008a). Findings for the stoichiometry of PNPase with the isolated recognition site from RNase E (Callaghan *et al.*, 2004), and recent crystallographic analysis of the *E. coli* PNPase/RNase E complex reveal an equimolar ratio (Nurmohamed *et al.*, 2009). In principle, three RNase E tetramers and four PNPase trimers could form a self-closing assembly composed of 12 protomers, satisfying all possible binding sites. The ideal composition of such an assembly is 12 : 12 : 24 : 12 (RNase E : PNPase : enolase : RhlB) (Marcaida *et al.*, 2006).

The group of minor components that bind to the degradosome to affect its composition and modulate its enzymatic activity includes polyphosphate kinase, poly(A) polymerase, ribosomal proteins and the molecular chaperones DnaK and GroEL (Miczak *et al.*, 1996; Butland *et al.*, 2005; Morita *et al.*, 2005; Regonesi *et al.*, 2006) and other DEAD-box helicases (SrmB, RhlE and CsdA) that may bind to sites outside the RhlB recognition region (Khemici & Carpousis, 2004). Another potential interaction may occur between the degradosome and the cytoskeleton protein MinD (a membrane-localized bacterial cytoskeletal protein), which may account for the apparent association of the degradosome with the cytoskeleton (Taghbalout & Rothfield, 2007).

The composition of the degradosome can also undergo changes depending on the conditions of growth or stress (Khemici *et al.*, 2004; Prud'homme-Genereux *et al.*, 2004; Morita *et al.*, 2005; Gao *et al.*, 2006). A different complex containing RNase E, Hfq and SgrS, a small regulatory RNA, is formed under conditions of phosphosugar stress (Morita *et al.*, 2005). The formation of the complex with Hfq and SgrS requires the same region of RNase E that is necessary for the formation of the canonical RNA degradosome, and evidence suggests that the degradosome is remodelled as a consequence of the new interaction. There is evidence that RNase E can form a 'cold-shock' RNA degradosome in which the helicase RhlB is replaced by CsdA, another DEAD-box RNA helicase (Khemici *et al.*, 2004; Prud'homme-Genereux *et al.*, 2004). The compositional changes in the degradosome following cold exposure may account, in part, for changes in mRNA stability associated with cold shock response. The PNPase content of the degradosome can change in response to phosphosugar stress, temperature shock and the growth stage (Beran & Simons, 2001; Liou *et al.*, 2001). Surprisingly, RNase E from *P. syringae* interacts with the hydrolytic exoribonuclease RNase R instead of PNPase and with another DEAD-box helicase, RhlE (Purusharth *et al.*, 2005).

Degradosome composition and function may also be modulated through its interactions with the RNase E inhibitory proteins RraA and RraB, which interact with the C-terminal half of RNase E, thereby altering the composition of the degradosome, namely the amount of PNPase, RhlB and enolase bound to RNase E. RraB expression gave rise to degradosomes that contained the noncanonical components DnaK and CsdA.

The global effects of mutations in degradosome constituents on mRNA levels have been evaluated using microarrays (Bernstein *et al.*, 2004). This work reported that the functions of all degradosome constituents are necessary for normal mRNA turnover and that assembled degradosome components work in concert to regulate the transcripts of some *E. coli* metabolic pathways, but not others. This suggests the existence of structural features or biochemical factors that distinguish among different classes of mRNAs targeted for degradation.

Archaea are microscopic, single-celled organisms with no nucleus, no mitochondria and no chloroplasts. Regarding mRNA, they are more similar to bacteria than to eukaryotes: mRNA does not have introns, it is polycistronic, is not modified and does not have long stabilizing poly(A) tails at the 3′ end (Brown & Coleman, 1975; Brown & Reeve, 1986). However, in *Sulfolobus* and *Methanothermobacter*, the existence of an archaeal exosome with characteristics of the eukaryotic exosome was demonstrated (Evguenieva-Hackenberg *et al.*, 2003; Farhoud *et al.*, 2005). The exosome is a multiprotein complex involved in the maintenance of the

correct levels of mRNA in eukaryotic cells (van Hoof & Parker, 1999) (see also below the section on RNA degradation on eukaryotic microorganisms). The exosome of the archaeon *S. solfataricus* is a protein complex with a dual function: it is an RNA-tailing and RNA-degrading enzyme because it has both phosphorolytic and polyadenylating activity (Lorentzen *et al.*, 2005; Portnoy *et al.*, 2005). It is formed by a hexameric ring consisting of three dimers of the orthologues of Rrp41 and Rrp42, and is responsible for phosphorolytic RNA degradation (Lorentzen *et al.*, 2005). It is able to synthesize heteropolymeric RNA tails, and, generally, RNA synthesis by the hexameric ring is more efficient than RNA phosphorolysis (Evguenieva-Hackenberg *et al.*, 2008). The Rrp41 orthologue contains the active site; however, the ring structure is necessary for the activity of the complex (Lorentzen *et al.*, 2005). On the top of the ring there are three polypeptides with RNA-binding domains that are orthologues of Rrp4 (which contains S1 and KH domains) and/or Csl4 (which contains S1 and Zn-ribbon domains) (Buttner *et al.*, 2005; Lorentzen *et al.*, 2007). Recently, the structure of the *S. solfataricus* exosome was resolved (Lu *et al.*, 2010). The structure showed that the RNA-binding ring is flexible, which may be important for the unwinding of secondary structures (Lu *et al.*, 2010). The structure of the archaeal nine-subunit exosome is very similar to the one present in Eukarya and to PNPase (Lorentzen *et al.*, 2005, 2007; Liu *et al.*, 2006b). However, the archaeal exosome contains at least one additional subunit with an unknown function, a protein designated DnaG (Evguenieva-Hackenberg *et al.*, 2003), which can participate in 5S rRNA gene maturation. The *S. solfataricus* exosome is able to degrade synthetic and natural RNA efficiently, which is in accordance with its proposed role as a major complex of 3′ to 5′ exoribonucleases in the cell. Moreover, the genome of *S. solfataricus* does not contain genes for other predicted 3′–5′ exoribonucleases. In the absence of triphosphate at the 5′ end, the mRNA degradation can also occur in the 5′–3′ direction (Hasenohrl *et al.*, 2008). In this case, the degradation is probably performed by the RNase J1/J2 homologue, which is identical to the Mbl-like RNase (Koonin *et al.*, 2001).

However, in halophilic and many methanogenic archaea genomes, it is not possible to find the orthologues of exosomal subunits, which indicates that the mechanism for RNA degradation may be different in these archaea (Koonin *et al.*, 2001). Moreover, in archaea without an exosome, there is no post-transcriptional modification of the RNA molecules, and no tails are added to RNAs (Portnoy *et al.*, 2005; Portnoy & Schuster, 2006). In halophilic archaea, there is an RNase R-like protein that is not found in methanogenic archaea (Portnoy & Schuster, 2006). Like in *Mycoplasma*, these archaea also have a minimal genome, and, for this reason, the RNase R homologue may be the only enzyme responsible for the exoribonucleolytic activity, because both exosome and PNPase are absent (Zuo & Deutscher, 2001). *Haloferax volcanii* is a representative halophilic archaeon. It was shown that RNase R is required for viability in *H. volcanni*, and therefore, plays an important role in the mechanism of RNA degradation independent of polyadenylation (Portnoy *et al.*, 2005; Portnoy & Schuster, 2006).

The RNases in action

Processing and degradation of RNAs

Processing of RNAs

All rRNA and tRNA species are transcribed as precursor molecules that further undergo a series of modifications to achieve the mature molecules (Deutscher, 2009). Here, we will focus on the importance of RNases in the processing events during the maturation of rRNA and tRNA effectors. We will also refer to their role in the quality control of these processes.

In prokaryotes, the 70S ribosomes are constituted of two subunits: 30S and 50S particles. The smaller subunit comprises a 16S rRNA molecule and 21 proteins, and the larger subunit comprises a 23S and a 5S rRNA molecules plus 33 proteins. rRNAs are transcribed as precursor molecules that are processed and modified while assembly is occurring. In *E. coli*, there are seven rRNA operons comprising the three rRNA molecules always displayed in the same order: the 16S gene at the 5′ end, followed by the 23S, and finally by the 5S rRNA gene at the 3′ end (Deutscher, 2009). During transcription, RNase III cleaves double-stranded structures in the pre-rRNAs, releasing the fragments that will be subsequently cleaved to generate the 16S, 23S and 5S rRNA genes (Robertson *et al.*, 1968; Gegenheimer & Apirion, 1975).

RNase E further reduces the extra 115 nt from the 17S rRNA gene (16S rRNA gene precursor) to 66 at the 5′ end, resulting in a 16.3S intermediate. Finally, RNase G (also termed RNase M16) converts the 5′ end to the mature molecule (Hayes & Vasseur, 1976; Dahlberg *et al.*, 1978; Li *et al.*, 1999b). In *B. subtilis*, the 5′–3′ exoribonuclease RNase J1 is involved in rRNA processing (Even *et al.*, 2005; Britton *et al.*, 2007; de la Sierra-Gallay *et al.*, 2008). The 3′ maturation enzyme remains to be characterized both in *E. coli* and in *B. subtilis*. In *P. syringae* the 3′–5′ exonuclease RNase R seems to be acting to directly induce the maturation of the 3′ terminus of the 16S rRNA gene (Cheng & Deutscher, 2002, 2005; Deutscher, 2006, 2009; Purusharth *et al.*, 2007).

The *E. coli* 23S rRNA gene precursor is released, harboring three or seven 5′ and seven to nine 3′ extra residues. The 3′ maturation requires RNase T for completion (Li *et al.*, 1999a). In *B. subtilis* the RNase III family Mini-III dimeric enzyme is responsible for the simultaneous maturation of both 5′ and 3′ sides of the double-stranded stalk that flanks

the mature 23S rRNA gene (Olmedo & Guzman, 2008; Redko *et al.*, 2008). *Salmonella* constitutes an interesting case where RNase III removes IVS in a way that the mature rRNA molecule results from two fragments (Burgin *et al.*, 1990).

The *E. coli* 5S rRNA gene derives from a 9S precursor, which is endonucleolytically cleaved by RNase E, releasing an intermediate molecule with three additional nucleotides at both ends (Ghora & Apirion, 1978; Misra & Apirion, 1979). The 5′ maturation is still uncharacterized, while RNase T is again responsible for removing (at least) the least two 3′ residues (Li & Deutscher, 1995). *Bacillus subtilis* almost repeats the mechanism of maturation of the 23S for the 5S rRNA gene, but in this case, RNase M5 cleaves the double-stranded region, simultaneously inducing the maturation of the 5′ and 3′ ends (Sogin *et al.*, 1977).

rRNA degradation takes place whenever errors (e.g. improper structure conformations, or misordered addition of proteins) occur and also in response to stress conditions (Deutscher, 2009). Quality control mechanisms occur at levels that are almost negligible in fast-growing cells, but are nevertheless essential as they avoid the accumulation of defective ribosomes. RNase LS may participate in the 23S rRNA gene degradation; PNPase, together with an RNA helicase or RNase R, may also be involved, because they are the only ones that can degrade structured RNAs. In addition to these, any process that leads to damaged cell membranes induces drastic RNA degradation, because it promotes the release of the nonspecific endoribonuclease RNase I from the periplasm into the cells (Cheng & Deutscher, 2005; Otsuka & Yonesaki, 2005; Deutscher, 2009).

tRNAs are vital adaptors for the decoding of the genome into proteins, and contribute up to 20% of the total RNA in the cell (Dittmar *et al.*, 2004; Hartmann *et al.*, 2009). Both *E. coli* K12 and *B. subtilis* bear 86 tRNA genes in their genome, many of them associated into operons (Fournier & Ozeki, 1985; Inokuchi & Yamao, 1995; Dittmar *et al.*, 2004). Introns are rarely found and are present only in the anticodon loop of some tRNAs in bacteria, but occur extensively in archaea (Vogel & Hess, 2001; Marck & Grosjean, 2002, 2003). Two endoribonucleases mainly process the pre-tRNAs: RNase P, which almost universally generates 5′ mature ends (Evans *et al.*, 2006; Randau *et al.*, 2008), and RNase Z, which cleaves the CCA-less pre-tRNAs (see the sections on RNase P and RNase Z for details on these enzymes). All tRNA molecules must have a CCA signal at their 3′ end to allow aminoacylation by the tRNA nucleotidyltransferase. That can be achieved, either by removing all extra nucleotides, when it is already present in the sequence, or cutting after the discriminator nucleotide (Li & Deutscher, 1995; Hartmann *et al.*, 2009). The CCA motif varies from absent in eukarya to being present in all genes of *E. coli*, about 2/3 of the *B. subtilis* pre-tRNAs, and from 0% to 100% in archaea (Hartmann *et al.*, 2009). Two main modes of 3′ maturation have been described so far: a one-step endonucleolytic cleavage by the universally conserved RNase Z homodimer (Dutta & Deutscher, 2009) and a multistep process involving both endo- and exonucleases (Li *et al.*, 1998; Hartmann *et al.*, 2009).

For instance, in *E. coli* where all genes encode the CCA sequence, maturation usually begins with an RNase E cut at the 3′ end (eventually aided by PNPase or RNase II), followed by 5′ processing by RNase P, and a final 3′ exonucleolytic trimming to expose the CCA sequence. The trimming reaction is carried out by RNase II, RNase D, or more effectively, RNase T or RNase PH (Li & Deutscher, 2002; Ow & Kushner, 2002).

Even though RNase Z is not essential for *E. coli*, it is encoded in its genome and has been shown to be able to shut down growth when overexpressed (Takaku & Nashimoto, 2008).

In *B. subtilis* all the CCA-less tRNAs are processed by the RNase Z and all the CCA-containing tRNAs are envisaged to follow a multistep maturation pathway, although the endonuclease responsible for the first step has not yet been found (Pellegrini *et al.*, 2003). RNase PH is the main exo involved in the trimming process (Wen *et al.*, 2005).

tRNAs have several constraints because they must be sufficiently similar to be processed, and able to fit within the ribosome, but must be sufficiently different to ensure correct loading with specific amino acids and recognize exclusively the codon(s) for their anticodon sequence (Hopper *et al.*, 2010). Modifications are of absolute importance for folding stabilization avoiding rapid decay, fidelity and efficiency of aminoacylation and/or proper binding to the ribosomes (Hou & Perona, 2010; Phizicky & Alfonzo, 2010). Indeed, about 100 modifications have been described for tRNAs so far (Czerwoniec *et al.*, 2009; Hopper *et al.*, 2010). Although tRNAs are stable, they have quality control mechanisms for eliminating defective species, and it seems at least partially dependent on polyadenylation by poly(A) polymerase (and removal by polynucleotide phosphorylase). RNase R has also been shown to participate in tRNA quality control mechanisms in a *B. subtilis* conditional CCA mutant strain. In this sense, flawed stable RNA molecules would behave like unstable RNAs being rapidly degraded by similar mechanisms (Li *et al.*, 2002; Campos-Guillen *et al.*, 2010).

tmRNA is a hybrid/bifunctional RNA molecule that shares the characteristics of both tRNA structural folds involving the 3′ and 5′ ends (Hayes & Keiler, 2010) – and mRNA – bearing a sequence that encodes for an ORF, consisting of a peptide signal for proteolytic degradation, ended with UAA termination codons. The tmRNA maturation is similar to the mechanism described above regarding tRNA processing. However, it was shown that RNase R is quite important for the maturation of the 3′ end of the

tmRNA, even more relevantly under cold-shock conditions (Cairrão *et al.*, 2003). SmpB is a small basic protein that binds to tmRNA with a high affinity and specificity (Karzai *et al.*, 1999; Dulebohn *et al.*, 2006), and specifically recognizes paused ribosomes near the 3′ end of truncated mRNAs (Janssen & Hayes, 2009). This RNA-binding protein is a regulator for the tmRNA-based quality control system in the cells, because it can prevent tmRNA degradation by RNase R (Hong *et al.*, 2005).

RNA degradation mechanisms

The same RNA molecule can be degraded by different pathways depending on the stress conditions or the growth phase. Thus, the degradation pathways are not universal. However, the interplay between the different factors involved in RNA decay emphasizes the role of RNases in the degradation of multiple substrates (Fig. 3).

In this section, we illustrate various examples of the relevant mechanisms of mRNAs and sRNAs degradation mainly in *E. coli*, but we also refer to examples from *B. subtilis*.

pyrF-orfF

The dicistronic transcript from *pyrF-orfF* contains *pyrF*, encoding orotidine-5′-monophosphatase decarboxylase, and an ORF (*orfF*) encoding a polypeptide of unknown function (Donovan & Kushner, 1983; Jensen *et al.*, 1984; Turnbough *et al.*, 1987). The full-length transcript is rapidly cleaved into a series of breakdown products, and at least 18 endonucleolytic cleavage sites have been mapped throughout the full-length mRNA (Arraiano *et al.*, 1997). Moreover, it seems that the *pyrF-orfF* transcript may be degraded by more than one enzymatic pathway depending on where the initial cleavage occurs. Therefore, some fragments seem to be degraded in a 5′–3′ direction, while other degradation products are processively cleaved in a 3′–5′ direction. The results obtained by Arraiano *et al.* (1997) provided, for the first time, support to the hypothesis that multiple decay pathways are involved in the decay of a single transcript. It thus seems reasonable to assume that *in vivo* there are a variety of ways in which a particular mRNA can be degraded. Which pathway is used may be related to the particular context in which one or more of the decay-mediating factors has access to the mRNA.

trxA

The *E. coli trxA* gene, which encodes for thioredoxin, is transcribed as a monocistronic message of 493 nucleotides. In the study of the *trxA* decay multiple mutant strains were constructed deficient in RNase E (*rne* – previously known as *ams*), PNPase (*pnp*) and RNase II (*rnb*) (Arraiano *et al.*, 1988). Northern and S1 analysis showed that full-length transcripts are initially processed by endonucleolytic cleavages (Arraiano *et al.*, 1993). The complete degradation of the initially cleaved transcripts occurs through progression of endonucleolytic steps in the 3′–5′ direction, followed by exonucleolytic degradation by RNase II and PNPase. This was the first report of a progression of endonucleolytic cleavages in a 3′–5′ direction during the degradation of a full-length transcript.

rpsO

The *rpsO* gene encodes for the *E. coli* ribosomal protein S15. The degradation of *rpsO* mRNA is accomplished by several independent pathways, including the RNase E-dependent endonucleolytic pathway and a pathway that requires the polyadenylation of transcripts (Braun *et al.*, 1996). The stability of the *rpsO* transcript is mainly controlled by RNase E. After RNase E cleavage, the mRNA lacking the 3′-terminal RNA secondary structure becomes an ideal substrate for PNPase (Braun *et al.*, 1996). When the primary pathway of decay mediated by RNase E is inactive, the exoribonucleolytic poly(A)-dependent degradation of *rpsO* mRNA is stimulated (Hajnsdorf *et al.*, 1995; Marujo *et al.*, 2003; Folichon *et al.*, 2005). It was shown that RNase R is the main enzyme involved in the poly(A)-dependent degradation of the *rpsO* mRNA (Andrade *et al.*, 2009a) and that RNase II protects the full-length *rpsO* mRNA from degradation by removing the poly(A) tails (Marujo *et al.*, 2000). Elongated *rpsO* transcripts harboring poly(A) tails of increased length are specifically recognized by RNase R and strongly accumulate in the absence of this exonuclease. Because this enzyme is able to degrade dsRNAs, the 3′ oligo(A)-extension may stimulate the binding of RNase R, allowing the complete degradation of the *rpsO* mRNA. The RNA chaperone Hfq can protect the *rpsO* mRNA from exonucleolytic degradation by PNPase and RNase II, and from cleavage by RNase E (Folichon *et al.*, 2003). Moreover, it was shown recently that in the absence of this chaperone, stabilization of *rpsO* mRNA occurs, with a concomitant decrease in its level, indicating that the change in the mRNA levels in the *hfq* mutant does not result from the modification of RNA stability, but probably from changes in transcriptional activity (Le Derout *et al.*, 2010).

rpsT

The *rps*T gene encodes the *E. coli* ribosomal protein S20. This gene is transcribed from two promoters (P1 and P2) and terminates at a Rho-independent terminator, yielding two monocistronic mRNA species: P1 (447 nt) and P2 (356 nt) (Mackie & Parsons, 1983). The first step of the *rpsT* decay is carried out by RNase E and there are several lines of evidence indicating that this step is independent of polyadenylation (Mackie, 1991; Coburn & Mackie, 1996b, 1998). However,

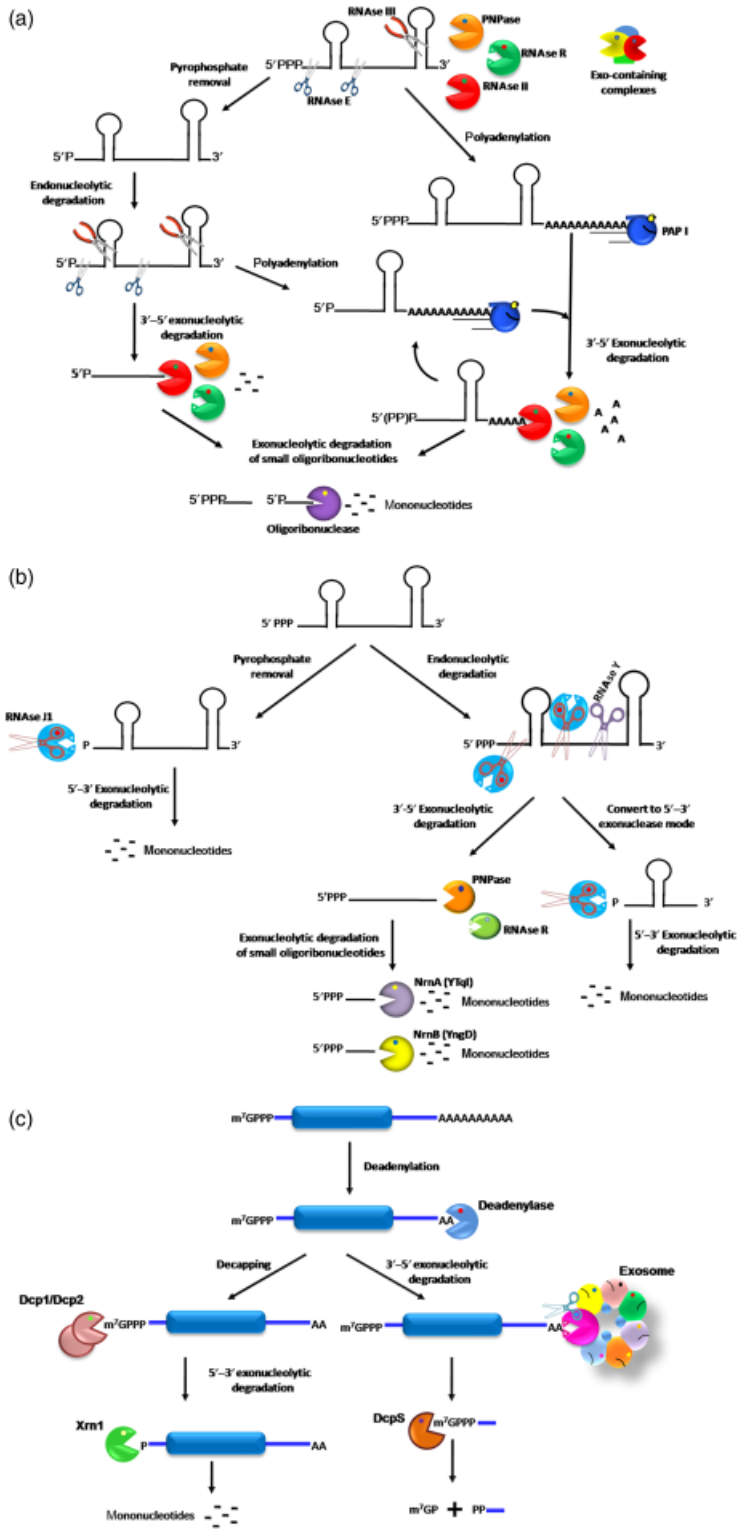

Fig. 3. Mechanisms of decay. (a) Model of RNA degradation pathways in *Escherichia coli*. The decay of the majority of transcripts starts with an endoribonucleolytic cleavage by RNase E. This endoribonuclease prefers a monophosphorylated 5′ end, but not in a strict way, and several RNAs escaping this rule have been described (Kime *et al.*, 2009). RNase III is another enzyme responsible for the initial endoribonucleolytic cleavage of structured RNAs. However, unlike RNase E (that only cleaves single-stranded RNAs), RNase III cleaves dsRNAs. After endoribonucleolytic cleavages, the linear transcripts are rapidly degraded by the 3′–5′ degradative exoribonucleases, RNase II, RNase R and PNPase. RNase R, unlike RNase II and PNPase, is efficient against highly structured RNAs. PNPase, in association with other proteins, namely RNA helicases, can also unwind RNA duplexes. A minor pathway in the cell is the exoribonucleolytic degradation of full-length transcripts. Poly(A) polymerase (PAP I) adds a poly(A) tail to the short 3′ overhang. These tails provide a 'toe-hold' to which exoribonucleases can bind. Cycles of polyadenylation and exoribonucleolytic digestion can overcome RNA secondary structures. The small oligoribonucleotides (two to five nucleotides) released by exoribonucleases are finally degraded to mononucleotides by oligoribonuclease (Andrade *et al.*, 2009b). (b) Model of RNA degradation pathways in *Bacillus subtilis*. In *B. subtilis*, the main enzyme responsible for RNA decay is RNase J1. RNase J1 has both an endoribonucleolytic and a 5′–3′ exoribonucleolytic activity (Mathy *et al.*, 2007). RNase J2 has endoribonucleolytic cleavage activities and specificities similar to RNase J1 and normally they form a complex. RNAs can be degraded from the 5′ end by the 5′–3′ exoribonuclease activity of RNase J1, or first, they can be endonucleolytically cleaved by RNase J1 or by RNase Y (Shahbabian *et al.*, 2009). The products from this endoribonucleolytic cleavage can then be degraded by the 3′–5′ exoribonucleases, PNPase and RNase R, or by the 5′–3′ exoribonuclease activity of RNase J1 (Bechhofer, 2009). The small oligoribonucleotides released by the 3′–5′ exoribonucleases are finally degraded to mononucleotides by the NrnA (YTqI) or the NrnB (YngD) enzymes (Fang *et al.*, 2009). (c) Model of RNA degradation in eukaryotes. In yeast, the mRNA decay is initiated with the shortening of the poly(A) tail at the 3′ end (deadenylation). After deadenylation, there are two possible degradation pathways for the transcripts. One is the removal of the 5′ cap structure of the transcripts by the Dcp1p/Dcp2p decapping complex, leaving the RNA molecule accessible to the Xrn1 5′–3′ exoribonuclease, which rapidly degrades the uncapped RNA. The other pathway is the 3′–5′ exoribonucleolytic degradation by the exosome, a multiprotein complex in which the Rrp44 is the only active RNase (Houseley & Tollervey, 2009). Recently, it was demonstrated that Rrp44 can degrade RNA in both an exo- and an endoribonucleolytic manner (Schaeffer *et al.*, 2009). The capped oligonucleotides produced from the exosome RNA decay are hydrolyzed by the DcpS scavenger decapping enzyme (Liu & Kiledjian, 2006a).

PAP I, PNPase, ATP and phosphate are necessary to catalyze the degradation of the smaller intermediates generated by RNase E cleavage (Coburn & Mackie, 1998). On the other hand, RNase II inhibits PNPase-mediated degradation of transcripts by removing the poly(A) tails added by PAP I. The same had also been observed with *rpsO* (Coburn & Mackie,

1998; Marujo *et al.*, 2000). Therefore, RNase II paradoxically protects these RNAs from degradation by PNPase.

malEF

The polycistronic malEFG operon of *E. coli* encodes three proteins involved in the transport of maltodextrins. The *malEF* intercistronic region contains two REP sequences (Newbury *et al.*, 1987) that protect the transcript from 3′–5′ exonucleolytic degradation (Higgins *et al.*, 1988). RNase R and PNPase are shown to play a major role in the degradation of the sRNA fragments resulting from the RNase E cleavage (Khemici & Carpousis, 2004; Cheng & Deutscher, 2005). PNPase degradation of the *malEF* transcript is only accomplished in the presence of RNase E and RhlB, indicating that the degradosome complex participates in this degradation (Stickney *et al.*, 2005). RhlB unwinds the folded RNA and passes it to PNPase (Coburn *et al.*, 1999; Khemici & Carpousis, 2004). Polyadenylation of the *malEF* REP sequences by PAP I seems to be a crucial factor in the degradation of these sequences because they accumulate to high levels in *pcn*B mutants (Khemici & Carpousis, 2004).

ompA

The *ompA* gene is transcribed as a monocistronic mRNA and encodes the major protein of *E. coli* outer membrane OmpA (von Gabain *et al.*, 1983). It was demonstrated previously that *ompA* stability is growth rate dependent and that shorter generation times in the exponential phase corresponded to longer *ompA* mRNA half-lives (Nilsson *et al.*, 1984). The degradation of this mRNA is initiated by an RNase E cleavage in the 5′ UTR stem-loops (Melefors & von Gabain, 1988; Arnold *et al.*, 1998). Then, exonucleolytic degradation and polyadenylation seem to account for the elimination of breakdown products (O'Hara *et al.*, 1995; Mohanty & Kushner, 1999; Andrade *et al.*, 2006). The presence of only one of the exoribonucleases (RNase II, RNase R or PNPase) may be sufficient to remove most of the decay intermediates (Cheng & Deutscher, 2005). Furthermore, the exonucleolytic activity on the full-length *ompA* transcript was shown to be growth phase regulated (Andrade *et al.*, 2006). The sRNA MicA, first known as SraD, is the principal post-transcriptional regulator of the *ompA* expression (Rasmussen *et al.*, 2005; Udekwu *et al.*, 2005). This antisense sRNA, when present in high levels, blocks ribosome binding at the *ompA* mRNA translation start site and subsequently destabilizes this mRNA. Moreover, the MicA-mediated decay of *ompA* mRNA depends on Hfq (Rasmussen *et al.*, 2005; Udekwu *et al.*, 2005). Therefore, the levels of *ompA* are also dependent on the levels of MicA. Because OmpA is one of the main outer membrane proteins in *E. coli*, it is fundamental to have a strict regulation in order to maintain the homeostasis of the cell.

pac

Penicillin amidase, encoded by the *pac* gene, is an important enzyme for industry because it is used in the production of semi-synthetic penicillins. The degradation of this mRNA seems to be initiated by an endonucleolytic cleavage because the most remarkable stabilization of the *E. coli pac* mRNA was obtained in the RNase E mutant. RNase III seems to play no role in the degradation of this transcript. The RNase E cleavage is followed by the exonucleolytic degradation by RNase II, RNase R and/or PNPase. Single deletions of any of these exoribonucleases were unable to stabilize this mRNA most probably because of their redundant effect (Viegas *et al.*, 2005).

trp

In the last few years, the degradation of the *B. subtilis* tryptophan operon, *trp*, has been studied in detail. This operon was used recently for the study of the cleavage specificity of the RNase J1 endonuclease (Deikus & Bechhofer, 2009). The *trp* operon is regulated at the level of transcription termination (Babitzke & Gollnick, 2001; Henkin & Yanofsky, 2002), which is controlled by binding of the *trp* RNA-binding attenuation protein (TRAP) to the *trp* leader RNA. When the supply of intracellular tryptophan is low, the *trp* operon genes are transcribed from a constitutive promoter and more tryptophan is generated. When the intracellular supply of tryptophan is sufficient, the TRAP protein complex binds to a specific region of the *trp* leader sequence. This binding results in the formation of a stem-loop structure that induces transcription termination, generating a 140 nt *trp* leader RNA.

The degradation of this *trp* leader RNA is initiated by an RNase J1 endonucleolytic cleavage at a single-stranded AU-rich region upstream of the 3′ transcription terminator (Deikus *et al.*, 2008). This cleavage is followed by a 5′–3′ degradation of the downstream fragment by the exonucleolytic activity of the RNase J1 (Deikus *et al.*, 2008) and a 3′–5′ degradation of the upstream fragment by PNPase (Deikus *et al.*, 2004). The PNPase action is essential for the efficient release and recycling of TRAP (Deikus *et al.*, 2004).

sRNAs

RNases also play a very important role in the regulation of sRNAs. These RNAs have received considerable attention over the past decade because they can be crucial for the post-transcriptional control of gene expression (Storz *et al.*, 2004; Viegas & Arraiano, 2008). In order to understand the action of these sRNAs, it is fundamental to study the processing and turnover of these molecules.

sRNA MicA and RybB are stationary-phase regulators and belong to the group of sRNAs that control outer membrane permeability. RybB controls the expression of outer membrane proteins OmpC and OmpW (Guillier

et al., 2006; Johansen *et al.*, 2006) and MicA controls the expression of OmpA (Rasmussen *et al.*, 2005; Udekwu *et al.*, 2005). In *E. coli*, MicA and RybB are destabilized by PNPase in the stationary phase (Andrade & Arraiano, 2008). Moreover, PNPase can degrade MicA in a degradosome-independent manner. Polyadenylation of MicA by PAP I appears not to be essential for PNPase action on this sRNA. The 3′ exoribonucleases RNase II and RNase R appear not to be required for the degradation of MicA.

In *S. typhimurium*, the sRNAs MicA, SraL, CsrB and CsrC are also mainly degraded by PNPase in the late stationary phase. In the case of CsrB and CsrC, the absence of this exoribonuclease also induced a change in degradation patterns with the accumulation of several decay intermediates (Viegas *et al.*, 2007).

The antisense RNA CopA inhibits the replication of plasmid R1 by binding to the target region, CopT, that is located within the *repA* mRNA. This binding blocks the synthesis of the replication initiator protein RepA (Stougaard *et al.*, 1981; Givskov & Molin, 1984). The decay of CopA is initiated by an endonucleolytic cleavage by RNase E, followed by the addition of a poly(A) tail. The poly(A) tails facilitate degradation by PNPase and RNase II (Söderbom *et al.*, 1997). Both PNPase and RNase II were able to degrade the processed transcript (Söderbom & Wagner, 1998).

ColE1 RNAI is the copy number regulator of the plasmid ColE1 (Lin-Chao & Cohen, 1991). PNPase, PAP I, RNase E and RNase III have been demonstrated to play roles in ColE1 RNAI decay (Lin-Chao & Cohen, 1991; Xu *et al.*, 1993; Xu & Cohen, 1995; Binnie *et al.*, 1999). Two degradation pathways have been suggested for this RNA (Binnie *et al.*, 1999). The primary pathway starts with RNase E cleavage, followed by PAP I polyadenylation and PNPase-mediated degradation. The second mechanism begins with the polyadenylation of RNAI, followed by RNase III cleavage and a subsequent exonucleolytic attack. In the absence of RNase E, RNase III and PAP I, the antisense RNAI continues to disappear, showing that yet other enzymes are able to catalyze its decay.

The replication of the ColE2 plasmid requires a plasmid-coded initiator protein, Rep. ColE2 RNAI controls *rep* expression by the blockage of translation (Takechi *et al.*, 1994). ColE2 RNAI degradation starts with RNase E cleavage at the 5′ end. PAP I polyadenylates the 3′ ends of degradation intermediates and both RNase II and PNPase act in further exoribonucleolytic degradation (Nishio & Itoh, 2008). Because PNPase and RNase II prefer a single-stranded 'toe-hold' to bind the 3′ end of the mRNA, PAP I generates a binding site for these exoribonucleases by adding a poly(A) tail to the 3′ end of the mRNA. Thus, cycles of polyadenylation and exoribonucleolytic attack contribute towards the correct degradation of the mRNA after the initial cleavage.

The *hok*/*sok* system mediates plasmid R1 stabilization by killing plasmid-free cells. Sok antisense RNA inhibits the translation of the *hok* mRNA, a toxic protein mRNA (Gerdes *et al.*, 1990). As Sok RNA is highly unstable, the pool of free Sok RNA decays rapidly in plasmid-free cells. The decay of Sok RNA leads to Hok protein synthesis and killing of the plasmid-free cells (Dam Mikkelsen & Gerdes, 1997). Like in the other antisense RNAs described previously, the initial step of Sok RNA decay is performed by RNase E in the single-stranded 5′ end. RNase E cleavage products are rapidly degraded from their 3′ ends by PNPase using a PAP I-dependent mechanism. Sok RNA, as well as CopA, is destabilized when RNase II is absent.

RNA degradation in eukaryotes

Because this publication has focused mainly on RNA degradation in prokaryotes, it was not the purpose of this chapter to provide a complete overview of RNA metabolism in eukaryotic cells but only pinpoints some interesting links between the systems. For a more comprehensive overview of the RNA degradation pathways in eukaryotes, readers can refer to publications focused on eukaryotes (Doma & Parker, 2007; Amaral *et al.*, 2008; Rougemaille *et al.*, 2008; Shyu *et al.*, 2008; Houseley & Tollervey, 2009; Moore & Proudfoot, 2009).

RNA degradation in eukaryotes is much more complex and involves more factors than those in prokaryotes (Houseley & Tollervey, 2009). The eukaryotic cell is divided into two main parts: the nucleus and the cytoplasm, and RNA degradation is important in both compartments. Compartmentalization causes considerable change in mRNA's fate; eukaryotic RNAs have to survive in the cell much longer than prokaryotic messengers, and the molecule synthesized in the nucleolus has to be transported to the cytoplasm for protein production. In the nucleus, aberrant transcripts are selectively degraded; RNases also act in multiple processing steps and remove the processing byproducts and a myriad of noncoding cryptic transcripts. The balance between the rate of transcription and RNA degradation regulates messenger levels. In the cytoplasm, the transcripts are translated to the proteins; therefore, in this compartment, it is very important to check the translational abilities of RNAs and remove incorrect molecules that can cause the production of aberrant proteins (Doma & Parker, 2007). In the cytoplasm, differences in the degradation rate can influence protein expression. A set of factors can affect the lifetime of the transcript including RNA-binding proteins that bind to the RNAs, and sRNAs that can drive transcripts to degradation or cause translational silencing (siRNA and miRNA) (Eulálio *et al.*, 2008; Carthew & Sontheimer, 2009).

It has been considered that in eukaryotes, the RNA degradation is mainly exonucleolytic (Fig. 3), while in prokaryotes, endonucleases have a significant impact on degradation process. In the best-studied model – yeast *S. cerevisiae* – the main enzymes involved in the degradation are exoribonucleases. Degradation in the 5′–3′ direction is performed by the Xrn1 protein in the cytoplasm and the Rat1 enzyme in the nucleus (Fritz *et al.*, 2004; Meyer *et al.*, 2004). The main yeast 3′–5′ hydrolytic exonuclease is Rrp44/(Dis3) from the RNase II family. In the nucleus, there is also another 3′–5′ exonuclease: Rrp6. Rrp44 interacts with the nine-protein ring-shapes complex to generate a ribonucleolytically active exosome, where Rrp44 is the only active RNase (Liu *et al.*, 2006b; Dziembowski *et al.*, 2007). The exosome ring is homologous to the archaeal complex with phosphorolytic nuclease activity and to the bacterial PNPase (Lorentzen *et al.*, 2007). Surprisingly, this huge protein machine lost its phosphorolytic activity in the evolution and in most eukaryotes can induce RNA degradation only when cooperating with the active component Rrp44 (Dziembowski *et al.*, 2007). Recent structural studies showed that even if the Rrp44 protein by itself is able to degrade RNA, it seems that the substrates that are delivered to this nuclease first have to pass the channel in the exosome ring structure (Bonneau *et al.*, 2009).

Research performed in the last few years proved that involvement of endonuclease activity in the RNA degradation process in eukaryotes was underestimated. Among the other examples (Huntzinger *et al.*, 2008; Eberle *et al.*, 2009), the most evident was the discovery of the endonucleolytic activity of the exosome complex; this activity is carried by the PIN domain localized in the N-terminal part of the Rrp44 protein. Rrp44, the only active component of the yeast exosome, can degrade RNA in both an exo- and an endonucleolytic manner. Because the homologues of Rrp44 from other eukaryotes also have PIN domains, it seems that endonucleolytic activity is the common feature in its RNA degradation (Lebreton *et al.*, 2008; Schaeffer *et al.*, 2009).

For a long time, the function of polyadenylation in the RNA degradation process was considered as one of the most striking differences between the eukaryotic and the prokaryotic RNA metabolism. In the eukaryotes, long poly(A) tails added by the poly(A) polymerase to the 3′ end of newly created transcripts have been considered as RNA-stabilizing elements while in the prokaryotic cell polyadenylation leads to transcript degradation. Surprisingly, it was discovered that in eukaryotes, polyadenylation can also drive RNAs to decay. The TRAMP complex composed of poly(A) polymerase, helicase and an RNA-binding protein is able to add short poly(A) tails to the aberrant transcripts, targeting them to induce rapid degradation (LaCava *et al.*, 2005). This showed that the poly(A)-dependent RNA degradation mechanism active in prokaryotes is still present in eukaryotic cells.

Last discoveries in the field of RNA degradation in eukaryotes showed that we can find much more similarities to prokaryotic systems than was previously expected. The degradation pathways in eukaryotes are obviously more complex and different in many aspects, but at the same time, many mechanisms are very similar. We can find homologues of prokaryotic enzymes that serve important functions in eukaryotic systems such as bacterial RNase II and RNase R homologue Rrp44, RNase D homologue Rrp6, the exosome ring that is structurally very similar to PNPase and others. Moreover, we can find strikingly similar mechanisms even if they are performed by factors without obvious homology. A key example is the prokaryotic antiviral defense system CRISPR, which resembles the eukaryotic RNAi mechanism (Hale *et al.*, 2009). Another example is the 5′–3′ direction exoribonucleolytic degradation pathway, which is very important in eukaryotic RNA metabolism. In the last few years, it became clear that, in spite of earlier beliefs, this pathway in prokaryotes also exists, but enzymes that are involved are not homologues of the eukaryotic ones (Mathy *et al.*, 2007). This and many other examples clearly show that evolution has led to the development of similar solutions regarding degradation mechanisms.

Eukaryotic organelles are structures of endosymbiotic prokaryotic origin; they possess their own usually reduced genome, which is expressed and transcribed, and RNAs are processed and degraded. The expression of proteins encoded in the organellar genome is, in most cases, crucial for energy management in eukaryotic cells. Many questions still remain about the RNA degradation pathways in organelles, mostly because they seem to be different in different organisms and so it is hard to find the general rules that can be applied to all systems. Nonetheless, RNA metabolic pathways in the organelles retained some characteristics of the prokaryotic ancestors. RNA degradation in chloroplasts seems to be most similar to prokaryotic process. In the higher plant genomes, we can find sequences of homologues of bacterial nucleases RNase E and RNase J that are localized in chloroplasts (Lange *et al.*, 2009). The degradation process, similar to that in bacteria, starts with endonucleolytic cleavage and is then accelerated by polyadenylation and exonucleolytic degradation by PNPase. There is also an RNase R homologue that was shown to play a role in rRNA processing (Bollenbach *et al.*, 2005).

RNA degradation pathways in the mitochondria seem to be more divergent in different organisms. Interestingly, and in contrast to the situation in chloroplasts, degradation pathways in the mitochondria are supposed to be mostly exonucleolytic. In plants, the main player seems to be PNPase, which degrades polyadenylated RNA molecules in the mitochondria (Holec *et al.*, 2006). In contrast, in yeast *S. cerevisiae*, there is no mitochondrial PNPase; instead, the main degrading machinery is the mitochondrial

degradosome complex (mtEXO), which digests RNA in the 3′–5′ direction and is composed of the homologue of RNase II-Dis3 protein and the conserved RNA DEAD-box helicase Suv3 (Dziembowski *et al.*, 2003; Malecki *et al.*, 2007). Additionally, it was suggested that there is one more potential enzyme Pet127 that can degrade RNA in the 5′–3′ direction (Fekete *et al.*, 2008). Surprisingly, there is no polyadenylaton in yeast mitochondria; instead, stabilizing functions are served by the proteins that bind to the 3′ and 5′ untranslated ends of the RNA molecules. Degradation of transcripts in human mitochondria is not well characterized. Although the data on this topic are not consistent, it seems that a homologue of bacterial PNPase is present in the mitochondria, and it was found recently that it can form a complex with the human homologue of Suv3 helicase. Suv3 is involved in RNA degradation and removal of aberrant and cryptic transcripts; the exact function of this protein is still not clear (Szczesny *et al.*, 2010). Transcripts in human mitochondria are stably polyadenylated, which, in contrast to the situation in plant mitochondria, suggests a stabilization role for poly(A) tails (Tomecki *et al.*, 2004). On the other hand, scientists also discovered polyadenylated degradation byproducts, which suggests that polyadenylation can trigger or aid transcripts' degradation; therefore, it seems that polyadenylation in human mitochondria can serve both functions (Slomovic *et al.*, 2005; Szczesny *et al.*, 2010).

Concluding remarks

Maintenance of optimal levels of RNAs at any time and under any circumstance is an extremely difficult task to achieve and requires great coordination among all the factors involved in this control. It is also assumed that there is a cross-talk between transcription and degradation to maintain the balance that is best for the survival of microorganisms. There are several examples where this is obvious, and when a specific message is more transcribed, it is also more stabilized, and vice versa.

Transcripts can have a different half-life under different growth conditions to rapidly carry out the necessary changes and adjust to adequate RNA levels. The same RNA can have a 'preferred' decay pathway, but there are examples where there are alternative degradation pathways for the same transcript, depending on which enzyme cleaves first. After cleavage, the RNA breakdown product(s) can have a distinct half-life depending on sequence and structure. Therefore, the structural characteristics of RNA stability and instability predetermine the 'fate' of an RNA, but the environment and the consequent levels and nature of the degradative enzymes will also play a determinant role in its turnover. For instance, the mRNAs expressed in heterologous systems can have a very different half-life than if they are expressed in their own microorganism. The directionality of the decay process depends on the transcript analyzed. Once we characterize the enzymes from one microorganism, we can design strategies to stabilize RNAs. Mutants have been instrumental in characterizing degradation pathways and in changing the turnover of specific transcripts, especially because a limited number of RNases intervene in the maturation and degradation of RNAs.

There are fundamental principles that govern RNA decay in all organisms. Evolution has resulted in similar functions performed by different enzymes. For instance, in *E. coli*, RNase E is one of the major endonucleases, but this enzyme is absent in *B. subtilis*. In *B. subtilis*, RNase J1 seems to take over the same function, and this enzyme is not present in *E. coli*. RNase J1 has been shown to have both endo and 5′–3′ exo activities. In yeast, 5′–3′ decay is prominent, and Rrp44/Dis3, an RNase II family enzyme, has dual endo and 3′–5′ exo activities, being an example of an optimized 'RNA degradation machine'. Sometimes, RNases also combine into complexes to speed up the decay process or confer specificity to certain targets.

It is fascinating to know that RNases themselves are strictly regulated proteins and have mechanisms to adapt them to the environment and to the levels of the other RNases. For instance, RNase R is highly increased under cold shock; the levels of PNPase and RNase II are inter-regulated and the level of RNase E is autoregulated.

Recent studies demonstrate that, between prokaryotic and eukaryotic systems, the RNA degradation mechanisms have much more similarities than expected. The mechanism of RNAi in eukaryotes has shown the power of RNA degradation mechanisms involving RNases. It is now obvious that the modulation of RNA levels and their respective proteins can be rapidly achieved. In prokaryotes, it was already known that antisense RNAs could be quite important for the control of gene expression. Moreover, the recently discovered CRISP RNAs (Karginov & Hannon, 2010), which can be considered a bacterial RNAi mechanism, have lent an extra level of complexity to the study of RNAs and bacterial RNA degradation mechanisms. It is very stimulating to work in a field of research still full of surprises! This is a thorough review, but in a few years, we are sure that there will be much more to say!

It is our hope that this review conveys some of the current excitement in research on RNA and serves as a source of inspiration for scientists entering this field.

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References

Afonyushkin T, Vecerek B, Moll I, Blasi U & Kaberdin VR (2005) Both RNase E and RNase III control the stability of *sodB* mRNA upon translational inhibition by the small regulatory RNA RyhB. *Nucleic Acids Res* **33**: 1678–1689.

Agrawal N, Dasaradhi PV, Mohmmed A, Malhotra P, Bhatnagar RK & Mukherjee SK (2003) RNA interference: biology, mechanism, and applications. *Microbiol Mol Biol R* **67**: 657–685.

Alifano P, Rivellini F, Piscitelli C, Arraiano CM, Bruni CB & Carlomagno MS (1994) Ribonuclease E provides substrates for ribonuclease P-dependent processing of a polycistronic mRNA. *Gene Dev* **8**: 3021–3031.

Altman S, Wesolowski D, Guerrier-Takada C & Li Y (2005) RNase P cleaves transient structures in some riboswitches. *P Natl Acad Sci USA* **102**: 11284–11289.

Amaral PP, Dinger ME, Mercer TR & Mattick JS (2008) The eukaryotic genome as an RNA machine. *Science* **319**: 1787–1789.

Amblar M & Arraiano CM (2005) A single mutation in *Escherichia coli* ribonuclease II inactivates the enzyme without affecting RNA binding. *FEBS J* **272**: 363–374.

Amblar M, Viegas SC, Lopez P & Arraiano CM (2004) Homologous and heterologous expression of RNase III from *Lactococcus lactis*. *Biochem Bioph Res Co* **323**: 884–890.

Amblar M, Barbas A, Fialho AM & Arraiano CM (2006) Characterization of the functional domains of *Escherichia coli* RNase II. *J Mol Biol* **360**: 921–933.

Amblar M, Barbas A, Gomez-Puertas P & Arraiano CM (2007) The role of the S1 domain in exoribonucleolytic activity: substrate specificity and multimerization. *RNA* **13**: 317–327.

Andrade JM & Arraiano CM (2008) PNPase is a key player in the regulation of small RNAs that control the expression of outer membrane proteins. *RNA* **14**: 543–551.

Andrade JM, Cairrao F & Arraiano CM (2006) RNase R affects gene expression in stationary phase: regulation of *ompA*. *Mol Microbiol* **60**: 219–228.

Andrade JM, Hajnsdorf E, Régnier P & Arraiano CM (2009a) The poly(A)-dependent degradation pathway of *rpsO* mRNA is primarily mediated by RNase R. *RNA* **15**: 316–326.

Andrade JM, Pobre V, Silva IJ, Domingues S & Arraiano CM (2009b) The role of 3′–5′ exoribonucleases in RNA degradation. *Prog Mol Biol Transl Sci* **85**: 187–229.

Apirion D & Lassar AB (1978) A conditional lethal mutant of *Escherichia coli* which affects the processing of ribosomal RNA. *J Biol Chem* **253**: 1738–1742.

Arnold TE, Yu J & Belasco JG (1998) mRNA stabilization by the *ompA* 5′ untranslated region: two protective elements hinder distinct pathways for mRNA degradation. *RNA* **4**: 319–330.

Arraiano C, Yancey SD & Kushner SR (1993) Identification of endonucleolytic cleavage sites involved in decay of *Escherichia coli trxA* mRNA. *J Bacteriol* **175**: 1043–1052.

Arraiano CM, Yancey SD & Kushner SR (1988) Stabilization of discrete mRNA breakdown products in *ams pnp rnb* multiple mutants of *Escherichia coli* K-12. *J Bacteriol* **170**: 4625–4633.

Arraiano CM, Cruz AA & Kushner SR (1997) Analysis of the *in vivo* decay of the *Escherichia coli* dicistronic *pyrF-orfF* transcript: evidence for multiple degradation pathways. *J Mol Biol* **268**: 261–272.

Arraiano CM, Barbas A & Amblar M (2008) Characterizing ribonucleases *in vitro* examples of synergies between biochemical and structural analysis. *Method Enzymol* **447**: 131–160.

Asha PK, Blouin RT, Zaniewski R & Deutscher MP (1983) Ribonuclease BN: identification and partial characterization of a new tRNA processing enzyme. *P Natl Acad Sci USA* **80**: 3301–3304.

Awano N, Inouye M & Phadtare S (2008) RNase activity of polynucleotide phosphorylase is critical at low temperature in *Escherichia coli* and is complemented by RNase II. *J Bacteriol* **190**: 5924–5933.

Awano N, Rajagopal V, Arbing M, Patel S, Hunt J, Inouye M & Phadtare S (2010) *Escherichia coli* RNase R has dual activities, helicase and RNase. *J Bacteriol* **192**: 1344–1352.

Babitzke P & Gollnick P (2001) Posttranscription initiation control of tryptophan metabolism in *Bacillus subtilis* by the *trp* RNA-binding attenuation protein (TRAP), anti-TRAP, and RNA structure. *J Bacteriol* **183**: 5795–5802.

Babitzke P & Kushner SR (1991) The Ams (altered mRNA stability) protein and ribonuclease E are encoded by the same structural gene of *Escherichia coli*. *P Natl Acad Sci USA* **88**: 1–5.

Babitzke P, Granger L, Olszewski J & Kushner SR (1993) Analysis of mRNA decay and rRNA processing in *Escherichia coli* multiple mutants carrying a deletion in RNase III. *J Bacteriol* **175**: 229–239.

Baer MF, Wesolowski D & Altman S (1989) Characterization *in vitro* of the defect in a temperature-sensitive mutant of the protein subunit of RNase P from *Escherichia coli*. *J Bacteriol* **171**: 6862–6866.

Baker KE & Mackie GA (2003) Ectopic RNase E sites promote bypass of 5′-end-dependent mRNA decay in *Escherichia coli*. *Mol Microbiol* **47**: 75–88.

Barbas A, Andrade JM, Fialho AM & Arraiano CM (2006) Ribonucleases e controlo pós-transcripcional da expressão génica. *O Mundo do RNA – Novos Desafios e Perspectivas Futuras* (Arraiano CM & Fialho AM, eds), pp. 119–139. Lidel Edições Técnicas, Lisbon, Portugal.

Barbas A, Matos RG, Amblar M, Lopez-Vinas E, Gomez-Puertas P & Arraiano CM (2008) New insights into the mechanism of RNA degradation by ribonuclease II: identification of the residue responsible for setting the RNase II end-product. *J Biol Chem* **283**: 13070–13076.

Barbas A, Matos RG, Amblar M, Lopez-Vinas E, Gomez-Puertas P & Arraiano CM (2009) Determination of key residues for catalysis and RNA cleavage specificity: one mutation turns RNase II into a 'SUPER-ENZYME'. *J Biol Chem* **284**: 20486–20498.

Bardwell JC, Régnier P, Chen SM, Nakamura Y, Grunberg-Manago M & Court DL (1989) Autoregulation of RNase III operon by mRNA processing. *EMBO J* **8**: 3401–3407.

Barnett TC, Bugrysheva JV & Scott JR (2007) Role of mRNA stability in growth phase regulation of gene expression in the group A *Streptococcus*. *J Bacteriol* **189**: 1866–1873.

Bechhofer DH (2009) Messenger RNA decay and maturation in *Bacillus subtilis*. *Prog Mol Biol Transl Sci* **85**: 231–273.

Belitsky BR, Gustafsson MC, Sonenshein AL & Von Wachenfeldt C (1997) An lrp-like gene of *Bacillus subtilis* involved in branched-chain amino acid transport. *J Bacteriol* **179**: 5448–5457.

Beran RK & Simons RW (2001) Cold-temperature induction of *Escherichia coli* polynucleotide phosphorylase occurs by reversal of its autoregulation. *Mol Microbiol* **39**: 112–125.

Bermúdez-Cruz RM, Fernandez-Ramírez F, Kameyama-Kawabe L & Montañez C (2005) Conserved domains in polynucleotide phosphorylase among eubacteria. *Biochimie* **87**: 737–745.

Bernstein JA, Lin PH, Cohen SN & Lin-Chao S (2004) Global analysis of *Escherichia coli* RNA degradosome function using DNA microarrays. *P Natl Acad Sci USA* **101**: 2758–2763.

Binnie U, Wong K, McAteer S & Masters M (1999) Absence of RNase III alters the pathway by which RNAI, the antisense inhibitor of ColE1 replication, decays. *Microbiology* **145**: 3089–3100.

Blaszczyk J, Tropea JE, Bubunenko M, Routzahn KM, Waugh DS, Court DL & Ji X (2001) Crystallographic and modeling studies of RNase III suggest a mechanism for double-stranded RNA cleavage. *Structure* **9**: 1225–1236.

Blaszczyk J, Gan J, Tropea JE, Court DL, Waugh DS & Ji X (2004) Noncatalytic assembly of ribonuclease III with double-stranded RNA. *Structure* **12**: 457–466.

Bollenbach TJ, Lange H, Gutierrez R, Erhardt M, Stern DB & Gagliardi D (2005) RNR1, a 3′–5′ exoribonuclease belonging to the RNR superfamily, catalyzes 3′ maturation of chloroplast ribosomal RNAs in *Arabidopsis thaliana*. *Nucleic Acids Res* **33**: 2751–2763.

Bonneau F, Basquin J, Ebert J, Lorentzen E & Conti E (2009) The yeast exosome functions as a macromolecular cage to channel RNA substrates for degradation. *Cell* **139**: 547–559.

Bothwell AL, Garber RL & Altman S (1976) Nucleotide sequence and *in vitro* processing of a precursor molecule to *Escherichia coli* 4.5 S RNA. *J Biol Chem* **251**: 7709–7716.

Bralley P & Jones GH (2003) Overexpression of the polynucleotide phosphorylase gene (pnp) of *Streptomyces antibioticus* affects mRNA stability and poly(A) tail length but not ppGpp levels. *Microbiology* **149**: 2173–2182.

Bralley P, Gust B, Chang S, Chater KF & Jones GH (2006) RNA 3′-tail synthesis in *Streptomyces*: *in vitro* and *in vivo* activities of RNase PH, the SCO3896 gene product and polynucleotide phosphorylase. *Microbiology* **152**: 627–636.

Braun F, Hajnsdorf E & Régnier P (1996) Polynucleotide phosphorylase is required for the rapid degradation of the RNase E-processed *rps*O mRNA of *Escherichia coli* devoid of its 3′ hairpin. *Mol Microbiol* **19**: 997–1005.

Briani F, Del Favero M, Capizzuto R *et al.* (2007) Genetic analysis of polynucleotide phosphorylase structure and functions. *Biochimie* **89**: 145–157.

Briani F, Curti S, Rossi F, Carzaniga T, Mauri P & Dehò G (2008) Polynucleotide phosphorylase hinders mRNA degradation upon ribosomal protein S1 overexpression in *Escherichia coli*. *RNA* **14**: 2417–2429.

Britton RA, Wen T, Schaefer L *et al.* (2007) Maturation of the 5′ end of *Bacillus subtilis* 16S rRNA by the essential ribonuclease YkqC/RNase J1. *Mol Microbiol* **63**: 127–138.

Brown JW & Reeve JN (1986) Polyadenylated RNA isolated from the archaebacterium *Halobacterium halobium*. *J Bacteriol* **166**: 686–688.

Brown S & Coleman G (1975) Messenger ribonucleic acid content of *Bacillus amyloliquefaciens* throughout its growth cycle compared with *Bacillus subtilis* 168. *J Mol Biol* **96**: 345–352.

Bugrysheva JV & Scott JR (2009) The ribonucleases J1 and J2 are essential for growth and have independent roles in mRNA decay in *Streptococcus pyogenes*. *Mol Microbiol* **75**: 731–743.

Burgin AB, Parodos K, Lane DJ & Pace NR (1990) The excision of intervening sequences from *Salmonella* 23S ribosomal RNA. *Cell* **60**: 405–414.

Butland G, Peregrin-Alvarez JM, Li J *et al.* (2005) Interaction network containing conserved and essential protein complexes in *Escherichia coli*. *Nature* **433**: 531–537.

Buttner K, Wenig K & Hopfner KP (2005) Structural framework for the mechanism of archaeal exosomes in RNA processing. *Mol Cell* **20**: 461–471.

Cairrão F & Arraiano CM (2006) The role of endoribonucleases in the regulation of RNase R. *Biochem Bioph Res Co* **343**: 731–737.

Cairrão F, Chora A, Zilhão R, Carpousis AJ & Arraiano CM (2001) RNase II levels change according to the growth conditions: characterization of *gmr*, a new *Escherichia coli* gene involved in the modulation of RNase II. *Mol Microbiol* **39**: 1550–1561.

Cairrão F, Cruz A, Mori H & Arraiano CM (2003) Cold shock induction of RNase R and its role in the maturation of the quality control mediator SsrA/tmRNA. *Mol Microbiol* **50**: 1349–1360.

Calin-Jageman I & Nicholson AW (2003) RNA structure-dependent uncoupling of substrate recognition and cleavage by *Escherichia coli* ribonuclease III. *Nucleic Acids Res* **31**: 2381–2392.

Callaghan AJ, Aurikko JP, Ilag LL *et al.* (2004) Studies of the RNA degradosome-organizing domain of the *Escherichia coli* ribonuclease RNase E. *J Mol Biol* **340**: 965–979.

Callaghan AJ, Marcaida MJ, Stead JA, McDowall KJ, Scott WG & Luisi BF (2005a) Structure of *Escherichia coli* RNase E catalytic domain and implications for RNA turnover. *Nature* **437**: 1187–1191.

Callaghan AJ, Redko Y, Murphy LM *et al.* (2005b) 'Zn-link': a metal-sharing interface that organizes the quaternary structure and catalytic site of the endoribonuclease, RNase E. *Biochemistry* **44**: 4667–4675.

Campos-Guillen J, Arvizu-Gomez JL, Jones GH & Olmedo-Alvarez G (2010) Characterization of tRNACys processing in a conditional *Bacillus subtilis* CCase mutant reveals the

participation of RNase R in its quality control. *Microbiology* **156**: 2102–2111.
Cannistraro VJ & Kennell D (1989) Purification and characterization of ribonuclease M and mRNA degradation in *Escherichia coli*. *Eur J Biochem* **181**: 363–370.
Cannistraro VJ & Kennell D (1991) RNase I*, a form of RNase I, and mRNA degradation in *Escherichia coli*. *J Bacteriol* **173**: 4653–4659.
Cannistraro VJ & Kennell D (1994) The processive reaction mechanism of ribonuclease II. *J Mol Biol* **243**: 930–943.
Cannistraro VJ & Kennell D (1999) The reaction mechanism of ribonuclease II and its interaction with nucleic acid secondary structures. *Biochim Biophys Acta* **1433**: 170–187.
Cardenas PP, Carrasco B, Sanchez H, Deikus G, Bechhofer DH & Alonso JC (2009) *Bacillus subtilis* polynucleotide phosphorylase 3′-to-5′ DNase activity is involved in DNA repair. *Nucleic Acids Res* **37**: 4157–4169.
Carpousis AJ, Vanzo NF & Raynal LC (1999) mRNA degradation. A tale of poly(A) and multiprotein machines. *Trends Genet* **15**: 24–28.
Carpousis AJ, Luisi BF & McDowall KJ (2009) Endonucleolytic initiation of mRNA decay in *Escherichia coli*. *Prog Mol Biol Transl Sci* **85**: 91–135.
Carthew RW & Sontheimer EJ (2009) Origins and mechanisms of miRNAs and siRNAs. *Cell* **136**: 642–655.
Carzaniga T, Briani F, Zangrossi S, Merlino G, Marchi P & Dehò G (2009) Autogenous regulation of *Escherichia coli* polynucleotide phosphorylase expression revisited. *J Bacteriol* **191**: 1738–1748.
Celesnik H, Deana A & Belasco JG (2007) Initiation of RNA decay in *Escherichia coli* by 5′ pyrophosphate removal. *Mol Cell* **27**: 79–90.
Cerritelli SM & Crouch RJ (2009) Ribonuclease H: the enzymes in eukaryotes. *FEBS J* **276**: 1494–1505.
Chandran V & Luisi BF (2006) Recognition of enolase in the *Escherichia coli* RNA degradosome. *J Mol Biol* **358**: 8–15.
Chandran V, Poljak L, Vanzo NF *et al.* (2007) Recognition and cooperation between the ATP-dependent RNA helicase RhlB and ribonuclease RNase E. *J Mol Biol* **367**: 113–132.
Charpentier X, Faucher SP, Kalachikov S & Shuman HA (2008) Loss of RNase R induces competence development in *Legionella pneumophila*. *J Bacteriol* **190**: 8126–8136.
Chen JL, Nolan JM, Harris ME & Pace NR (1998) Comparative photocross-linking analysis of the tertiary structures of *Escherichia coli* and *Bacillus subtilis* RNase P RNAs. *EMBO J* **17**: 1515–1525.
Cheng ZF & Deutscher MP (2002) Purification and characterization of the *Escherichia coli* exoribonuclease RNase R. Comparison with RNase II. *J Biol Chem* **277**: 21624–21629.
Cheng ZF & Deutscher MP (2003) Quality control of ribosomal RNA mediated by polynucleotide phosphorylase and RNase R. *P Natl Acad Sci USA* **100**: 6388–6393.
Cheng ZF & Deutscher MP (2005) An important role for RNase R in mRNA decay. *Mol Cell* **17**: 313–318.
Cheng ZF, Zuo Y, Li Z, Rudd KE & Deutscher MP (1998) The *vacB* gene required for virulence in *Shigella flexneri* and *Escherichia coli* encodes the exoribonuclease RNase R. *J Biol Chem* **273**: 14077–14080.
Choi JM, Park EY, Kim JH, Chang SK & Cho Y (2004) Probing the functional importance of the hexameric ring structure of RNase PH. *J Biol Chem* **279**: 755–764.
Choonee N, Even S, Zig L & Putzer H (2007) Ribosomal protein L20 controls expression of the *Bacillus subtilis infC* operon via a transcription attenuation mechanism. *Nucleic Acids Res* **35**: 1578–1588.
Chung DH, Min Z, Wang BC & Kushner SR (2010) Single amino acid changes in the predicted RNase H domain of *Escherichia coli* RNase G lead to complementation of RNase E deletion mutants. *RNA* **16**: 1371–1385.
Clements MO, Eriksson S, Thompson A, Lucchini S, Hinton JC, Normark S & Rhen M (2002) Polynucleotide phosphorylase is a global regulator of virulence and persistency in *Salmonella enterica*. *P Natl Acad Sci USA* **99**: 8784–8789.
Coburn GA & Mackie GA (1996a) Overexpression, purification, and properties of *Escherichia coli* ribonuclease II. *J Biol Chem* **271**: 1048–1053.
Coburn GA & Mackie GA (1996b) Differential sensitivities of portions of the mRNA for ribosomal protein S20 to 3′-exonucleases dependent on oligoadenylation and RNA secondary structure. *J Biol Chem* **271**: 15776–15781.
Coburn GA & Mackie GA (1998) Reconstitution of the degradation of the mRNA for ribosomal protein S20 with purified enzymes. *J Mol Biol* **279**: 1061–1074.
Coburn GA, Miao X, Briant DJ & Mackie GA (1999) Reconstitution of a minimal RNA degradosome demonstrates functional coordination between a 3′ exonuclease and a DEAD-box RNA helicase. *Gene Dev* **13**: 2594–2603.
Commichau FM, Rothe FM, Herzberg C *et al.* (2009) Novel activities of glycolytic enzymes in *Bacillus subtilis*: interactions with essential proteins involved in mRNA processing. *Mol Cell Proteomics* **8**: 1350–1360.
Condon C & Putzer H (2002) The phylogenetic distribution of bacterial ribonucleases. *Nucleic Acids Res* **30**: 5339–5346.
Condon C, Brechemier-Baey D, Beltchev B, Grunberg-Manago M & Putzer H (2001) Identification of the gene encoding the 5S ribosomal RNA maturase in *Bacillus subtilis*: mature 5S rRNA is dispensable for ribosome function. *RNA* **7**: 242–253.
Condon C, Rourera J, Brechemier-Baey D & Putzer H (2002) Ribonuclease M5 has few, if any, mRNA substrates in *Bacillus subtilis*. *J Bacteriol* **184**: 2845–2849.
Court DL (1993) RNA processing and degradation by RNase III. *Control of Messenger RNA Stability* (Belasco GJ & Brawerman G, ed), pp. 71–116. Academic Press, New York.
Cudny H & Deutscher MP (1980) Apparent involvement of ribonuclease D in the 3′ processing of tRNA precursors. *P Natl Acad Sci USA* **77**: 837–841.
Cudny H, Zaniewski R & Deutscher MP (1981) *Escherichia coli* RNase D. Catalytic properties and substrate specificity. *J Biol Chem* **256**: 5633–5637.

Czerwoniec A, Dunin-Horkawicz S, Purta E *et al.* (2009) MODOMICS: a database of RNA modification pathways. 2008 update. *Nucleic Acids Res* **37**: D118–D121.

Dahlberg AE, Dahlberg JE, Lund E *et al.* (1978) Processing of the 5′ end of *Escherichia coli* 16S ribosomal RNA. *P Natl Acad Sci USA* **75**: 3598–3602.

Dam Mikkelsen N & Gerdes K (1997) Sok antisense RNA from plasmid R1 is functionally inactivated by RNase E and polyadenylated by poly(A) polymerase I. *Mol Microbiol* **26**: 311–320.

Dasgupta S, Fernandez L, Kameyama L, Inada T, Nakamura Y, Pappas A & Court DL (1998) Genetic uncoupling of the dsRNA-binding and RNA cleavage activities of the *Escherichia coli* endoribonuclease RNase III – the effect of dsRNA binding on gene expression. *Mol Microbiol* **28**: 629–640.

Datta AK & Niyogi K (1975) A novel oligoribonuclease of *Escherichia coli*. II. Mechanism of action. *J Biol Chem* **250**: 7313–7319.

Davies JF II, Hostomska Z, Hostomsky Z, Jordan SR & Matthews DA (1991) Crystal structure of the ribonuclease H domain of HIV-1 reverse transcriptase. *Science* **252**: 88–95.

Deana A, Celesnik H & Belasco JG (2008) The bacterial enzyme RppH triggers messenger RNA degradation by 5′ pyrophosphate removal. *Nature* **451**: 355–358.

Deikus G & Bechhofer DH (2009) *Bacillus subtilis trp* Leader RNA: RNase J1 endonuclease cleavage specificity and PNPase processing. *J Biol Chem* **284**: 26394–26401.

Deikus G, Babitzke P & Bechhofer DH (2004) Recycling of a regulatory protein by degradation of the RNA to which it binds. *P Natl Acad Sci USA* **101**: 2747–2751.

Deikus G, Condon C & Bechhofer DH (2008) Role of *Bacillus subtilis* RNase J1 endonuclease and 5′-exonuclease activities in *trp* leader RNA turnover. *J Biol Chem* **283**: 17158–17167.

de la Sierra-Gallay IL, Pellegrini O & Condon C (2005) Structural basis for substrate binding, cleavage and allostery in the tRNA maturase RNase Z. *Nature* **433**: 657–661.

de la Sierra-Gallay IL, Zig L, Jamalli A & Putzer H (2008) Structural insights into the dual activity of RNase J. *Nat Struct Mol Biol* **15**: 206–212.

Del Favero M, Mazzantini E, Briani F, Zangrossi S, Tortora P & Dehò G (2008) Regulation of *Escherichia coli* polynucleotide phosphorylase by ATP. *J Biol Chem* **283**: 27355–27359.

Deutscher MP (1990) Ribonucleases, tRNA nucleotidyltransferase, and the 3′ processing of tRNA. *Prog Nucleic Acid Re* **39**: 209–240.

Deutscher MP (2006) Degradation of RNA in bacteria: comparison of mRNA and stable RNA. *Nucleic Acids Res* **34**: 659–666.

Deutscher MP (2009) Maturation and degradation of ribosomal RNA in bacteria. *Prog Mol Biol Transl Sci* **85**: 369–391.

Deutscher MP & Marlor CW (1985) Purification and characterization of *Escherichia coli* RNase T. *J Biol Chem* **260**: 7067–7071.

Deutscher MP & Reuven NB (1991) Enzymatic basis for hydrolytic versus phosphorolytic mRNA degradation in *Escherichia coli* and *Bacillus subtilis*. *P Natl Acad Sci USA* **88**: 3277–3280.

Deutscher MP, Marlor CW & Zaniewski R (1985) RNase T is responsible for the end-turnover of tRNA in *Escherichia coli*. *P Natl Acad Sci USA* **82**: 6427–6430.

Deutscher MP, Marshall GT & Cudny H (1988) RNase PH: an *Escherichia coli* phosphate-dependent nuclease distinct from polynucleotide phosphorylase. *P Natl Acad Sci USA* **85**: 4710–4714.

Dittmar KA, Mobley EM, Radek AJ & Pan T (2004) Exploring the regulation of tRNA distribution on the genomic scale. *J Mol Biol* **337**: 31–47.

Diwa A, Bricker AL, Jain C & Belasco JG (2000) An evolutionarily conserved RNA stem-loop functions as a sensor that directs feedback regulation of RNase E gene expression. *Gene Dev* **14**: 1249–1260.

Doma MK & Parker R (2007) RNA quality control in eukaryotes. *Cell* **131**: 660–668.

Domingues S, Matos RG, Reis FP, Fialho AM, Barbas A & Arraiano CM (2009) Biochemical characterization of the RNase II family of exoribonucleases from the human pathogens *Salmonella typhimurium* and *Streptococcus pneumoniae*. *Biochemistry* **48**: 11848–11857.

Donovan WP & Kushner SR (1983) Cloning and physical analysis of the *pyr*F gene (coding for orotidine-5′-phosphate decarboxylase) from *Escherichia coli* K-12. *Gene* **25**: 39–48.

Donovan WP & Kushner SR (1986) Polynucleotide phosphorylase and ribonuclease II are required for cell viability and mRNA turnover in *Escherichia coli* K-12. *P Natl Acad Sci USA* **83**: 120–124.

Drider D & Condon C (2004) The continuing story of endoribonuclease III. *J Mol Microb Biotech* **8**: 195–200.

Drider D, Santos JM, Arraiano CM & Lopez P (1998) RNA processing is involved in the post-transcriptional control of the citQRP operon from *Lactococcus lactis* biovar *diacetylactis*. *Mol Gen Genet* **258**: 9–15.

Drider D, Garcia-Quintans N, Santos JM, Arraiano CM & Lopez P (1999) A comparative analysis of the citrate permease P mRNA stability in *Lactococcus lactis* biovar diacetylactis and *Escherichia coli*. *FEMS Microbiol Lett* **172**: 115–122.

Dulebohn DP, Cho HJ & Karzai AW (2006) Role of conserved surface amino acids in binding of SmpB protein to SsrA RNA. *J Biol Chem* **281**: 28536–28545.

Dutta T & Deutscher MP (2009) Catalytic properties of RNase BN/RNase Z from *Escherichia coli*: RNase BN is both an exo- and endoribonuclease. *J Biol Chem* **284**: 15425–15431.

Dutta T & Deutscher MP (2010) Mode of action of RNase BN/RNase Z on tRNA precursors: RNase BN does not remove the CCA sequence from tRNA. *J Biol Chem*, in press.

Dziembowski A, Piwowarski J, Hoser R *et al.* (2003) The yeast mitochondrial degradosome. Its composition, interplay between RNA helicase and RNase activities and the role in mitochondrial RNA metabolism. *J Biol Chem* **278**: 1603–1611.

Dziembowski A, Lorentzen E, Conti E & Seraphin B (2007) A single subunit, Dis3, is essentially responsible for yeast exosome core activity. *Nat Struct Mol Biol* **14**: 15–22.

Eberle AB, Lykke-Andersen S, Muhlemann O & Jensen TH (2009) SMG6 promotes endonucleolytic cleavage of nonsense mRNA in human cells. *Nat Struct Mol Biol* **16**: 49–55.

Erova TE, Kosykh VG, Fadl AA, Sha J, Horneman AJ & Chopra AK (2008) Cold shock exoribonuclease R (VacB) is involved in *Aeromonas hydrophila* pathogenesis. *J Bacteriol* **190**: 3467–3474.

Eulálio A, Huntzinger E & Izaurralde E (2008) Getting to the root of miRNA-mediated gene silencing. *Cell* **132**: 9–14.

Evans D, Marquez SM & Pace NR (2006) RNase P: interface of the RNA and protein worlds. *Trends Biochem Sci* **31**: 333–341.

Even S, Pellegrini O, Zig L, Labas V, Vinh J, Brechemmier-Baey D & Putzer H (2005) Ribonucleases J1 and J2: two novel endoribonucleases in *B. subtilis* with functional homology to *E. coli* RNase E. *Nucleic Acids Res* **33**: 2141–2152.

Evguenieva-Hackenberg E & Klug G (2000) RNase III processing of intervening sequences found in helix 9 of 23S rRNA in the alpha subclass of Proteobacteria. *J Bacteriol* **182**: 4719–4729.

Evguenieva-Hackenberg E & Klug G (2009) RNA degradation in Archaea and Gram-negative bacteria different from *Escherichia coli*. *Prog Mol Biol Transl Sci* **85**: 275–317.

Evguenieva-Hackenberg E, Schiltz E & Klug G (2002) Dehydrogenases from all three domains of life cleave RNA. *J Biol Chem* **277**: 46145–46150.

Evguenieva-Hackenberg E, Walter P, Hochleitner E, Lottspeich F & Klug G (2003) An exosome-like complex in *Sulfolobus solfataricus*. *EMBO Rep* **4**: 889–893.

Evguenieva-Hackenberg E, Roppelt V, Finsterseifer P & Klug G (2008) Rrp4 and Csl4 are needed for efficient degradation but not for polyadenylation of synthetic and natural RNA by the archaeal exosome. *Biochemistry* **47**: 13158–13168.

Ezraty B, Dahlgren B & Deutscher MP (2005) The RNase Z homologue encoded by *Escherichia coli elaC* gene is RNase BN. *J Biol Chem* **280**: 16542–16545.

Falaleeva MV, Chetverina HV, Ugarov VI, Uzlova EA & Chetverin AB (2008) Factors influencing RNA degradation by *Thermus thermophilus* polynucleotide phosphorylase. *FEBS J* **275**: 2214–2226.

Fang M, Zeisberg WM, Condon C, Ogryzko V, Danchin A & Mechold U (2009) Degradation of nanoRNA is performed by multiple redundant RNases in *Bacillus subtilis*. *Nucleic Acids Res* **37**: 5114–5125.

Farhoud MH, Wessels HJ, Steenbakkers PJ *et al.* (2005) Protein complexes in the archaeon *Methanothermobacter thermautotrophicus* analyzed by blue native/SDS-PAGE and mass spectrometry. *Mol Cell Proteomics* **4**: 1653–1663.

Fekete Z, Ellis TP, Schonauer MS & Dieckmann CL (2008) Pet127 governs a $5' \rightarrow 3'$-exonuclease important in maturation of apocytochrome b mRNA in *Saccharomyces cerevisiae*. *J Biol Chem* **283**: 3767–3772.

Fiedler TJ, Vincent HA, Zuo Y, Gavrialov O & Malhotra A (2004) Purification and crystallization of *Escherichia coli* oligoribonuclease. *Acta Crystallogr D* **60**: 736–739.

Folichon M, Arluison V, Pellegrini O, Huntzinger E, Régnier P & Hajnsdorf E (2003) The poly(A) binding protein Hfq protects RNA from RNase E and exoribonucleolytic degradation. *Nucleic Acids Res* **31**: 7302–7310.

Folichon M, Marujo PE, Arluison V, Le Derout J, Pellegrini O, Hajnsdorf E & Régnier P (2005) Fate of mRNA extremities generated by intrinsic termination: detailed analysis of reactions catalyzed by ribonuclease II and poly(A) polymerase. *Biochimie* **87**: 819–826.

Fonseca P, Moreno R & Rojo F (2008) Genomic analysis of the role of RNase R in the turnover of *Pseudomonas putida* mRNAs. *J Bacteriol* **190**: 6258–6263.

Fournier MJ & Ozeki H (1985) Structure and organization of the transfer ribonucleic acid genes of *Escherichia coli* K-12. *Microbiol Rev* **49**: 379–397.

Frazão C, McVey CE, Amblar M, Barbas A, Vonrhein C, Arraiano CM & Carrondo MA (2006) Unravelling the dynamics of RNA degradation by ribonuclease II and its RNA-bound complex. *Nature* **443**: 110–114.

Freire P, Amaral JD, Santos JM & Arraiano CM (2006) Adaptation to carbon starvation: RNase III ensures normal expression levels of bolA1p mRNA and sigma(S). *Biochimie* **88**: 341–346.

Fritz DT, Bergman N, Kilpatrick WJ, Wilusz CJ & Wilusz J (2004) Messenger RNA decay in mammalian cells: the exonuclease perspective. *Cell Biochem Biophys* **41**: 265–278.

Fusi P, Tedeschi G, Aliverti A, Ronchi S, Tortora P & Guerritore A (1993) Ribonucleases from the extreme thermophilic archaebacterium *S. solfataricus*. *Eur J Biochem* **211**: 305–310.

Gan J, Tropea JE, Austin BP, Court DL, Waugh DS & Ji X (2006) Structural insight into the mechanism of double-stranded RNA processing by ribonuclease III. *Cell* **124**: 355–366.

Gao J, Lee K, Zhao M *et al.* (2006) Differential modulation of *E. coli* mRNA abundance by inhibitory proteins that alter the composition of the degradosome. *Mol Microbiol* **61**: 394–406.

García-Mena J, Das A, Sánchez-Trujillo A, Portier C & Montanez C (1999) A novel mutation in the KH domain of polynucleotide phosphorylase affects autoregulation and mRNA decay in *Escherichia coli*. *Mol Microbiol* **33**: 235–248.

Geer LY, Domrachev M, Lipman DJ & Bryant SH (2002) CDART: protein homology by domain architecture. *Genome Res* **12**: 1619–1623.

Gegenheimer P & Apirion D (1975) *Escherichia coli* ribosomal ribonucleic acids are not cut from an intact precursor molecule. *J Biol Chem* **250**: 2407–2409.

Gerdes K, Thisted T & Martinussen J (1990) Mechanism of post-segregational killing by the *hok/sok* system of plasmid R1: *sok* antisense RNA regulates formation of a *hok* mRNA species correlated with killing of plasmid-free cells. *Mol Microbiol* **4**: 1807–1818.

Ghora BK & Apirion D (1978) Structural analysis and *in vitro* processing to p5 rRNA of a 9S RNA molecule isolated from an *rne* mutant of *E. coli*. *Cell* **15**: 1055–1066.

Ghosh S & Deutscher MP (1999) Oligoribonuclease is an essential component of the mRNA decay pathway. *P Natl Acad Sci USA* **96**: 4372–4377.

Gimple O & Schon A (2001) *In vitro* and *in vivo* processing of cyanelle tmRNA by RNase P. *Biol Chem* **382**: 1421–1429.

Givskov M & Molin S (1984) Copy mutants of plasmid R1: effects of base pair substitutions in the *cop*A gene on the replication control system. *Mol Gen Genet* **194**: 286–292.

Gobert A, Gutmann B, Taschner A *et al.* (2010) A single *Arabidopsis* organellar protein has RNase P activity. *Nat Struct Mol Biol* **17**: 740–744.

Godefroy T (1970) Kinetics of polymerization and phosphorolysis reactions of *Escherichia coli* polynucleotide phosphorylase. Evidence for multiple binding of polynucleotide in phosphorolysis. *Eur J Biochem* **14**: 222–231.

Grossman D & van Hoof A (2006) RNase II structure completes group portrait of 3′ exoribonucleases. *Nat Struct Mol Biol* **13**: 760–761.

Guillier M, Gottesman S & Storz G (2006) Modulating the outer membrane with small RNAs. *Gene Dev* **20**: 2338–2348.

Haddad N, Burns CM, Bolla JM, Prévost H, Federighi M, Drider D & Cappelier JM (2009) Long-term survival of *Campylobacter jejuni* at low temperatures is dependent on polynucleotide phosphorylase activity. *Appl Environ Microb* **75**: 7310–7318.

Hahn J, Luttinger A & Dubnau D (1996) Regulatory inputs for the synthesis of ComK, the competence transcription factor of *Bacillus subtilis*. *Mol Microbiol* **21**: 763–775.

Hajnsdorf E, Steier O, Coscoy L, Teysset L & Régnier P (1994) Roles of RNase E, RNase II and PNPase in the degradation of the *rpsO* transcripts of *Escherichia coli*: stabilizing function of RNase II and evidence for efficient degradation in an *ams pnp rnb* mutant. *EMBO J* **13**: 3368–3377.

Hajnsdorf E, Braun F, Haugel-Nielsen J & Régnier P (1995) Polyadenylylation destabilizes the *rps*O mRNA of *Escherichia coli*. *P Natl Acad Sci USA* **92**: 3973–3977.

Hale CR, Zhao P, Olson S *et al.* (2009) RNA-guided RNA cleavage by a CRISPR RNA–Cas protein complex. *Cell* **139**: 945–956.

Hall TA & Brown JW (2002) Archaeal RNase P has multiple protein subunits homologous to eukaryotic nuclear RNase P proteins. *RNA* **8**: 296–306.

Hankins JS, Zappavigna C, Prud'homme-Genereux A & Mackie GA (2007) Role of RNA structure and susceptibility to RNase E in regulation of a cold shock mRNA, cspA mRNA. *J Bacteriol* **189**: 4353–4358.

Harlow LS, Kadziola A, Jensen KF & Larsen S (2004) Crystal structure of the phosphorolytic exoribonuclease RNase PH from *Bacillus subtilis* and implications for its quaternary structure and tRNA binding. *Protein Sci* **13**: 668–677.

Hartmann RK, Heinrich J, Schlegl J & Schuster H (1995) Precursor of C4 antisense RNA of bacteriophages P1 and P7 is a substrate for RNase P of *Escherichia coli*. *P Natl Acad Sci USA* **92**: 5822–5826.

Hartmann RK, Gossringer M, Spath B, Fischer S & Marchfelder A (2009) The making of tRNAs and more – RNase P and tRNase Z. *Prog Mol Biol Transl Sci* **85**: 319–368.

Hasenohrl D, Lombo T, Kaberdin V, Londei P & Blasi U (2008) Translation initiation factor a/eIF2(-gamma) counteracts 5′ to 3′ mRNA decay in the archaeon *Sulfolobus solfataricus*. *P Natl Acad Sci USA* **105**: 2146–2150.

Hayes CS & Keiler KC (2010) Beyond ribosome rescue: tmRNA and co-translational processes. *FEBS Lett* **584**: 413–419.

Hayes F & Vasseur M (1976) Processing of the 17-S *Escherichia coli* precursor RNA in the 27-S pre-ribosomal particle. *Eur J Biochem* **61**: 433–442.

Henkin TM (2008) Riboswitch RNAs: using RNA to sense cellular metabolism. *Gene Dev* **22**: 3383–3390.

Henkin TM & Yanofsky C (2002) Regulation by transcription attenuation in bacteria: how RNA provides instructions for transcription termination/antitermination decisions. *Bioessays* **24**: 700–707.

Herskovitz MA & Bechhofer DH (2000) Endoribonuclease RNase III is essential in *Bacillus subtilis*. *Mol Microbiol* **38**: 1027–1033.

Higgins CF, McLaren RS & Newbury SF (1988) Repetitive extragenic palindromic sequences, mRNA stability and gene expression: evolution by gene conversion? A review. *Gene* **72**: 3–14.

Holec S, Lange H, Kuhn K, Alioua M, Borner T & Gagliardi D (2006) Relaxed transcription in *Arabidopsis* mitochondria is counterbalanced by RNA stability control mediated by polyadenylation and polynucleotide phosphorylase. *Mol Cell Biol* **26**: 2869–2876.

Holzmann J, Frank P, Loffler E, Bennett KL, Gerner C & Rossmanith W (2008) RNase P without RNA: identification and functional reconstitution of the human mitochondrial tRNA processing enzyme. *Cell* **135**: 462–474.

Hong SJ, Tran QA & Keiler KC (2005) Cell cycle-regulated degradation of tmRNA is controlled by RNase R and SmpB. *Mol Microbiol* **57**: 565–575.

Hopper AK, Pai DA & Engelke DR (2010) Cellular dynamics of tRNAs and their genes. *FEBS Lett* **584**: 310–317.

Hou YM & Perona JJ (2010) Stereochemical mechanisms of tRNA methyltransferases. *FEBS Lett* **584**: 278–286.

Houseley J & Tollervey D (2009) The many pathways of RNA degradation. *Cell* **136**: 763–776.

Huntzinger E, Boisset S, Saveanu C *et al.* (2005) *Staphylococcus aureus* RNAIII and the endoribonuclease III coordinately regulate *spa* gene expression. *EMBO J* **24**: 824–835.

Huntzinger E, Kashima I, Fauser M, Sauliere J & Izaurralde E (2008) SMG6 is the catalytic endonuclease that cleaves mRNAs containing nonsense codons in metazoan. *RNA* **14**: 2609–2617.

Hutchison CA, Peterson SN, Gill SR *et al.* (1999) Global transposon mutagenesis and a minimal *Mycoplasma* genome. *Science* **286**: 2165–2169.

Inokuchi H & Yamao F (1995) Structure and expression of prokaryotic tRNA genes. *tRNA: Structure, Biosynthesis, and Function* (Söll D & RajBhandary UL, eds), pp. 17–30. ASM Press, Washington, DC.

Irie M (1997) RNase T1/RNase T2 family RNases. *Ribonucleases: Structure and Functions* (D'Allessio GRJ, ed), pp. 101–124. Academic Press, San Diego, CA.

Ishii R, Nureki O & Yokoyama S (2003) Crystal structure of the tRNA processing enzyme RNase PH from *Aquifex aeolicus*. *J Biol Chem* **278**: 32397–32404.

Ishii R, Minagawa A, Takaku H, Takagi M, Nashimoto M & Yokoyama S (2005) Crystal structure of the tRNA 3′ processing endoribonuclease tRNase Z from *Thermotoga maritima*. *J Biol Chem* **280**: 14138–14144.

Itaya M, Omori A, Kanaya S, Crouch RJ, Tanaka T & Kondo K (1999) Isolation of RNase H genes that are essential for growth of *Bacillus subtilis* 168. *J Bacteriol* **181**: 2118–2123.

Jagannath A & Wood M (2007) RNA interference based gene therapy for neurological disease. *Brief Funct Genomic Proteomic* **6**: 40–49.

Jäger S, Fuhrmann O, Heck C, Hebermehl M, Schiltz E, Rauhut R & Klug G (2001) An mRNA degrading complex in *Rhodobacter capsulatus*. *Nucleic Acids Res* **29**: 4581–4588.

Jäger S, Evguenieva-Hackenberg E & Klug G (2004) Temperature-dependent processing of the *cspA* mRNA in *Rhodobacter capsulatus*. *Microbiology* **150**: 687–695.

Jain C & Belasco JG (1995) Autoregulation of RNase E synthesis in *Escherichia coli*. *Nucleic Acids Symp Ser* **33**: 85–88.

Janssen BD & Hayes CS (2009) Kinetics of paused ribosome recycling in *Escherichia coli*. *J Mol Biol* **394**: 251–267.

Jarrige A, Brechemier-Baey D, Mathy N, Duche O & Portier C (2002) Mutational analysis of polynucleotide phosphorylase from *Escherichia coli*. *J Mol Biol* **321**: 397–409.

Jarrige AC, Mathy N & Portier C (2001) PNPase autocontrols its expression by degrading a double-stranded structure in the *pnp* mRNA leader. *EMBO J* **20**: 6845–6855.

Jaskiewicz L & Filipowicz W (2008) Role of Dicer in posttranscriptional RNA silencing. *Curr Top Microbiol* **320**: 77–97.

Jensen KF, Larsen JN, Schack L & Sivertsen A (1984) Studies on the structure and expression of *Escherichia coli pyr*C, *pyr*D, and *pyr*F using the cloned genes. *Eur J Biochem* **140**: 343–352.

Jensen KF, Andersen JT & Poulsen P (1992) Overexpression and rapid purification of the *orfE*/*rph* gene product, RNase PH of *Escherichia coli*. *J Biol Chem* **267**: 17147–17152.

Jiang X & Belasco JG (2004) Catalytic activation of multimeric RNase E and RNase G by 5′-monophosphorylated RNA. *P Natl Acad Sci USA* **101**: 9211–9216.

Jiang X, Diwa A & Belasco JG (2000) Regions of RNase E important for 5′-end-dependent RNA cleavage and autoregulated synthesis. *J Bacteriol* **182**: 2468–2475.

Jinek M & Doudna JA (2009) A three-dimensional view of the molecular machinery of RNA interference. *Nature* **457**: 405–412.

Johansen J, Rasmussen AA, Overgaard M & Valentin-Hansen P (2006) Conserved small non-coding RNAs that belong to the sigmaE regulon: role in down-regulation of outer membrane proteins. *J Mol Biol* **364**: 1–8.

Kaberdin VR & Blasi U (2006) Translation initiation and the fate of bacterial mRNAs. *FEMS Microbiol Rev* **30**: 967–979.

Kaberdin VR, Miczak A, Jakobsen JS, Lin-Chao S, McDowall KJ & von Gabain A (1998) The endoribonucleolytic N-terminal half of *Escherichia coli* RNase E is evolutionarily conserved in *Synechocystis* sp. and other bacteria but not the C-terminal half, which is sufficient for degradosome assembly. *P Natl Acad Sci USA* **95**: 11637–11642.

Kane JF (1995) Effects of rare codon clusters on high-level expression of heterologous proteins in *Escherichia coli*. *Curr Opin Biotech* **6**: 494–500.

Karginov FV & Hannon GJ (2010) The CRISPR system: small RNA-guided defense in bacteria and archaea. *Mol Cell* **37**: 7–19.

Karzai AW, Susskind MM & Sauer RT (1999) SmpB, a unique RNA-binding protein essential for the peptide-tagging activity of SsrA (tmRNA). *EMBO J* **18**: 3793–3799.

Kelly KO & Deutscher MP (1992) Characterization of *Escherichia coli* RNase PH. *J Biol Chem* **267**: 17153–17158.

Kelly KO, Reuven NB, Li Z & Deutscher MP (1992) RNase PH is essential for tRNA processing and viability in RNase-deficient *Escherichia coli* cells. *J Biol Chem* **267**: 16015–16018.

Khemici V & Carpousis AJ (2004) The RNA degradosome and poly(A) polymerase of *Escherichia coli* are required *in vivo* for the degradation of small mRNA decay intermediates containing REP-stabilizers. *Mol Microbiol* **51**: 777–790.

Khemici V, Toesca I, Poljak L, Vanzo NF & Carpousis AJ (2004) The RNase E of *Escherichia coli* has at least two binding sites for DEAD-box RNA helicases: functional replacement of RhlB by RhlE. *Mol Microbiol* **54**: 1422–1430.

Khemici V, Poljak L, Luisi BF & Carpousis AJ (2008) The RNase E of *Escherichia coli* is a membrane-binding protein. *Mol Microbiol* **70**: 799–813.

Kime L, Jourdan SS, Stead JA, Hidalgo-Sastre A & McDowall KJ (2009) Rapid cleavage of RNA by RNase E in the absence of 5′-monophosphate stimulation. *Mol Microbiol* **76**: 590–604.

Kirsebom LA & Trobro S (2009) RNase P RNA-mediated cleavage. *IUBMB Life* **61**: 189–200.

Kirsebom LA, Baer MF & Altman S (1988) Differential effects of mutations in the protein and RNA moieties of RNase P on the efficiency of suppression by various tRNA suppressors. *J Mol Biol* **204**: 879–888.

Kitamura S, Fujishima K, Sato A, Tsuchiya D, Tomita M & Kanai A (2010) Characterization of RNase HII substrate recognition using RNase HII-Argonaute chimeric enzymes from *Pyrococcus furiosus*. *Biochem J* **426**: 337–344.

Ko JH & Altman S (2007) OLE RNA, an RNA motif that is highly conserved in several extremophilic bacteria, is a substrate for and can be regulated by RNase P RNA. *P Natl Acad Sci USA* **104**: 7815–7820.

Ko JH, Han K, Kim Y *et al.* (2008) Dual function of RNase E for control of M1 RNA biosynthesis in *Escherichia coli*. *Biochemistry* **47**: 762–770.

Koonin EV, Wolf YI & Aravind L (2001) Prediction of the archaeal exosome and its connections with the proteasome and the translation and transcription machineries by a comparative-genomic approach. *Genome Res* **11**: 240–252.

Koslover DJ, Callaghan AJ, Marcaida MJ, Garman EF, Martick M, Scott WG & Luisi BF (2008) The crystal structure of the *Escherichia coli* RNase E apoprotein and a mechanism for RNA degradation. *Structure* **16**: 1238–1244.

Kulms D, Schafer G & Hahn U (1995) SaRD, a new protein isolated from the extremophile archaeon *Sulfolobus acidocaldarius*, is a thermostable ribonuclease with DNA-binding properties. *Biochem Bioph Res Co* **214**: 646–652.

LaCava J, Houseley J, Saveanu C, Petfalski E, Thompson E, Jacquier A & Tollervey D (2005) RNA degradation by the exosome is promoted by a nuclear polyadenylation complex. *Cell* **121**: 713–724.

Lalonde MS, Zuo Y, Zhang J, Gong X, Wu S, Malhotra A & Li Z (2007) Exoribonuclease R in *Mycoplasma genitalium* can carry out both RNA processing and degradative functions and is sensitive to RNA ribose methylation. *RNA* **13**: 1957–1968.

Lamontagne B, Larose S, Boulanger J & Elela SA (2001) The RNase III family: a conserved structure and expanding functions in eukaryotic dsRNA metabolism. *Curr Issues Mol Biol* **3**: 71–78.

Lange H, Sement FM, Canaday J & Gagliardi D (2009) Polyadenylation-assisted RNA degradation processes in plants. *Trends Plant Sci* **14**: 497–504.

Lebreton A, Tomecki R, Dziembowski A & Seraphin B (2008) Endonucleolytic RNA cleavage by a eukaryotic exosome. *Nature* **456**: 993–996.

Le Derout J, Boni IV, Régnier P & Hajnsdorf E (2010) Hfq affects mRNA levels independently of degradation. *BMC Mol Biol* **11**: 17.

Lee K & Cohen SN (2003) A *Streptomyces coelicolor* functional orthologue of *Escherichia coli* RNase E shows shuffling of catalytic and PNPase-binding domains. *Mol Microbiol* **48**: 349–360.

Lee K, Bernstein JA & Cohen SN (2002) RNase G complementation of *rne* null mutation identifies functional interrelationships with RNase E in *Escherichia coli*. *Mol Microbiol* **43**: 1445–1456.

Lee K, Zhan X, Gao J *et al.* (2003) RraA. A protein inhibitor of RNase E activity that globally modulates RNA abundance in *E. coli*. *Cell* **114**: 623–634.

Li H & Nicholson AW (1996) Defining the enzyme binding domain of a ribonuclease III processing signal. Ethylation interference and hydroxyl radical footprinting using catalytically inactive RNase III mutants. *EMBO J* **15**: 1421–1433.

Li Y & Altman S (2003) A specific endoribonuclease, RNase P, affects gene expression of polycistronic operon mRNAs. *P Natl Acad Sci USA* **100**: 13213–13218.

Li Z & Deutscher MP (1995) The tRNA processing enzyme RNase T is essential for maturation of 5S RNA. *P Natl Acad Sci USA* **92**: 6883–6886.

Li Z & Deutscher MP (1996) Maturation pathways for *E. coli* tRNA precursors: a random multienzyme process *in vivo*. *Cell* **86**: 503–512.

Li Z & Deutscher MP (2002) RNase E plays an essential role in the maturation of *Escherichia coli* tRNA precursors. *RNA* **8**: 97–109.

Li Z, Zhan L & Deutscher MP (1996) *Escherichia coli* RNase T functions *in vivo* as a dimer dependent on cysteine 168. *J Biol Chem* **271**: 1133–1137.

Li Z, Pandit S & Deutscher MP (1998) 3′ Exoribonucleolytic trimming is a common feature of the maturation of small, stable RNAs in *Escherichia coli*. *P Natl Acad Sci USA* **95**: 2856–2861.

Li Z, Pandit S & Deutscher MP (1999a) Maturation of 23S ribosomal RNA requires the exoribonuclease RNase T. *RNA* **5**: 139–146.

Li Z, Pandit S & Deutscher MP (1999b) RNase G (CafA protein) and RNase E are both required for the 5′ maturation of 16S ribosomal RNA. *EMBO J* **18**: 2878–2885.

Li Z, Reimers S, Pandit S & Deutscher MP (2002) RNA quality control: degradation of defective transfer RNA. *EMBO J* **21**: 1132–1138.

Lin-Chao S & Cohen SN (1991) The rate of processing and degradation of antisense RNAI regulates the replication of ColE1-type plasmids *in vivo*. *Cell* **65**: 1233–1242.

Lin-Chao S, Wei CL & Lin YT (1999) RNase E is required for the maturation of SsrA RNA and normal SsrA RNA peptide-tagging activity. *P Natl Acad Sci USA* **96**: 12406–12411.

Lin PH & Lin-Chao S (2005) RhlB helicase rather than enolase is the beta-subunit of the *Escherichia coli* polynucleotide phosphorylase (PNPase)-exoribonucleolytic complex. *P Natl Acad Sci USA* **102**: 16590–16595.

Liou GG, Jane WN, Cohen SN, Lin NS & Lin-Chao S (2001) RNA degradosomes exist *in vivo* in *Escherichia coli* as multicomponent complexes associated with the cytoplasmic membrane via the N-terminal region of ribonuclease E. *P Natl Acad Sci USA* **98**: 63–68.

Liou GG, Chang HY, Lin CS & Lin-Chao S (2002) DEAD box RhlB RNA helicase physically associates with exoribonuclease PNPase to degrade double-stranded RNA independent of the degradosome-assembling region of RNase E. *J Biol Chem* **277**: 41157–41162.

Littauer YZ & Soreq H (1982) *Polynucleotide Phosphorylase*. Academic Press, New York.

Liu F & Altman S (1994) Differential evolution of substrates for an RNA enzyme in the presence and absence of its protein cofactor. *Cell* **77**: 1093–1100.

Liu F & Altman S (2009) *Ribonuclease P*. Springer-Verlag, New York.

Liu H & Kiledjian M (2006a) Decapping the message: a beginning or an end. *Biochem Soc T* **34**: 35–38.

Liu Q, Greimann JC & Lima CD (2006b) Reconstitution, activities, and structure of the eukaryotic RNA exosome. *Cell* **127**: 1223–1237.

Lombo TB & Kaberdin VR (2008) RNA processing in *Aquifex aeolicus* involves RNase E/G and an RNase P-like activity. *Biochem Bioph Res Co* **366**: 457–463.

Lopez PJ, Marchand I, Joyce SA & Dreyfus M (1999) The C-terminal half of RNase E, which organizes the *Escherichia coli* degradosome, participates in mRNA degradation but not rRNA processing *in vivo*. *Mol Microbiol* **33**: 188–199.

Lorentzen E, Walter P, Fribourg S, Evguenieva-Hackenberg E, Klug G & Conti E (2005) The archaeal exosome core is a hexameric ring structure with three catalytic subunits. *Nat Struct Mol Biol* **12**: 575–581.

Lorentzen E, Dziembowski A, Lindner D, Seraphin B & Conti E (2007) RNA channelling by the archaeal exosome. *EMBO Rep* **8**: 470–476.

Lu C, Ding F & Ke A (2010) Crystal structure of the *S. solfataricus* archaeal exosome reveals conformational flexibility in the RNA-binding ring. *PLoS One* **5**: e8739.

Lundberg U & Altman S (1995) Processing of the precursor to the catalytic RNA subunit of RNase P from *Escherichia coli*. *RNA* **1**: 327–334.

Luttinger A, Hahn J & Dubnau D (1996) Polynucleotide phosphorylase is necessary for competence development in *Bacillus subtilis*. *Mol Microbiol* **19**: 343–356.

Ma Y, Chan CY & He ML (2007) RNA interference and antiviral therapy. *World J Gastroentero* **13**: 5169–5179.

Mackie GA (1991) Specific endonucleolytic cleavage of the mRNA for ribosomal protein S20 of *Escherichia coli* requires the product of the *ams* gene *in vivo* and *in vitro*. *J Bacteriol* **173**: 2488–2497.

Mackie GA (1992) Secondary structure of the mRNA for ribosomal protein S20. Implications for cleavage by ribonuclease E. *J Biol Chem* **267**: 1054–1061.

Mackie GA (1998) Ribonuclease E is a 5′-end-dependent endonuclease. *Nature* **395**: 720–723.

Mackie GA (2000) Stabilization of circular rpsT mRNA demonstrates the 5′-end dependence of RNase E action *in vivo*. *J Biol Chem* **275**: 25069–25072.

Mackie GA & Parsons GD (1983) Tandem promoters in the gene for ribosomal protein S20. *J Biol Chem* **258**: 7840–7846.

MacRae IJ & Doudna JA (2007) Ribonuclease revisited: structural insights into ribonuclease III family enzymes. *Curr Opin Struc Biol* **17**: 138–145.

Mader U, Zig L, Kretschmer J, Homuth G & Putzer H (2008) mRNA processing by RNases J1 and J2 affects *Bacillus subtilis* gene expression on a global scale. *Mol Microbiol* **70**: 183–196.

Malecki M, Jedrzejczak R, Stepien PP & Golik P (2007) *In vitro* reconstitution and characterization of the yeast mitochondrial degradosome complex unravels tight functional interdependence. *J Mol Biol* **372**: 23–36.

Marcaida MJ, DePristo MA, Chandran V, Carpousis AJ & Luisi BF (2006) The RNA degradosome: life in the fast lane of adaptive molecular evolution. *Trends Biochem Sci* **31**: 359–365.

Marck C & Grosjean H (2002) tRNomics: analysis of tRNA genes from 50 genomes of Eukarya, Archaea, and Bacteria reveals anticodon-sparing strategies and domain-specific features. *RNA* **8**: 1189–1232.

Marck C & Grosjean H (2003) Identification of BHB splicing motifs in intron-containing tRNAs from 18 archaea: evolutionary implications. *RNA* **9**: 1516–1531.

Marszalkowski M, Willkomm DK & Hartmann RK (2008) 5′-End maturation of tRNA in *Aquifex aeolicus*. *Biol Chem* **389**: 395–403.

Marujo PE, Hajnsdorf E, Le Derout J, Andrade R, Arraiano CM & Régnier P (2000) RNase II removes the oligo(A) tails that destabilize the *rps*O mRNA of *Escherichia coli*. *RNA* **6**: 1185–1193.

Marujo PE, Braun F, Haugel-Nielsen J, Le Derout J, Arraiano CM & Régnier P (2003) Inactivation of the decay pathway initiated at an internal site by RNase E promotes poly(A)-dependent degradation of the *rps*O mRNA in *Escherichia coli*. *Mol Microbiol* **50**: 1283–1294.

Massire C, Jaeger L & Westhof E (1998) Derivation of the three-dimensional architecture of bacterial ribonuclease P RNAs from comparative sequence analysis. *J Mol Biol* **279**: 773–793.

Mathy N, Jarrige AC, Robert-Le Meur M & Portier C (2001) Increased expression of *Escherichia coli* polynucleotide phosphorylase at low temperatures is linked to a decrease in the efficiency of autocontrol. *J Bacteriol* **183**: 3848–3854.

Mathy N, Benard L, Pellegrini O, Daou R, Wen T & Condon C (2007) 5′-to-3′ exoribonuclease activity in bacteria: role of RNase J1 in rRNA maturation and 5′ stability of mRNA. *Cell* **129**: 681–692.

Mathy N, Hebert A, Mervelet P *et al.* (2010) *Bacillus subtilis* ribonucleases J1 and J2 form a complex with altered enzyme behaviour. *Mol Microbiol* **75**: 489–498.

Matos RG, Barbas A & Arraiano CM (2009) RNase R mutants elucidate the catalysis of structured RNA: RNA-binding domains select the RNAs targeted for degradation. *Biochem J* **423**: 291–301.

Matsunaga J, Simons EL & Simons RW (1996) RNase III autoregulation: structure and function of *rncO*, the posttranscriptional 'operator'. *RNA* **2**: 1228–1240.

Matus-Ortega ME, Regonesi ME, Pina-Escobedo A, Tortora P, Dehò G & García-Mena J (2007) The KH and S1 domains of *Escherichia coli* polynucleotide phosphorylase are necessary for autoregulation and growth at low temperature. *Biochim Biophys Acta* **1769**: 194–203.

McDowall KJ, Hernandez RG, Lin-Chao S & Cohen SN (1993) The *ams-1* and *rne-3071* temperature-sensitive mutations in the *ams* gene are in close proximity to each other and cause substitutions within a domain that resembles a product of the *Escherichia coli mre* locus. *J Bacteriol* **175**: 4245–4249.

McDowall KJ, Kaberdin VR, Wu SW, Cohen SN & Lin-Chao S (1995) Site-specific RNase E cleavage of oligonucleotides and inhibition by stem-loops. *Nature* **374**: 287–290.

Mechold U, Ogryzko V, Ngo S & Danchin A (2006) Oligoribonuclease is a common downstream target of lithium-

induced pAp accumulation in *Escherichia coli* and human cells. *Nucleic Acids Res* **34**: 2364–2373.

Mechold U, Fang G, Ngo S, Ogryzko V & Danchin A (2007) YtqI from *Bacillus subtilis* has both oligoribonuclease and pAp-phosphatase activity. *Nucleic Acids Res* **35**: 4552–4561.

Melefors O & von Gabain A (1988) Site-specific endonucleolytic cleavages and the regulation of stability of *E. coli omp*A mRNA. *Cell* **52**: 893–901.

Melefors O & von Gabain A (1991) Genetic studies of cleavage-initiated mRNA decay and processing of ribosomal 9S RNA show that the *Escherichia coli ams* and *rne* loci are the same. *Mol Microbiol* **5**: 857–864.

Meng W & Nicholson AW (2008) Heterodimer-based analysis of subunit and domain contributions to double-stranded RNA processing by *Escherichia coli* RNase III *in vitro*. *Biochem J* **410**: 39–48.

Meyer S, Temme C & Wahle E (2004) Messenger RNA turnover in eukaryotes: pathways and enzymes. *Crit Rev Biochem Mol* **39**: 197–216.

Mian IS (1997) Comparative sequence analysis of ribonucleases HII, III, II PH and D. *Nucleic Acids Res* **25**: 3187–3195.

Miczak A, Kaberdin VR, Wei CL & Lin-Chao S (1996) Proteins associated with RNase E in a multicomponent ribonucleolytic complex. *P Natl Acad Sci USA* **93**: 3865–3869.

Minagawa A, Takaku H, Takagi M & Nashimoto M (2004) A novel endonucleolytic mechanism to generate the CCA 3′ termini of tRNA molecules in *Thermotoga maritima*. *J Biol Chem* **279**: 15688–15697.

Misra TK & Apirion D (1979) RNase E, an RNA processing enzyme from *Escherichia coli*. *J Biol Chem* **254**: 11154–11159.

Mitchell P, Petfalski E, Shevchenko A, Mann M & Tollervey D (1997) The exosome: a conserved eukaryotic RNA processing complex containing multiple 3′ → 5′ exoribonucleases. *Cell* **91**: 457–466.

Mitra S & Bechhofer DH (1994) Substrate specificity of an RNase III-like activity from *Bacillus subtilis*. *J Biol Chem* **269**: 31450–31456.

Mohanty BK & Kushner SR (1999) Analysis of the function of *Escherichia coli* poly(A) polymerase I in RNA metabolism. *Mol Microbiol* **34**: 1094–1108.

Mohanty BK & Kushner SR (2000a) Polynucleotide phosphorylase, RNase II and RNase E play different roles in the *in vivo* modulation of polyadenylation in *Escherichia coli*. *Mol Microbiol* **36**: 982–994.

Mohanty BK & Kushner SR (2000b) Polynucleotide phosphorylase functions both as a 3′ right-arrow 5′ exonuclease and a poly(A) polymerase in *Escherichia coli*. *P Natl Acad Sci USA* **97**: 11966–11971.

Mohanty BK & Kushner SR (2002) Polyadenylation of *Escherichia coli* transcripts plays an integral role in regulating intracellular levels of polynucleotide phosphorylase and RNase E. *Mol Microbiol* **45**: 1315–1324.

Mohanty BK & Kushner SR (2003) Genomic analysis in *Escherichia coli* demonstrates differential roles for polynucleotide phosphorylase and RNase II in mRNA abundance and decay. *Mol Microbiol* **50**: 645–658.

Mohanty BK & Kushner SR (2006) The majority of *Escherichia coli* mRNAs undergo post-transcriptional modification in exponentially growing cells. *Nucleic Acids Res* **34**: 5695–5704.

Mohanty BK & Kushner SR (2010) Processing of the *Escherichia coli leuX* tRNA transcript, encoding tRNALeu5, requires either the 3′ → 5′ exoribonuclease polynucleotide phosphorylase or RNase P to remove the Rho-independent transcription terminator. *Nucleic Acids Res* **38**: 597–607.

Mohanty BK, Maples VF & Kushner SR (2004) The Sm-like protein Hfq regulates polyadenylation dependent mRNA decay in *Escherichia coli*. *Mol Microbiol* **54**: 905–920.

Moore MJ & Proudfoot NJ (2009) Pre-mRNA processing reaches back to transcription and ahead to translation. *Cell* **136**: 688–700.

Morita T, Maki K & Aiba H (2005) RNase E-based ribonucleoprotein complexes: mechanical basis of mRNA destabilization mediated by bacterial noncoding RNAs. *Gene Dev* **19**: 2176–2186.

Mudd EA & Higgins CF (1993) *Escherichia coli* endoribonuclease RNase E: autoregulation of expression and site-specific cleavage of mRNA. *Mol Microbiol* **9**: 557–568.

Mudd EA, Krisch HM & Higgins CF (1990) RNase E, an endoribonuclease, has a general role in the chemical decay of *Escherichia coli* mRNA: evidence that *rne* and *ams* are the same genetic locus. *Mol Microbiol* **4**: 2127–2135.

Nakamura A, Koide Y, Miyazaki H, Kitamura A, Masaki H, Beppu T & Uozumi T (1992) Gene cloning and characterization of a novel extracellular ribonuclease of *Bacillus subtilis*. *Eur J Biochem* **209**: 121–127.

Nashimoto M (1997) Distribution of both lengths and 5′ terminal nucleotides of mammalian pre-tRNA 3′ trailers reflects properties of 3′ processing endoribonuclease. *Nucleic Acids Res* **25**: 1148–1154.

Neubauer C, Gao Y-G, Andersen KR *et al.* (2009) The structural basis for mRNA recognition and cleavage by the ribosome-dependent endonuclease RelE. *Cell* **139**: 1084–1095.

Newbury SF, Smith NH & Higgins CF (1987) Differential mRNA stability controls relative gene expression within a polycistronic operon. *Cell* **51**: 1131–1143.

Nicholson AW (1999) Function, mechanism and regulation of bacterial ribonucleases. *FEMS Microbiol Rev* **23**: 371–390.

Nilsson G, Belasco JG, Cohen SN & von Gabain A (1984) Growth-rate dependent regulation of mRNA stability in *Escherichia coli*. *Nature* **312**: 75–77.

Nishio SY & Itoh T (2008) The effects of RNA degradation enzymes on antisense RNAI controlling ColE2 plasmid copy number. *Plasmid* **60**: 174–180.

Niyogi SK & Datta AK (1975) A novel oligoribonuclease of *Escherichia coli*. Isolation and properties. *J Biol Chem* **250**: 7307–7312.

Noirot-Gros MF, Dervyn E, Wu LJ, Mervelet P, Errington J, Ehrlich SD & Noirot P (2002) An expanded view of bacterial DNA replication. *P Natl Acad Sci USA* **99**: 8342–8347.

Nowotny M & Yang W (2006) Stepwise analyses of metal ions in RNase H catalysis from substrate destabilization to product release. *EMBO J* **25**: 1924–1933.

Nurmohamed S, Vaidialingam B, Callaghan AJ & Luisi BF (2009) Crystal structure of *Escherichia coli* polynucleotide phosphorylase core bound to RNase E, RNA and manganese: implications for catalytic mechanism and RNA degradosome assembly. *J Mol Biol* **389**: 17–33.

O'Hara EB, Chekanova JA, Ingle CA, Kushner ZR, Peters E & Kushner SR (1995) Polyadenylylation helps regulate mRNA decay in *Escherichia coli*. *P Natl Acad Sci USA* **92**: 1807–1811.

Oguro A, Kakeshita H, Nakamura K, Yamane K, Wang W & Bechhofer DH (1998) *Bacillus subtilis* RNase III cleaves both 5′- and 3′-sites of the small cytoplasmic RNA precursor. *J Biol Chem* **273**: 19542–19547.

Ohnishi Y, Nishiyama Y, Sato R, Kameyama S & Horinouchi S (2000) An oligoribonuclease gene in *Streptomyces griseus*. *J Bacteriol* **182**: 4647–4653.

Ohtani N, Haruki M, Morikawa M, Crouch RJ, Itaya M & Kanaya S (1999) Identification of the genes encoding Mn^{2+}-dependent RNase HII and Mg^{2+}-dependent RNase HIII from *Bacillus subtilis*: classification of RNases H into three families. *Biochemistry* **38**: 605–618.

Okada Y, Wachi M, Hirata A, Suzuki K, Nagai K & Matsuhashi M (1994) Cytoplasmic axial filaments in *Escherichia coli* cells: possible function in the mechanism of chromosome segregation and cell division. *J Bacteriol* **176**: 917–922.

Olmedo G & Guzman P (2008) Mini-III, a fourth class of RNase III catalyses maturation of the *Bacillus subtilis* 23S ribosomal RNA. *Mol Microbiol* **68**: 1073–1076.

Ono M & Kuwano M (1979) A conditional lethal mutation in an *Escherichia coli* strain with a longer chemical lifetime of messenger RNA. *J Mol Biol* **129**: 343–357.

Ono M & Kuwano M (1980) Chromosomal location of a gene for chemical longevity of messenger ribonucleic acid in a temperature-sensitive mutant of *Escherichia coli*. *J Bacteriol* **142**: 325–326.

Oppenheim AB, Kornitzer D, Altuvia S & Court DL (1993) Posttranscriptional control of the lysogenic pathway in bacteriophage lambda. *Prog Nucleic Acid Re* **46**: 37–49.

Ost KA & Deutscher MP (1991) *Escherichia coli orfE* (upstream of *pyrE*) encodes RNase PH. *J Bacteriol* **173**: 5589–5591.

Otsuka Y & Yonesaki T (2005) A novel endoribonuclease, RNase LS, in *Escherichia coli*. *Genetics* **169**: 13–20.

Oussenko IA, Sanchez R & Bechhofer DH (2002) *Bacillus subtilis* YhaM, a member of a new family of 3′-to-5′ exonucleases in gram-positive bacteria. *J Bacteriol* **184**: 6250–6259.

Oussenko IA, Abe T, Ujiie H, Muto A & Bechhofer DH (2005) Participation of 3′-to-5′ exoribonucleases in the turnover of *Bacillus subtilis* mRNA. *J Bacteriol* **187**: 2758–2767.

Ow MC & Kushner SR (2002) Initiation of tRNA maturation by RNase E is essential for cell viability in *E. coli*. *Gene Dev* **16**: 1102–1115.

Ow MC, Liu Q & Kushner SR (2000) Analysis of mRNA decay and rRNA processing in *Escherichia coli* in the absence of RNase E-based degradosome assembly. *Mol Microbiol* **38**: 854–866.

Ow MC, Liu Q, Mohanty BK, Andrew ME, Maples VF & Kushner SR (2002) RNase E levels in *Escherichia coli* are controlled by a complex regulatory system that involves transcription of the *rne* gene from three promoters. *Mol Microbiol* **43**: 159–171.

Ow MC, Perwez T & Kushner SR (2003) RNase G of *Escherichia coli* exhibits only limited functional overlap with its essential homologue, RNase E. *Mol Microbiol* **49**: 607–622.

Palanisamy SK, Fletcher C, Tanjung L, Katz ME & Cheetham BF (2009) Deletion of the C-terminus of polynucleotide phosphorylase increases twitching motility, a virulence characteristic of the anaerobic bacterial pathogen *Dichelobacter nodosus*. *FEMS Microbiol Lett* **302**: 39–45.

Peck-Miller KA & Altman S (1991) Kinetics of the processing of the precursor to 4.5 S RNA, a naturally occurring substrate for RNase P from *Escherichia coli*. *J Mol Biol* **221**: 1–5.

Pedersen K, Zavialov AV, Pavlov MY, Elf J, Gerdes K & Ehrenberg M (2003) The bacterial toxin RelE displays codon-specific cleavage of mRNAs in the ribosomal A site. *Cell* **112**: 131–140.

Pellegrini O, Nezzar J, Marchfelder A, Putzer H & Condon C (2003) Endonucleolytic processing of CCA-less tRNA precursors by RNase Z in *Bacillus subtilis*. *EMBO J* **22**: 4534–4543.

Pepe CM, Maslesa-Galic S & Simons RW (1994) Decay of the IS10 antisense RNA by 3′ exoribonucleases: evidence that RNase II stabilizes RNA-OUT against PNPase attack. *Mol Microbiol* **13**: 1133–1142.

Pertzev AV & Nicholson AW (2006b) Characterization of RNA sequence determinants and antideterminants of processing reactivity for a minimal substrate of *Escherichia coli* ribonuclease III. *Nucleic Acids Res* **34**: 3708–3721.

Perwez T & Kushner SR (2006a) RNase Z in *Escherichia coli* plays a significant role in mRNA decay. *Mol Microbiol* **60**: 723–737.

Phizicky EM & Alfonzo JD (2010) Do all modifications benefit all tRNAs? *FEBS Lett* **584**: 265–271.

Piazza F, Zappone M, Sana M, Briani F & Dehò G (1996) Polynucleotide phosphorylase of *Escherichia coli* is required for the establishment of bacteriophage P4 immunity. *J Bacteriol* **178**: 5513–5521.

Portier C & Regnier P (1984) Expression of the *rpsO* and *pnp* genes: structural analysis of a DNA fragment carrying their control regions. *Nucleic Acids Res* **12**: 6091–6102.

Portier C, Dondon L, Grunberg-Manago M & Régnier P (1987) The first step in the functional inactivation of the *Escherichia coli* polynucleotide phosphorylase messenger is a ribonuclease III processing at the 5′ end. *EMBO J* **6**: 2165–2170.

Portnoy V & Schuster G (2006) RNA polyadenylation and degradation in different Archaea; roles of the exosome and RNase R. *Nucleic Acids Res* **34**: 5923–5931.

Portnoy V, Evguenieva-Hackenberg E, Klein F, Walter P, Lorentzen E, Klug G & Schuster G (2005) RNA polyadenylation in Archaea: not observed in *Haloferax* while the exosome polynucleotidylates RNA in *Sulfolobus*. *EMBO Rep* **6**: 1188–1193.

Prud'homme-Genereux A, Beran RK, Iost I, Ramey CS, Mackie GA & Simons RW (2004) Physical and functional interactions among RNase E, polynucleotide phosphorylase and the cold-shock protein, CsdA: evidence for a 'cold shock degradosome'. *Mol Microbiol* **54**: 1409–1421.

Pruijn GJ (2005) Doughnuts dealing with RNA. *Nat Struct Mol Biol* **12**: 562–564.

Purusharth RI, Klein F, Sulthana S *et al.* (2005) Exoribonuclease R interacts with endoribonuclease E and an RNA helicase in the psychrotrophic bacterium *Pseudomonas syringae* Lz4W. *J Biol Chem* **280**: 14572–14578.

Purusharth RI, Madhuri B & Ray MK (2007) Exoribonuclease R in *Pseudomonas syringae* is essential for growth at low temperature and plays a novel role in the 3′ end processing of 16 and 5 S ribosomal RNA. *J Biol Chem* **282**: 16267–16277.

Py B, Causton H, Mudd EA & Higgins CF (1994) A protein complex mediating mRNA degradation in *Escherichia coli*. *Mol Microbiol* **14**: 717–729.

Py B, Higgins CF, Krisch HM & Carpousis AJ (1996) A DEAD-box RNA helicase in the *Escherichia coli* RNA degradosome. *Nature* **381**: 169–172.

Randau L, Schroder I & Soll D (2008) Life without RNase P. *Nature* **453**: 120–123.

Rasmussen AA, Eriksen M, Gilany K, Udesen C, Franch T, Petersen C & Valentin-Hansen P (2005) Regulation of *omp*A mRNA stability: the role of a small regulatory RNA in growth phase-dependent control. *Mol Microbiol* **58**: 1421–1429.

Redko Y, Bechhofer DH & Condon C (2008) Mini-III, an unusual member of the RNase III family of enzymes, catalyses 23S ribosomal RNA maturation in *B. subtilis*. *Mol Microbiol* **68**: 1096–1106.

Régnier P & Grunberg-Manago M (1990) RNase III cleavages in non-coding leaders of *Escherichia coli* transcripts control mRNA stability and genetic expression. *Biochimie* **72**: 825–834.

Régnier P & Portier C (1986) Initiation, attenuation and RNase III processing of transcripts from the *Escherichia coli* operon encoding ribosomal protein S15 and polynucleotide phosphorylase. *J Mol Biol* **187**: 23–32.

Regonesi ME, Briani F, Ghetta A, Zangrossi S, Ghisotti D, Tortora P & Deho G (2004) A mutation in polynucleotide phosphorylase from *Escherichia coli* impairing RNA binding and degradosome stability. *Nucleic Acids Res* **32**: 1006–1017.

Regonesi ME, Del Favero M, Basilico F *et al.* (2006) Analysis of the *Escherichia coli* RNA degradosome composition by a proteomic approach. *Biochimie* **88**: 151–161.

Reva ON, Weinel C, Weinel M, Bohm K, Stjepandic D, Hoheisel JD & Tummler B (2006) Functional genomics of stress response in *Pseudomonas putida* KT2440. *J Bacteriol* **188**: 4079–4092.

Robert-Le Meur M & Portier C (1992) *E.coli* polynucleotide phosphorylase expression is autoregulated through an RNase III-dependent mechanism. *EMBO J* **11**: 2633–2641.

Robert-Le Meur M & Portier C (1994) Polynucleotide phosphorylase of *Escherichia coli* induces the degradation of its RNase III processed messenger by preventing its translation. *Nucleic Acids Res* **22**: 397–403.

Robertson HD, Webster RE & Zinder ND (1968) Purification and properties of ribonuclease III from *Escherichia coli*. *J Biol Chem* **243**: 82–91.

Robertson HD, Altman S & Smith JD (1972) Purification and properties of a specific *Escherichia coli* ribonuclease which cleaves a tyrosine transfer ribonucleic acid precursor. *J Biol Chem* **247**: 5243–5251.

Rosenzweig JA, Weltman G, Plano GV & Schesser K (2005) Modulation of *Yersinia* type three secretion system by the S1 domain of polynucleotide phosphorylase. *J Biol Chem* **280**: 156–163.

Rosenzweig JA, Chromy B, Echeverry A *et al.* (2007) Polynucleotide phosphorylase independently controls virulence factor expression levels and export in *Yersinia* spp. *FEMS Microbiol Lett* **270**: 255–264.

Rott R, Zipor G, Portnoy V, Liveanu V & Schuster G (2003) RNA polyadenylation and degradation in cyanobacteria are similar to the chloroplast but different from *Escherichia coli*. *J Biol Chem* **278**: 15771–15777.

Rougemaille M, Villa T, Gudipati RK & Libri D (2008) mRNA journey to the cytoplasm: attire required. *Biol Cell* **100**: 327–342.

Sakai T, Nakamura N, Umitsuki G, Nagai K & Wachi M (2007) Increased production of pyruvic acid by *Escherichia coli* RNase G mutants in combination with *cra* mutations. *Appl Microbiol Biot* **76**: 183–192.

Sanson B & Uzan M (1995) Post-transcriptional controls in bacteriophage T4: roles of the sequence-specific endoribonuclease RegB. *FEMS Microbiol Rev* **17**: 141–150.

Santos JM, Drider D, Marujo PE, Lopez P & Arraiano CM (1997) Determinant role of *E. coli* RNase III in the decay of both specific and heterologous mRNAs. *FEMS Microbiol Lett* **157**: 31–38.

Schaeffer D, Tsanova B, Barbas A *et al.* (2009) The exosome contains domains with specific endoribonuclease, exoribonuclease and cytoplasmic mRNA decay activities. *Nat Struct Mol Biol* **16**: 56–62.

Schiffer S, Rosch S & Marchfelder A (2002) Assigning a function to a conserved group of proteins: the tRNA 3′-processing enzymes. *EMBO J* **21**: 2769–2777.

Schilling O, Ruggeberg S, Vogel A *et al.* (2004) Characterization of an *Escherichia coli elaC* deletion mutant. *Biochem Bioph Res Co* **320**: 1365–1373.

Seif E & Altman S (2008) RNase P cleaves the adenine riboswitch and stabilizes *pbuE* mRNA in *Bacillus subtilis*. *RNA* **14**: 1237–1243.

Sello JK & Buttner MJ (2008) The oligoribonuclease gene in *Streptomyces coelicolor* is not transcriptionally or translationally coupled to *adp*A, a key *bld*A target. *FEMS Microbiol Lett* **286**: 60–65.

Shahbabian K, Jamalli A, Zig L & Putzer H (2009) RNase Y, a novel endoribonuclease, initiates riboswitch turnover in *Bacillus subtilis*. *EMBO J* **28**: 3523–3533.

Shehi E, Serina S, Fumagalli G *et al.* (2001) The Sso7d DNA-binding protein from *Sulfolobus solfataricus* has ribonuclease activity. *FEBS Lett* **497**: 131–136.

Shi Z, Yang WZ, Lin-Chao S, Chak KF & Yuan HS (2008) Crystal structure of *Escherichia coli* PNPase: central channel residues are involved in processive RNA degradation. *RNA* **14**: 2361–2371.

Shibata HS, Minagawa A, Takaku H, Takagi M & Nashimoto M (2006) Unstructured RNA is a substrate for tRNase Z. *Biochemistry* **45**: 5486–5492.

Shiraishi H & Shimura Y (1986) Mutations affecting two distinct functions of the RNA component of RNase P. *EMBO J* **5**: 3673–3679.

Shyu AB, Wilkinson MF & van Hoof A (2008) Messenger RNA regulation: to translate or to degrade. *EMBO J* **27**: 471–481.

Sim SH, Yeom JH, Shin C *et al.* (2010) *Escherichia coli* ribonuclease III activity is downregulated by osmotic stress: consequences for the degradation of *bdm* mRNA in biofilm formation. *Mol Microbiol* **75**: 413–425.

Slomovic S, Laufer D, Geiger D & Schuster G (2005) Polyadenylation and degradation of human mitochondrial RNA: the prokaryotic past leaves its mark. *Mol Cell Biol* **25**: 6427–6435.

Slomovic S, Portnoy V, Yehudai-Resheff S, Bronshtein E & Schuster G (2008) Polynucleotide phosphorylase and the archaeal exosome as poly(A)-polymerases. *Biochim Biophys Acta* **1779**: 247–255.

Smith D, Burgin AB, Haas ES & Pace NR (1992) Influence of metal ions on the ribonuclease P reaction. Distinguishing substrate binding from catalysis. *J Biol Chem* **267**: 2429–2436.

Smith JK, Hsieh J & Fierke CA (2007) Importance of RNA–protein interactions in bacterial ribonuclease P structure and catalysis. *Biopolymers* **87**: 329–338.

Söderbom F & Wagner EG (1998) Degradation pathway of *Cop*A, the antisense RNA that controls replication of plasmid R1. *Microbiology* **144**: 1907–1917.

Söderbom F, Binnie U, Masters M & Wagner EG (1997) Regulation of plasmid R1 replication: PcnB and RNase E expedite the decay of the antisense RNA, *Cop*A. *Mol Microbiol* **26**: 493–504.

Sogin ML & Pace NR (1974) *In vitro* maturation of precursors of 5S ribosomal RNA from *Bacillus subtilis*. *Nature* **252**: 598–600.

Sogin ML, Pace B & Pace NR (1977) Partial purification and properties of a ribosomal RNA maturation endonuclease from *Bacillus subtilis*. *J Biol Chem* **252**: 1350–1357.

Sohlberg B, Huang J & Cohen SN (2003) The *Streptomyces coelicolor* polynucleotide phosphorylase homologue, and not the putative poly(A) polymerase, can polyadenylate RNA. *J Bacteriol* **185**: 7273–7278.

Song JJ, Smith SK, Hannon GJ & Joshua-Tor L (2004) Crystal structure of Argonaute and its implications for RISC slicer activity. *Science* **305**: 1434–1437.

Sousa S, Marchand I & Dreyfus M (2001) Autoregulation allows *Escherichia coli* RNase E to adjust continuously its synthesis to that of its substrates. *Mol Microbiol* **42**: 867–878.

Spickler C & Mackie GA (2000) Action of RNase II and polynucleotide phosphorylase against RNAs containing stem-loops of defined structure. *J Bacteriol* **182**: 2422–2427.

Stevens A & Niyogi SK (1967) Hydrolysis of oligoribonucleotides by an enzyme fraction from *Escherichia coli*. *Biochem Bioph Res Co* **29**: 550–555.

Stickney LM, Hankins JS, Miao X & Mackie GA (2005) Function of the conserved S1 and KH domains in polynucleotide phosphorylase. *J Bacteriol* **187**: 7214–7221.

Storz G, Opdyke JA & Zhang A (2004) Controlling mRNA stability and translation with small, noncoding RNAs. *Curr Opin Microbiol* **7**: 140–144.

Stougaard P, Molin S & Nordström K (1981) RNAs involved in copy-number control and incompatibility of plasmid R1. *P Natl Acad Sci USA* **78**: 6008–6012.

Subbarayan PR & Deutscher MP (2001) *Escherichia coli* RNase M is a multiply altered form of RNase I. *RNA* **7**: 1702–1707.

Sulewski M, Marchese-Ragona SP, Johnson KA & Benkovic SJ (1989) Mechanism of polynucleotide phosphorylase. *Biochemistry* **28**: 5855–5864.

Symmons MF, Jones GH & Luisi BF (2000) A duplicated fold is the structural basis for polynucleotide phosphorylase catalytic activity, processivity, and regulation. *Structure* **8**: 1215–1226.

Szczesny RJ, Borowski LS, Brzezniak LK, Dmochowska A, Gewartowski K, Bartnik E & Stepien PP (2010) Human mitochondrial RNA turnover caught in flagranti: involvement of hSuv3p helicase in RNA surveillance. *Nucleic Acids Res* **38**: 279–298.

Tadokoro T & Kanaya S (2009) Ribonuclease H: molecular diversities, substrate binding domains, and catalytic mechanism of the prokaryotic enzymes. *FEBS J* **276**: 1482–1493.

Taghbalout A & Rothfield L (2007) RNaseE and the other constituents of the RNA degradosome are components of the bacterial cytoskeleton. *P Natl Acad Sci USA* **104**: 1667–1672.

Taghbalout A & Rothfield L (2008) RNaseE and RNA helicase B play central roles in the cytoskeletal organization of the RNA degradosome. *J Biol Chem* **283**: 13850–13855.

Takaku H & Nashimoto M (2008) *Escherichia coli* tRNase Z can shut down growth probably by removing amino acids from aminoacyl-tRNAs. *Genes Cells* **13**: 1087–1097.

Takechi S, Yasueda H & Itoh T (1994) Control of ColE2 plasmid replication: regulation of Rep expression by a plasmid-coded antisense RNA. *Mol Gen Genet* **244**: 49–56.

Taraseviciene L, Miczak A & Apirion D (1991) The gene specifying RNase E (*rne*) and a gene affecting mRNA stability (*ams*) are the same gene. *Mol Microbiol* **5**: 851–855.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F & Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**: 4876–4882.

Tobe T, Sasakawa C, Okada N, Honma Y & Yoshikawa M (1992) *vacB*, a novel chromosomal gene required for expression of virulence genes on the large plasmid of *Shigella flexneri*. *J Bacteriol* **174**: 6359–6367.

Tock MR, Walsh AP, Carroll G & McDowall KJ (2000) The CafA protein required for the 5′-maturation of 16 S rRNA is a 5′-end-dependent ribonuclease that has context-dependent broad sequence specificity. *J Biol Chem* **275**: 8726–8732.

Tomecki R, Dmochowska A, Gewartowski K, Dziembowski A & Stepien PP (2004) Identification of a novel human nuclear-encoded mitochondrial poly(A) polymerase. *Nucleic Acids Res* **32**: 6001–6014.

Turnbough CL Jr, Kerr KH, Funderburg WR, Donahue JP & Powell FE (1987) Nucleotide sequence and characterization of the *pyr*F operon of *Escherichia coli* K12. *J Biol Chem* **262**: 10239–10245.

Udekwu KI, Darfeuille F, Vögel J, Reimegard J, Holmqvist E & Wagner EG (2005) Hfq-dependent regulation of OmpA synthesis is mediated by an antisense RNA. *Gene Dev* **19**: 2355–2366.

Umitsuki G, Wachi M, Takada A, Hikichi T & Nagai K (2001) Involvement of RNase G in *in vivo* mRNA metabolism in *Escherichia coli*. *Genes Cells* **6**: 403–410.

Uzan M (2001) Bacteriophage T4 RegB endoribonuclease. *Method Enzymol* **342**: 467–480.

Uzan M (2009) RNA processing and decay in bacteriophage T4. *Prog Mol Biol Transl Sci* **85**: 43–89.

van Hoof A & Parker R (1999) The exosome: a proteasome for RNA? *Cell* **99**: 347–350.

Vanzo NF, Li YS, Py B *et al.* (1998) Ribonuclease E organizes the protein interactions in the *Escherichia coli* RNA degradosome. *Gene Dev* **12**: 2770–2781.

Viegas SC & Arraiano CM (2008) Regulating the regulators: How ribonucleases dictate the rules in the control of small non-coding RNAs. *RNA Biol* **5**: 230–243.

Viegas SC, Schmidt D, Kasche V, Arraiano CM & Ignatova Z (2005) Effect of the increased stability of the penicillin amidase mRNA on the protein expression levels. *FEBS Lett* **579**: 5069–5073.

Viegas SC, Pfeiffer V, Sittka A, Silva IJ, Vogel J & Arraiano CM (2007) Characterization of the role of ribonucleases in *Salmonella* small RNA decay. *Nucleic Acids Res* **35**: 7651–7664.

Vincent HA & Deutscher MP (2006) Substrate recognition and catalysis by the exoribonuclease RNase R. *J Biol Chem* **281**: 29769–29775.

Vincent HA & Deutscher MP (2009) The roles of individual domains of RNase R in substrate binding and exoribonuclease activity. The nuclease domain is sufficient for digestion of structured RNA. *J Biol Chem* **284**: 486–494.

Viswanathan M, Dower KW & Lovett ST (1998) Identification of a potent DNase activity associated with RNase T of *Escherichia coli*. *J Biol Chem* **273**: 35126–35131.

Vogel A, Schilling O, Niecke M, Bettmer J & Meyer-Klaucke W (2002) ElaC encodes a novel binuclear zinc phosphodiesterase. *J Biol Chem* **277**: 29078–29085.

Vogel J & Hess WR (2001) Complete 5′ and 3′ end maturation of group II intron-containing tRNA precursors. *RNA* **7**: 285–292.

Vogel J, Argaman L, Wagner EG & Altuvia S (2004) The small RNA IstR inhibits synthesis of an SOS-induced toxic peptide. *Curr Biol* **14**: 2271–2276.

von Gabain A, Belasco JG, Schottel JL, Chang AC & Cohen SN (1983) Decay of mRNA in *Escherichia coli*: investigation of the fate of specific segments of transcripts. *P Natl Acad Sci USA* **80**: 653–657.

Wachi M, Umitsuki G, Shimizu M, Takada A & Nagai K (1999) *Escherichia coli cafA* gene encodes a novel RNase, designated as RNase G, involved in processing of the 5′ end of 16S rRNA. *Biochem Bioph Res Co* **259**: 483–488.

Wagner EG, Altuvia S & Romby P (2002) Antisense RNAs in bacteria and their genetic elements. *Adv Genet* **46**: 361–398.

Wagner S & Klug G (2007) An archaeal protein with homology to the eukaryotic translation initiation factor 5A shows ribonucleolytic activity. *J Biol Chem* **282**: 13966–13976.

Wang W & Bechhofer DH (1996) Properties of a *Bacillus subtilis* polynucleotide phosphorylase deletion strain. *J Bacteriol* **178**: 2375–2382.

Wang W & Bechhofer DH (1997) *Bacillus subtilis* RNase III gene: cloning, function of the gene in *Escherichia coli*, and construction of *Bacillus subtilis* strains with altered *rnc* loci. *J Bacteriol* **179**: 7379–7385.

Wen T, Oussenko IA, Pellegrini O, Bechhofer DH & Condon C (2005) Ribonuclease PH plays a major role in the exonucleolytic maturation of CCA-containing tRNA precursors in *Bacillus subtilis*. *Nucleic Acids Res* **33**: 3636–3643.

Willkomm DK, Feltens R & Hartmann RK (2002) tRNA maturation in *Aquifex aeolicus*. *Biochimie* **84**: 713–722.

Winkler WC & Breaker RR (2005) Regulation of bacterial gene expression by riboswitches. *Annu Rev Microbiol* **59**: 487–517.

Worrall JA & Luisi BF (2007) Information available at cut rates: structure and mechanism of ribonucleases. *Curr Opin Struc Biol* **17**: 128–137.

Worrall JA, Gorna M, Crump NT *et al.* (2008a) Reconstitution and analysis of the multienzyme *Escherichia coli* RNA degradosome. *J Mol Biol* **382**: 870–883.

Worrall JA, Howe FS, McKay AR, Robinson CV & Luisi BF (2008b) Allosteric activation of the ATPase activity of the *Escherichia coli* RhlB RNA helicase. *J Biol Chem* **283**: 5567–5576.

Wu J, Jiang Z, Liu M, Gong X, Wu S, Burns CM & Li Z (2009) Polynucleotide phosphorylase protects *Escherichia coli* against oxidative stress. *Biochemistry* **48**: 2012–2020.

Xu A, Jao DL & Chen KY (2004) Identification of mRNA that binds to eukaryotic initiation factor 5A by affinity co-purification and differential display. *Biochem J* **384**: 585–590.

Xu F & Cohen SN (1995) RNA degradation in *Escherichia coli* regulated by 3′ adenylation and 5′ phosphorylation. *Nature* **374**: 180–183.

Xu F, Lin-Chao S & Cohen SN (1993) The *Escherichia coli pcn*B gene promotes adenylylation of antisense RNAI of ColE1-type plasmids *in vivo* and degradation of RNAI decay intermediates. *P Natl Acad Sci USA* **90**: 6756–6760.

Yamaguchi Y & Inouye M (2009) mRNA interferases, sequence-specific endoribonucleases from the toxin-antitoxin systems. *Prog Mol Biol Transl Sci* **85**: 467–500.

Yang L & Altman S (2007) A noncoding RNA in *Saccharomyces cerevisiae* is an RNase P substrate. *RNA* **13**: 682–690.

Yang W, Hendrickson WA, Crouch RJ & Satow Y (1990) Structure of ribonuclease H phased at 2 Å resolution by MAD analysis of the selenomethionyl protein. *Science* **249**: 1398–1405.

Yao S, Blaustein JB & Bechhofer DH (2007) Processing of *Bacillus subtilis* small cytoplasmic RNA: evidence for an additional endonuclease cleavage site. *Nucleic Acids Res* **35**: 4464–4473.

Yehudai-Resheff S, Hirsh M & Schuster G (2001) Polynucleotide phosphorylase functions as both an exonuclease and a poly(A) polymerase in spinach chloroplasts. *Mol Cell Biol* **21**: 5408–5416.

Zangrossi S, Briani F, Ghisotti D, Regonesi ME, Tortora P & Dehò G (2000) Transcriptional and post-transcriptional control of polynucleotide phosphorylase during cold acclimation in *Escherichia coli*. *Mol Microbiol* **36**: 1470–1480.

Zhang JR & Deutscher MP (1988a) Cloning, characterization, and effects of overexpression of the *Escherichia coli rnd* gene encoding RNase D. *J Bacteriol* **170**: 522–527.

Zhang JR & Deutscher MP (1988b) Transfer RNA is a substrate for RNase D *in vivo*. *J Biol Chem* **263**: 17909–17912.

Zhang JR & Deutscher MP (1989) Analysis of the upstream region of the *Escherichia coli rnd* gene encoding RNase D. Evidence for translational regulation of a putative tRNA processing enzyme. *J Biol Chem* **264**: 18228–18233.

Zhang K & Nicholson AW (1997) Regulation of ribonuclease III processing by double-helical sequence antideterminants. *P Natl Acad Sci USA* **94**: 13437–13441.

Zhang X, Zhu L & Deutscher MP (1998) Oligoribonuclease is encoded by a highly conserved gene in the 3′-5′ exonuclease superfamily. *J Bacteriol* **180**: 2779–2781.

Zhu LQ, Gangopadhyay T, Padmanabha KP & Deutscher MP (1990) *Escherichia coli rna* gene encoding RNase I: cloning, overexpression, subcellular distribution of the enzyme, and use of an *rna* deletion to identify additional RNases. *J Bacteriol* **172**: 3146–3151.

Zilhão R, Camelo L & Arraiano CM (1993) DNA sequencing and expression of the gene *rnb* encoding *Escherichia coli* ribonuclease II. *Mol Microbiol* **8**: 43–51.

Zilhão R, Caillet J, Regnier P & Arraiano CM (1995a) Precise physical mapping of the *Escherichia coli rnb* gene, encoding ribonuclease II. *Mol Gen Genet* **248**: 242–246.

Zilhão R, Régnier P & Arraiano CM (1995b) The role of endonucleases in the expression of ribonuclease II in *Escherichia coli*. *FEMS Microbiol Lett* **130**: 237–244.

Zilhão R, Cairrão F, Régnier P & Arraiano CM (1996a) PNPase modulates RNase II expression in *Escherichia coli*: implications for mRNA decay and cell metabolism. *Mol Microbiol* **20**: 1033–1042.

Zilhão R, Plumbridge J, Hajnsdorf E, Regnier P & Arraiano CM (1996b) *Escherichia coli* RNase II: characterization of the promoters involved in the transcription of *rnb*. *Microbiology* **142**: 367–375.

Zuo Y & Deutscher MP (2001) Exoribonuclease superfamilies: structural analysis and phylogenetic distribution. *Nucleic Acids Res* **29**: 1017–1026.

Zuo Y & Deutscher MP (2002) The physiological role of RNase T can be explained by its unusual substrate specificity. *J Biol Chem* **277**: 29654–29661.

Zuo Y, Wang Y & Malhotra A (2005) Crystal structure of *Escherichia coli* RNase D, an exoribonuclease involved in structured RNA processing. *Structure* **13**: 973–984.

Zuo Y, Vincent HA, Zhang J, Wang Y, Deutscher MP & Malhotra A (2006) Structural basis for processivity and single-strand specificity of RNase II. *Mol Cell* **24**: 149–156.

Zuo Y, Zheng H, Wang Y *et al*. (2007) Crystal structure of RNase T, an exoribonuclease involved in tRNA maturation and end turnover. *Structure* **15**: 417–428.

The crucial role of PNPase in the degradation of small RNAs that are not associated with Hfq

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The crucial role of PNPase in the degradation of small RNAs that are not associated with Hfq

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ABSTRACT

The transient existence of small RNAs free of binding to the RNA chaperone Hfq is part of the normal dynamic lifecycle of a sRNA. Small RNAs are extremely labile when not associated with Hfq, but the mechanism by which Hfq stabilizes sRNAs has been elusive. In this work we have found that polynucleotide phosphorylase (PNPase) is the major factor involved in the rapid degradation of small RNAs, especially those that are free of binding to Hfq. The levels of MicA, GlmY, RyhB, and SgrS RNAs are drastically increased upon PNPase inactivation in Hfq$^-$ cells. In the absence of Hfq, all sRNAs are slightly shorter than their full-length species as result of 3′-end trimming. We show that the turnover of Hfq-free small RNAs is growth-phase regulated, and that PNPase activity is particularly important in stationary phase. Indeed, PNPase makes a greater contribution than RNase E, which is commonly believed to be the main enzyme in the decay of small RNAs. Lack of poly(A) polymerase I (PAP I) is also found to affect the rapid degradation of Hfq-free small RNAs, although to a lesser extent. Our data also suggest that when the sRNA is not associated with Hfq, the degradation occurs mainly in a target-independent pathway in which RNase III has a reduced impact. This work demonstrated that small RNAs free of Hfq binding are preferably degraded by PNPase. Overall, our data highlight the impact of 3′-exonucleolytic RNA decay pathways and re-evaluates the degradation mechanisms of Hfq-free small RNAs.

Keywords: Hfq; MicA; PNPase; small noncoding RNAs; RNase E

INTRODUCTION

The bacterial Hfq is a member of the Sm/Lsm superfamily of proteins involved in RNA metabolism (Wilusz and Wilusz 2005). It is a global regulator of cell physiology with particular impact on stress responses and affects the virulence traits of many pathogens (Tsui et al. 1994; Chao and Vogel 2010). Hfq plays a relevant role as a mediator of small noncoding RNA−mRNA interactions (Valentin-Hansen et al. 2004; Waters and Storz 2009). Base-pairing of small RNAs with their target mRNAs can alter mRNA translation and/or stability. The majority of small RNAs act as inhibitors of translation, usually triggering mRNA decay, although some other sRNAs act as positive regulators (Massé et al. 2003; Vecerek et al. 2007; Soper et al. 2010).

Hfq forms a stable hexamer with a ring-shaped structure displaying two distinct RNA-binding surfaces (Brennan and Link 2007). Biochemical and structural data support that the Hfq hexamer can bind simultaneously the sRNA on its proximal face and mRNA on its distal face, increasing the probability of RNA–RNA interactions in order to form a heteroduplex (Schumacher et al. 2002; Lease and Woodson 2004; Mikulecky et al. 2004; Link et al. 2009). In agreement, it has been suggested that the Hfq hexamer forms a ternary complex with oligo A_{18} and the small RNA $DsrA_{DII}$ with a stoichiometry of 1:1:1 (Updegrove et al. 2011). Hfq can also work as a RNA chaperone and induce structural rearrangement of the RNA molecules to enable the contact between the two partner RNAs (Moll et al. 2003b; Geissmann and Touati 2004; Afonyushkin et al. 2005; Arluison et al. 2007).

Bacterial small RNAs that act as repressors bind at or near the ribosome binding site (RBS) of the target mRNA blocking its translation (Morita et al. 2006; Bouvier et al. 2008). Most of the time, this promotes cleavages in the mRNA, not only on the vicinity of the duplex (as happens with *ompA* mRNA/MicA) (Udekwu et al. 2005), but also downstream into the coding region (as reported for *sodB* mRNA/RyhB) (Prévost et al. 2011). Ribonuclease (RNase) III is an important endonuclease in the degradation of sRNA coupled to their target mRNAs (Afonyushkin et al. 2005;

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Deltcheva et al. 2011). However, other sRNAs mediate the destabilization of the target mRNA in an RNase E-dependent manner (Massé et al. 2003; Afonyushkin et al. 2005; Morita et al. 2005; Udekwu et al. 2005). RNase E is a single-stranded RNA endonuclease involved in mRNA decay in *Escherichia coli* (Arraiano et al. 2010). Hfq can associate with RNase E and sRNA in ribonucleoprotein complexes that are thought to make the degradation of target mRNAs more efficient (Aiba 2007).

Hfq is also found to interact with other proteins involved in mRNA decay. One of these proteins is the poly(A) polymerase I (PAP I), responsible for the majority of polyadenylation in *E. coli* cells (Régnier and Hajnsdorf 2009). Hfq is suggested to regulate polyadenylation by stimulating PAP I activity on mRNA (Hajnsdorf and Régnier 2000; Folichon et al. 2005). In the absence of Hfq, the poly(A) levels are reduced and the poly(A) tails are suggested to become smaller (Le Derout et al. 2003; Mohanty et al. 2004). Hfq was also shown to interact with the polynucleotide phosphorylase (PNPase) (Mohanty et al. 2004), a major 3′–5′ exonuclease involved in RNA degradation (Andrade et al. 2009b). PNPase responds to environmental stimuli, and its activity is modulated by metabolites such as ATP, citrate, and cyclic di-GMP (Del Favero et al. 2008; Nurmohamed et al. 2011; Tuckerman et al. 2011). We have previously shown that PNPase is a key factor in the turnover of small RNAs controlling the expression of outer membrane proteins in the stationary phase of growth (Andrade and Arraiano 2008). It was recently suggested that PNPase can also have a protector role for some sRNAs in exponentially growing cells (De Lay and Gottesman 2011). However, the details of the interplay between PNPase and Hfq in the function of sRNA are still not clear.

The interaction of Hfq with small RNAs is dynamic. Small RNAs compete for access to Hfq, and it was shown that the expression of unrelated sRNAs can dissociate Hfq–sRNA complexes already formed (Fender et al. 2010; Hussein and Lim 2011). The transient existence of small RNAs free from Hfq binding is thus part of the normal dynamic lifecycle of a sRNA. In addition, variations in the Hfq expression levels or in the availability of the free pool of Hfq can result in the reduction of Hfq–sRNA complexes. A small RNA that is not associated with Hfq is rapidly degraded, although the mechanism by which Hfq stabilizes small RNAs is not yet fully understood. RNase E was considered to be responsible for the rapid degradation of the small RNAs and was shown to compete with Hfq for access the same RNA sequences (Massé et al. 2003; Moll et al. 2003a). However, the activity of RNase E may not be as generalized; for example, RNase E was not found to be important for the in vivo degradation of OxyS upon Hfq inactivation (Basineni et al. 2009).

In this work we have characterized the degradation of small RNAs that are unassociated with Hfq. We have constructed multiple *hfq* mutants defective in RNases or in the poly(A) polymerase, and studied the impact of these factors in the expression of several small RNAs. We have shown that small RNAs in their Hfq-free state are rapidly degraded by PNPase, particularly in the stationary phase of growth. Moreover, PNPase was found to be more relevant than RNase E or RNase III in the degradation of small RNAs when these were not associated to Hfq. PNPase-mediated degradation of small RNAs is also found to be an active regulatory pathway in the cells expressing Hfq. Together, results show that PNPase has a predominant role in the degradation of Hfq-free small RNAs.

RESULTS

Inactivation of PNPase markedly increases the levels of small RNAs not associated with Hfq

It is commonly believed that the RNA chaperone Hfq protects regulatory RNAs from RNase E endonucleolytic cleavages (Massé et al. 2003; Moll et al. 2003a). Nevertheless, we have previously showed that 3′–5′ exonucleolytic activity can be a determinant for the degradation of small RNAs, even in the presence of Hfq (Andrade and Arraiano 2008). In this work, we wanted to characterize the role of 3′–5′ exonucleases in the degradation of small RNAs that are not associated with Hfq. Therefore, we have performed most of our studies in strains lacking Hfq.

In this work we have analyzed four well-characterized *E. coli* sRNAs: MicA, SgrS, RyhB, and GlmY. Most of these small RNAs are induced under specific conditions of stress and in the stationary phase. Therefore, we decided to focus our work on this growth phase. Total RNA was extracted from stationary-phase cultures, and the steady-state levels of these small RNAs were analyzed by Northern blotting. Inactivation of Hfq resulted in the high reduction of all of the small RNAs analyzed when compared with the wild-type strain (Fig. 1, middle). Only GlmY levels seemed not to be so strongly affected by the absence of Hfq in the conditions analyzed. Subsequently, a set of multiple mutants lacking both Hfq and one of the main 3′–5′ exonucleases (PNPase, RNase II, or RNase R) was constructed. RNase II and RNase R had a reduced impact on this regulation; RNase II mutant was only shown to change GlmY levels, and the absence of RNase R did not affect the levels of any of these small RNAs. Upon inactivation of PNPase in cells lacking Hfq, it was possible to detect a very strong signal for all of the small RNAs analyzed. These results indicate that PNPase is a major factor controlling the levels of small RNA that are not associated with Hfq.

In Hfq$^-$ PNPase$^-$ cells we observed the accumulation of a slightly shorter form of all of the small RNAs (here designated, respectively, by MicA*, SgrS*, RyhB*, and GlmY*)

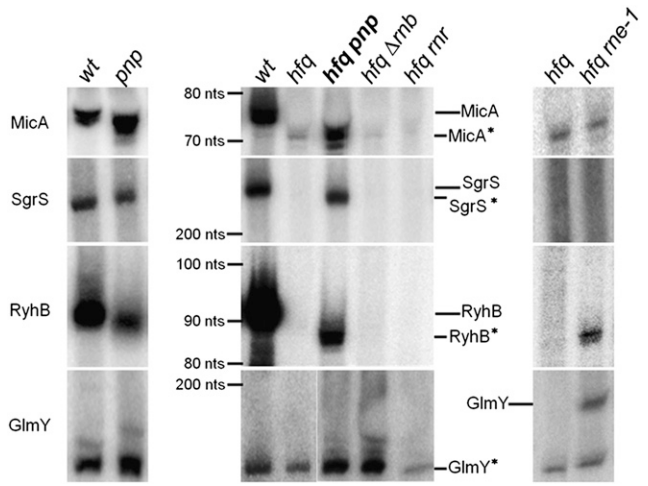

FIGURE 1. PNPase strongly affects the levels of several small RNAs that are not bound to Hfq. Small RNA expression was analyzed by Northern blot. (*Left*) The levels of MicA, SgrS, Ryhb, and GlmY were analyzed in the wild-type (hfq^+ pnp^+) and a PNPase mutant (hfq^+ pnp^-). Total RNA was extracted from stationary-phase cultures grown at 37°C as mentioned in the Materials and Methods. (*Middle*) Hfq mutants lacking one of the 3′–5′ exoribonucleases PNPase (*pnp*), RNase II (*rnb*), and RNase R (*rnr*) were compared with wild-type (*wt*) and *hfq* single mutant. (*Right*) To study the impact of the essential RNase E (*rne*), the double *hfq rne*-1 mutant was grown at 30°C until it reached stationary phase and then shifted to the nonpermissive temperature of 44°C for inactivation of the thermosensitive RNase E. Samples were withdrawn after 5 min of incubation. For comparison, the single *hfq* mutant was treated in the same conditions. Specific [32P]-labeled probes were used to detect the small RNAs. Full-length small RNAs are clearly detected on wild type (except for GlmY), showing the expected sizes: MicA (74 nt), RyhB (90 nt), GlmY (180 nt), and SgrS (227 nt), as estimated from markers run along the gels. Small RNAs detected on *hfq* mutants (namely, in the *hfq pnp*) are slightly shorter than the corresponding full-length sRNAs; these shorter small RNAs are designated by an asterisk (*). The positions of both the full-length and the shorter small RNAs are indicated. 5S RNA or tmRNA were used as loading controls.

(Fig. 1, middle). These shorter sRNA are not detected or are barely perceptible in the wild-type strain. The shorter GlmY* (~140 nt) is an exception, being the predominant RNA detected in the wild type. This has been previously observed as the full-length GlmY (~180 nt) is rapidly processed in the 3′-end to originate the GlmY* species (Reichenbach et al. 2008; Urban and Vogel 2008). Overexpression of PNPase in the *hfq pnp* strain was shown to reverse the accumulation of MicA* (Supplemental Fig. S1). This result confirmed that PNPase is responsible for the higher levels of small RNAs found in the Hfq^- $PNPase^-$ cells.

We had previously demonstrated the involvement of PNPase in the degradation of MicA in stationary-phase cells harboring Hfq (Andrade and Arraiano 2008). To check the impact of PNPase in the regulation of all of these small RNAs in the presence of Hfq, we have analyzed the levels of the same small RNAs in the *pnp* single mutant and compared it with the wild-type strain (Fig. 1, left). Inactivation of PNPase in cells expressing Hfq resulted in higher levels of some of these sRNAs, namely, GlmY* and MicA. In contrast, other small RNAs such as RyhB and SgrS showed decreased amounts in the PNPase mutant strain. The reduction in the levels of other sRNAs following PNPase inactivation in exponential phase was recently observed and may potentially reflect an increase in the activity of other RNase(s) (De Lay and Gottesman 2011), but the genetic pathways involved in this regulation have not yet been elucidated. Here we show that in cells without Hfq the inactivation of PNPase (Hfq^- $PNPase^-$ cells) results in increasing levels of all of the sRNAs analyzed, but that this regulation is not universal in the presence of Hfq (Hfq^+ $PNPase^-$ cells). These results suggest that the binding of Hfq may impair the PNPase-dependent regulation of at least some small RNAs.

We also analyzed the effect of RNase E in the control of these regulatory RNAs in the absence of Hfq (Fig. 1, right). Only RyhB* and both GlmY/GlmY* levels were increased in the Hfq^- RNase E^- mutant, while MicA* and SgrS* levels did not change. This greatly contrasted with the strong stabilization of all of the small RNAs obtained in the Hfq^- $PNPase^-$ mutant. Hence, our results indicated that when these small RNAs are not associated with Hfq, they are clearly more vulnerable to degradation by PNPase than to cleavages by RNase E.

PNPase is a major factor in the rapid decay of the Hfq-free MicA*

Taking into account these results and our previous data on MicA (Andrade and Arraiano 2008), we decided to use this sRNA as the main model for further investigation. MicA (previously SraD) is an antisense RNA that down-regulates the expression level of outer membrane proteins OmpA (Rasmussen et al. 2005; Udekwu et al. 2005) and LamB (Bossi and Figueroa-Bossi 2007), as well as the members of the PhoPQ regulon (Coornaert et al. 2010).

To evaluate whether the higher MicA* levels in the absence of PNPase were the consequence of increased stability, we next analyzed the decay rates of MicA* in the Hfq^- $PNPase^-$ cells (Fig. 2). We also tested the potential role of RNase II and RNase R in the degradation of small RNAs in cells without Hfq. Stability measurements indicated that PNPase was found to be the only exoribonuclease significantly involved in the exo-degradation of MicA RNAs in stationary-phase cells lacking Hfq (Fig. 2). Neither RNase II nor RNase R was shown to significantly affect this decay. The *hfq pnp* double mutant showed a nearly fourfold stabilization of MicA* when compared with *hfq* single mutant. Accordingly, the increasing levels of MicA* in Hfq^- $PNPase^-$ cells are a consequence of its longer stability due to the inactivation

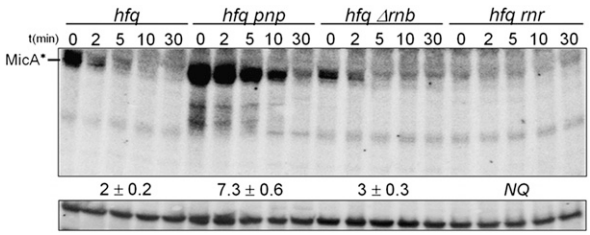

FIGURE 2. PNPase is the major exoribonuclease involved in the degradation of MicA*. Samples from stationary-phase cultures of *hfq* and its derivative exoribonuclease mutants (*hfq pnp*, *hfq Δrnb*, and *hfq rnr*) grown at 37°C were withdrawn after inhibition of transcription (timepoints are shown in minutes) and total RNA was analyzed by Northern blot. A specific riboprobe for MicA was used. A nonspecific band that cross-hybridized with the antisense MicA probe was used as loading control. This band migrates above MicA and disappears with a more stringent washing step of the membrane without affecting MicA signal (Andrade and Arraiano 2008). Hybridization with a 5S RNA riboprobe gave identical results. Only the MicA* RNA species is detected in the absence of Hfq. Half-lives were determined after PhosphorImager densitometry quantification showing that PNPase is the major exoribonuclease involved in the degradation of the Hfq-unprotected MicA*. (NQ) Not quantifiable.

of PNPase. These results indicate that PNPase has a major role in turning over MicA species that are not associated with Hfq.

Poly(A) polymerase I promotes the degradation of MicA*

Polyadenylation can promote RNA degradation by facilitating the exonucleolytic attack of an RNA substrate (Régnier and Hajnsdorf 2009). Therefore, we decided to analyze the impact of polyadenylation in the degradation of MicA and compare it with PNPase.

In stationary-phase cells expressing Hfq, the lack of poly(A) polymerase I (PAP I/*pcn*B) resulted in a modest increase in MicA half-life (only a 1.5-fold up-regulation) from 8.2 to 12.5 min, as detected by Northern blotting (Fig. 3A). On the other hand and in the same conditions, inactivation of PNPase resulted in a stronger stabilization of MicA (from 8.2 min in the wild type to 27.5 min in the *pnp* mutant). This indicates that in the presence of Hfq, PNPase activity against MicA surpasses the effect of PAP I polyadenylation-dependent pathways.

MicA is very unstable in the absence of Hfq; its half-life decreases from 8.2 min in the wild type to 2 min in the *hfq* mutant (Fig. 3A). To check whether PAP I could be involved in the rapid degradation of the MicA* in the absence of Hfq, we constructed the double *hfq pcnB* mutant. Two different *pcnB* mutations were used in this study: either a deletion Δ*pcnB* (O'Hara et al. 1995) or the *pcnB80* allele (Hajnsdorf et al. 1995) was transferred to the *hfq* mutant strain; measurement of MicA half-lives gave identical results for both strains. The double-mutant *hfq pcn*B was found to have a significant 2.5-fold more stable MicA* when compared with the single *hfq* mutant (increasing half-life from 2 to 5 min). In the absence of Hfq, the MicA* RNA is more susceptible to poly(A)-mediated decay in stationary-phase cells. This was surprising, as a Hfq$^-$ mutant was reported to have low levels of polyadenylation (Hajnsdorf and Régnier 2000; Le Derout et al. 2003; Mohanty et al. 2004). Notwithstanding the higher impact that poly(A) polymerase I displays in the degradation of the MicA* in the absence of Hfq, inactivation of PNPase still renders a more stable sRNA (Fig. 3A, bottom). Altogether, these results show that PNPase has a stronger effect than poly(A) polymerase I in the degradation of MicA RNAs, irrespective of the presence of Hfq.

We also determined the relative levels of other small RNAs in the *hfq* Δ*pncB* double mutant compared with the *hfq* single mutant (Fig. 3B). The levels of GlmY* were also increased by the lack of PAP I in the absence of Hfq. This was also confirmed to be a consequence of the higher stabilization of GlmY* in the *hfq pcn*B mutant (data not shown). GlmY* is known to be highly polyadenylated in cells harboring Hfq (Reichenbach et al. 2008; Urban and Vogel 2008). We have now shown that the lack of poly(A) polymerase I is an important factor affecting the sRNA decay in the absence of Hfq in stationary-phase cells. However, inactivation of PNPase in cells devoid of Hfq

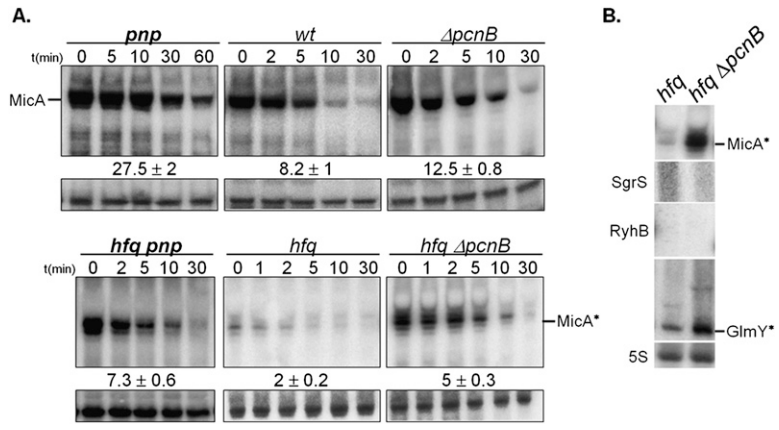

FIGURE 3. Lack of poly(A) polymerase I results in increasing levels of MicA*. (*A*) Impact of poly(A) polymerase I (*pcn*B) in the degradation of the small MicA RNA in Hfq$^+$ or Hfq$^-$ cells. Stationary-phase cultures of wild type and its derivatives *pnp*, Δ*pcn*B, *hfq pnp*, *hfq*, and *hfq* Δ*pcn*B strains were treated with rifampicin, and total RNA was analyzed by Northern blot. MicA was detected by use of a specific riboprobe. Only the shorter MicA* RNA is visible in the Hfq$^-$ cells. A nonspecific band cross-reacting with MicA probe was used as loading control. (*B*) The steady-state levels of several small RNAs from stationary-phase cultures of *hfq* and *hfq* Δ*pcn*B mutants were evaluated by Northern blot.

resulted in higher levels of all of the small RNAs analyzed, even the ones that were not affected by the lack of PAP I (namely RyhB* and SgrS*) (Figs. 1, 3B). Hence, PNPase activity against a small RNA that is not bound to Hfq does not necessarily require an active polyadenylation-dependent pathway.

In the absence of Hfq, MicA* is a substrate for PNPase, but not for RNase E or RNase III

RNase E was thought to be responsible for the rapid degradation of small RNAs not protected by Hfq (Massé et al. 2003; Moll et al. 2003a). Surprisingly, we found that MicA* levels did not change substantially between the *hfq* single mutant and the *hfq rne*-1 double mutant (Fig. 1, right). To analyze this observation further, we assayed MicA decay rates in both strains. As RNase E (*rne*) is essential in *E. coli*, we used a thermolabile allele (*rne*-1) and performed this set of experiments at the nonpermissive temperature (Fig. 4A).

From previous work, we have identified that RNase E is involved in the degradation of MicA in cells producing Hfq (Andrade and Arraiano 2008). Surprisingly, our results revealed that MicA* is not stabilized significantly when RNase E is inactivated in the absence of Hfq (Fig. 4A). This indicates that RNase E is not able to efficiently degrade MicA* unless Hfq is present in the cell. A similar RNase E/Hfq dependency was observed in OxyS turnover (Basineni et al. 2009).

To better assess the relative impact of RNase E and PNPase, we treated the culture of the *hfq pnp* mutant in the same conditions used to inactivate the thermosensitive RNase E (Fig. 4B). No significant changes were detected when Hfq and RNase E were inactive, but MicA steady-state levels are substantially higher upon inactivation of both Hfq and PNPase (an eightfold increase in the *hfq* mutant). This result clearly showed that in the absence of Hfq, PNPase is more important than RNase E in the degradation of this sRNA. This result is also substantiated by data from Figure 1.

The other main endonuclease involved in RNA degradation is RNase III (Arraiano et al. 2010). In vitro studies showed that *Salmonella* RNase III can cleave MicA when bound to its target *omp*A mRNA (Viegas et al. 2011). To further analyze the role of RNase III in cells without Hfq, we constructed and analyzed double mutants lacking both Hfq and RNase III. RNA extracted from stationary-phase cultures from the deletion mutant of *E. coli* RNase III (Δ*rnc*) and a double mutant lacking Hfq and RNase III (*hfq* Δ*rnc*) was analyzed by Northern blotting (Fig. 4C). Inactivation of RNase III in the presence of Hfq appeared to block the degradation of MicA (this RNA apparently did not decay even 240 min after transcription blocking). This clearly showed that *E. coli* RNase III is important in the control of MicA stability. However, MicA RNAs were barely detected in the double-mutant *hfq* Δ*rnc*, reflecting the results obtained with the single *hfq* mutant. To confirm this result we also tested another allele of RNase III, the *rnc*105; the double-mutant *hfq rnc*105 displayed identical results (data not shown). The strong decrease in MicA levels typically found in the absence of Hfq obviously reduce the number of duplexes formed between this sRNA and its target mRNAs, probably impairing RNase III activity against MicA. The MicA* levels found in the *hfq pnp* strain were higher (about a sixfold increase in the *hfq* single mutant) than the MicA* levels found in the *hfq* Δ*rnc* mutant (Fig. 4D). These results clearly indicated that PNPase was more important than RNase III in the elimination of MicA* from the cell. Overall, when MicA is not associated with Hfq, the 3′–5′ exoribonucleolytic degradation pathway mediated by PNPase is found to be more important in this degrada-

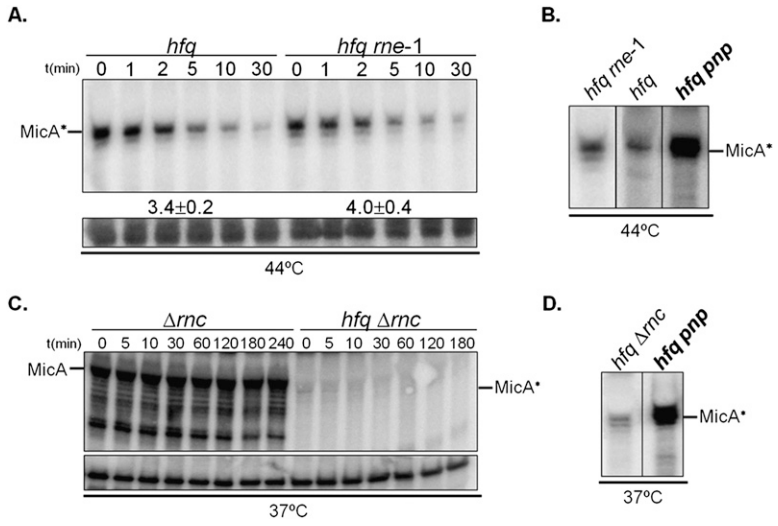

FIGURE 4. PNPase, but not RNase E or RNase III, degrades the Hfq-free MicA* RNA. (*A*) Northern blot detection of MicA RNA in Hfq$^-$ cells harboring or not harboring the *rne*-1 allele. Stationary-phase cultures were treated at 44°C for inactivation of the thermosensitive RNase E (as mentioned before). MicA RNA stability was analyzed by Northern blot with a specific riboprobe. (*B*) Northern blot analysis of MicA in Hfq$^-$ cells deficient in RNase E or PNPase. The double *hfq rne-1* mutant was grown at 30°C until stationary phase and then incubated at 44°C to inactivate RNase E. For comparison, the *hfq* and *hfq pnp* were treated in the same conditions. (*C*) Northern blot detection of MicA in stationary-phase cultures of Hfq$^-$ cells harboring or not harboring RNase III (*rnc*), respectively. A loading control corresponding to a nonspecific band that cross-reacted with MicA probe is shown in *below*. (*D*) Comparison of MicA* RNA steady-levels in Hfq$^-$ stationary-phase cells deficient in RNase III or PNPase grown at 37°C.

tion than any of the main endoribonucleases involved in RNA turnover.

Hfq is required for the optimal expression of the full-length MicA

The slightly smaller MicA* is the predominant form in the *hfq* mutant in stationary-phase cultures, but it is barely detected in the wild-type (Fig. 1). To examine whether this RNA pattern was dependent on a growth phase-specific regulation, we analyzed both the wild-type and the *hfq* strain along the growth curve (Fig. 5A; Supplemental Fig. S2). In exponential phase, the *hfq* mutant exhibited the full-length MicA, as well as additional shorter bands of similar intensity, apparently differing a few nucleotides in size. This pattern was growth dependent. In stationary phase there was a decrease in the amount of full-length MicA and what appeared to be a concomitant accumulation of the smaller MicA*. This greatly contrasted with the wild-type strain, where the full-length MicA was the most prominent band, irrespective of the growth phase analyzed. Moreover, supplying Hfq in *trans* from a plasmid complemented *hfq* deficiency on MicA expression and resulted in the strong accumulation of the full-length RNA and in the elimination of the shorter sized RNAs (Fig. 5A). These results indicated that Hfq determines MicA full-length expression along growth, particularly in the stationary phase.

The difference in size between the full-length MicA and MicA* is small, apparently in the range of from 3 to 4 nt, visible on Northern blotting. Such small variation must lie at one of the RNA extremities. Primer extension analysis was performed to evaluate which extremity was shortened (Fig. 5B). Stationary-phase cultures of both the wild-type (which expresses full-length MicA) and the *hfq* mutant

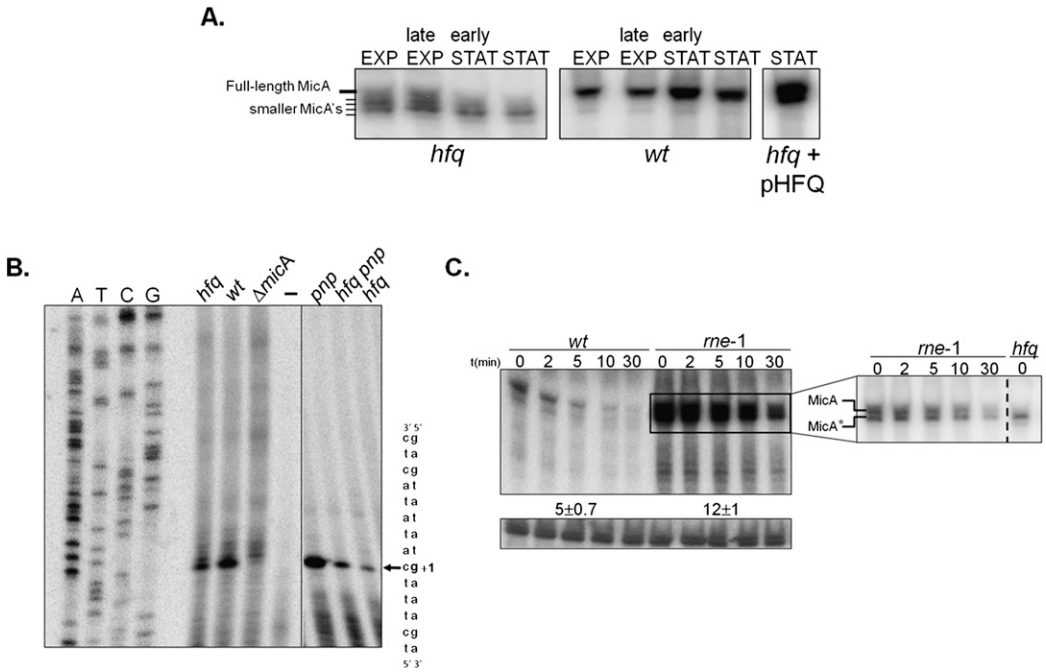

FIGURE 5. Hfq is required for the expression of the full-length MicA RNA. (*A*) Steady-state levels of MicA RNA along the growth curve. Culture samples of wild-type or *hfq* mutant bacteria were collected at exponential (EXP), late exponential, early stationary, and stationary phase (STAT) (corresponding to OD_{600} values of ~0.3, ~1.7, ~2.5, and ~5.5 for the wild-type and ~0.3, ~0.8, ~1.6, and ~2.3 for the *hfq* mutant, respectively). The growth curves for the wild-type and the *hfq* mutant strain are given in Supplemental Figure S2. A specific antisense MicA riboprobe was used to detect MicA. Stationary-phase cultures of the *hfq* mutant transformed with the overexpressing pHFQ plasmid show complementation and do not exhibit the heterogeneous population of MicA's typically found in the *hfq* single mutant. (*B*) Determination of the 5′-end of MicA. Total RNA from stationary-phase cells of wild-type, *hfq*, *pnp*, and *hfq pnp* strains was analyzed by primer extension with the [32P]-labeled primer MicA-PE. The same primer extension product (indicated by an arrow) is detected on all strains and absent from the deletion *micA* strain (Δ*micA*) and the negative control reaction (−) done without RNA. Part of the DNA sequence is indicated on the *right*. The transcription start site of MicA is indicated (+1) and is identical to the site described by Udekwu et al. (2005). The intensity of the primer extension product obtained is higher in the wild-type rather than the *hfq* mutant, in agreement with the higher amount of MicA detected in the wild-type strain (see Fig. 5A). (*C*) Northern blot detection of MicA in stationary-phase cultures of Hfq^+ cells upon inactivation of RNase E. Cultures of wild-type and an RNase E mutant strain were grown at 30°C until they reached stationary phase, and then shifted to the nonpermissive temperature of 44°C. After 5 min, transcription was blocked with the addition of rifampicin, and samples were withdrawn at times indicated. A specific riboprobe was used to detect MicA RNA. A nonspecific band that cross-hybridized with the antisense MicA probe was used as loading control. The *inset* corresponds to a shorter exposure of the membrane in which it is visible that both the full-length MicA and the shorter MicA* RNA are detected and stabilized upon inactivation of RNase E in Hfq^+ cells. The *hfq* mutant was used here as a control to clearly identify MicA* RNA.

strain (where MicA* is detected) showed accumulation of a band that matches the start of the MicA sequence. Furthermore, this same band was identified when testing either the *pnp* mutant or the *hfq pnp* double mutant, showing that the MicA RNAs that accumulate upon PNPase inactivation retain the same 5′ end as the wild-type MicA. An additional experimental approach using nuclease S1 mapping also determined the same 5′-end for both MicA species (Supplemental Fig. S3). Altogether, these findings supported that full-length MicA and MicA* have the same 5′-end, and that the difference in size is located at the 3′-end. This suggests that the smaller RNA species probably arises from 3′-end processing of the full-length MicA.

The MicA* RNA is expressed at very low levels in the wild type. This suggests that Hfq acts in order to prevent MicA* production or to ensure its rapid elimination. We decided to analyze the kinetics of decay and found that RNase E affected MicA* levels (Fig. 5C). Inactivation of RNase E (in cells harboring Hfq) resulted in the strong elevation of MicA levels in stationary phase. However, a shorter exposure of this gel revealed the detection not only of the full-length MicA, but as well, the smaller MicA* (inset in Fig. 5C). Both RNAs showed a twofold stabilization in the absence of RNase E. This indicated that even in the presence of Hfq, the shorter MicA* RNA fragment is produced in the cell.

Growth-phase regulation of small RNAs by PNPase

In the absence of Hfq, small RNAs are typically unstable and PNPase was found to be a major enzyme involved in the extensive degradation of MicA in stationary-phase cells. To check whether this could be generalized to other small RNAs, we extended this analysis to RyhB and SgrS. Since the RNA pattern of MicA changes along with growth in Hfq$^-$ cells (Fig. 5A), it is reasonable that different RNA degradation pathways might be involved in different stages of growth. To further analyze this, we decided to compare the small RNA stability between exponential and stationary-phase cultures.

In the absence of Hfq, all of the small RNAs analyzed were highly unstable, regardless of the growth phase that was analyzed (Fig. 6). As consequence of the extensive degradation occurring in the absence of Hfq, the MicA, RyhB, and SgrS levels were strongly reduced in the *hfq* mutant when compared with wild type, both in exponential and stationary-phase cultures. In contrast, all of these small RNAs were markedly stabilized in the stationary-phase cultures of the *hfq pnp* double mutant compared with the *hfq* strain. Interestingly, this regulation is not as common in exponentially growing cells. In fact, only SgrS was found to be stabilized in exponential-phase cultures of the *hfq pnp* mutant strain compared with the *hfq* strain (although this is significantly lower than the stabilization observed in stationary-phase cells). These results confirm PNPase as a major enzyme involved in the degradation of Hfq-free small RNAs in the cell.

Hfq deficiency resulted in the detection of shorter small RNAs that are stabilized upon further inactivation of PNPase (Figs. 1, 6). In exponential-growing cells without Hfq, only MicA was found to exhibit a heterogeneous-sized population (Figs. 5A, 6). From these fragments, MicA* is shown to be the most resistant and is even the predominant RNA species found in *hfq* mutants in the stationary phase. The smaller RyhB* and SgrS* RNAs were only detected in stationary-phase cells. Together, these results suggested a protection of the full-length sRNA by Hfq, which seems particularly important for sRNA expression in the stationary phase of growth.

To analyze whether PNPase is affecting the stability of small RNAs independently of Hfq, we further analyzed the

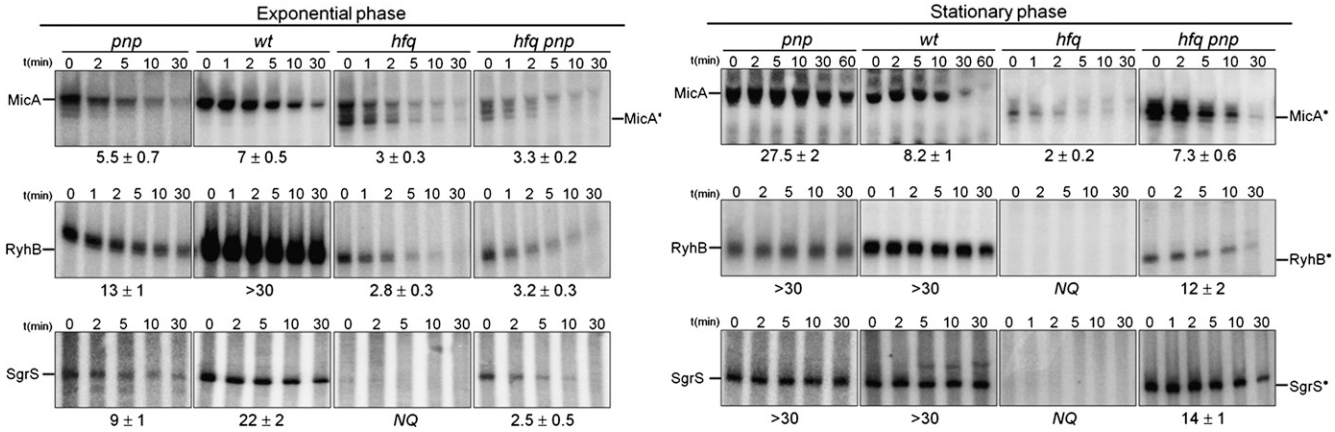

FIGURE 6. Growth-phase regulation of Hfq-free small RNAs by PNPase. Northern blot determination of MicA, RyhB, and SgrS RNA stabilities between the wild-type and its isogenic *pnp*, *hfq*, and *hfq pnp* mutants either in exponential-phase or stationary-phase cultures. Total RNA was extracted from culture samples withdrawn after inhibition of transcription with rifampicin (timepoints are shown in minutes). MicA, RyhB, and SgrS RNAs were detected by the use of specific radiolabeled probes and quantified by PhosphorImager analysis. The full-length small RNAs or their respective shorter forms (where detected) are indicated on the gels. (NQ) Not quantifiable.

decay rates of MicA, RyhB, and SgrS in the *pnp* single mutant. Inactivation of PNPase in exponential-phase cells producing Hfq resulted in reduced levels and decreased stability of the small RNAs. Similar results were reported with other small RNAs, suggesting that PNPase may somehow protect some sRNA in exponential phase (De Lay and Gottesman 2011). In fact, this is also observed in stationary-phase cultures, as inactivation of PNPase is also found to reduce RyhB and SgrS levels under this condition. Only MicA was shown to be a substrate for PNPase either in the absence or presence of Hfq. On the other hand, RyhB and SgrS were found to be preferably degraded by PNPase in the absence of Hfq and were greatly stabilized in the *hfq pnp* double mutant, particularly in the stationary phase of growth. Altogether, these results suggest that PNPase degrades small RNAs more efficiently in the absence of Hfq, although this turnover pathway is clearly active in cells with Hfq. The fact that this occurs in cells expressing Hfq may reflect the action of PNPase against small RNAs that are transiently in their Hfq-free state, a result of the dynamics of interaction with Hfq. PNPase-mediated degradation of small RNAs is suggested to be predominant in stationary-phase cells, as this regulation apparently is not so common in exponential-phase cells. Therefore, these results demonstrated that the degradation pathways of a same small RNA can be different between exponential and stationary phases and highlighted the role of PNPase in the growth-phase regulation of small RNAs.

DISCUSSION

This work demonstrated that the pool of small RNAs that are not associated with Hfq is preferably degraded by PNPase. Overall, our data highlight the impact of 3′–5′ exonucleolytic RNA decay pathways and re-evaluates the degradation mechanisms involved in the rapid decay of the Hfq-free small RNAs. The reduced levels of small RNAs typically found in the Hfq$^-$ strain were strongly increased upon inactivation of PNPase in stationary-phase cells (Fig. 1). This seems to be a general feature, since PNPase inactivation resulted in increasing levels of at least the MicA, SgrS, RyhB, and GlmY sRNAs. We only detected the accumulation of slightly shorter sRNAs rather than the full-length species, and this was shown to be the consequence of the higher stability of these fragments (Fig. 2).

The lack of poly(A) polymerase I was also found to impact the levels of small RNAs in the absence of Hfq, although to a lesser extent than PNPase (Fig. 3). These results were unexpected, as no significant differences in mRNA stability were detected between *hfq* and *hfq* Δ*pcn*B mutants (Mohanty et al. 2004). In contrast, our results clearly showed that in the absence of Hfq, the small RNA turnover can be affected by the lack of poly(A) polymerase I. The sRNAs found to be highly affected by polyadenylation (MicA* and GlmY*) were also found to be excellent substrates for PNPase. Nevertheless, PNPase activity against Hfq-unprotected small RNAs is not necessarily dependent on poly(A) polymerase I activity. RyhB* and SgrS* RNAs are not affected by polyadenylation, although their levels were highly increased upon PNPase inactivation, as observed in the double *hfq pnp* mutant (Figs. 1, 3B). Poly(A)-dependent pathways may thus not explain all the extraordinary impact of PNPase on sRNA turnover in the Hfq$^-$ cells.

Pioneer work on PNPase revealed its ability to synthesise RNA (Grunberg-Manago et al. 1955). Interestingly, it has been proposed that in the absence of Hfq, there is an increase in the biosynthetic activity of PNPase with heteropolynucleotide tails promoting RNA decay (Mohanty et al. 2004; Slomovic et al. 2008). Addition of these polynucleotide tails can potentially be responsible for PNPase notable impact on the degradation of sRNA in the absence of Hfq. RNase II (Marujo et al. 2000) and RNase R (Andrade et al. 2009a) are also major poly(A)-dependent exoribonucleases, but they were not found to be involved in the degradation of MicA (Fig. 2; Andrade and Arraiano 2008). Similar results were obtained regarding the degradation of RyhB (data not shown). Surprisingly, despite RNase R intrinsic ability to easily degrade structured RNAs on its own and its affinity to poly(A) tails, RNase R was not shown to be part of these decay pathways. The absence of RNase R resulted in the reduction of MicA* levels in cells without Hfq (Fig. 2). This might be a result of an indirect effect in which the activity of a MicA repressor is increased when RNase R is not functional. Although the protection of RNA by a ribonuclease seems paradoxical, a similar effect has been described either for RNase II or PNPase (Marujo et al. 2000; De Lay and Gottesman 2011). A major advantageous feature of PNPase in the degradation of small RNAs might be its ability to form complexes with other proteins, which can be particularly helpful in the elimination of such structured RNAs. However, we have already shown that PNPase activity on MicA can be independent of the degradosome assembly (Andrade and Arraiano 2008).

RNase E has a role in sRNA degradation (Massé et al. 2003; Morita et al. 2005; Suzuki et al. 2006; Viegas et al. 2007; Andrade and Arraiano 2008). However, our results demonstrated that its impact on Hfq$^-$ cells may not be as general as previously believed. RNase E depletion did not affect the levels of SgrS and MicA RNA. While in the presence of Hfq, both the full-length MicA and the MicA* RNAs are substrates for RNase E (Fig. 5C); this regulation is lost when Hfq is absent (Fig. 4A). This indicates that RNase E requires Hfq in order to degrade MicA. A similar RNase E dependency of Hfq to act on sRNA turnover was also reported in the growth-phase degradation of OxyS (Basineni et al. 2009). It is suggested that RNase E/Hfq cooperation (as observed in the mRNA decay mediated by sRNA) (Morita et al. 2005) can also be critical for the

degradation of some small noncoding RNAs, like MicA. Nevertheless, we recognize that RNase E can also impact the levels of some sRNAs independently of Hfq. As observed, both RyhB* and GlmY are RNase E substrates, even in cells lacking Hfq (Fig. 1).

A possible RNase III/Hfq pathway was also analyzed. RNase III inactivation results in extremely long-lived MicA; however, this is strictly dependent on the presence of Hfq, as this stabilization is completely lost in the *hfq* Δ*rnc* mutant (Fig. 4C). The low levels of MicA found in *hfq* mutants strongly decrease the probability of base-pairing with target mRNAs. The down-regulation in sRNA-target mRNA duplexes probably explains the impairment in RNase III activity on MicA, in agreement with in vitro studies (Viegas et al. 2011). Data suggest that the degradation of small RNAs that are not associated with Hfq mainly occurs in a target-independent pathway, in which RNase III has a reduced impact. The free pool of small RNAs is then preferably degraded by PNPase.

Hfq was thought to mainly protect sRNA from RNase E cleavages, as both proteins showed in vitro affinity for the same A/U-rich sequences in RNA (Moll et al. 2003a). However, it has been recently demonstrated that Hfq actually prefers to bind U-rich sequences at the 3′-end of small RNAs over internal A/U-rich sequences (Otaka et al 2011; Sauer and Weichenrieder 2011). Small RNAs, like MicA, usually display a short U-rich 3′-end sequence immediately downstream from a stem–loop as a consequence of Rho-independent transcription termination (Rasmussen et al. 2005; Udekwu et al. 2005). The physiological meaning of the high affinity of Hfq to this U-rich sequence can be the protection of the 3′-end of the RNA against degradation. Interestingly, our results showed that the 3′-ends of the small RNAs are shortened in the absence of interaction with Hfq (Figs. 1, 5B). Even though PNPase is observed to be the main exoribonuclease involved in the degradation of these shorter small RNAs, it does not seem to be the main reason for the initial 3′-end attack, as this is not prevented in a *pnp* background. RNase II and RNase R inactivation also did not suppress the shortening of MicA. Data suggested that other (exo)nucleases would be responsible for the 3′-end trimming of the small RNAs when they are Hfq free. The transcriptional terminator stem–loop of the small RNAs may function as a physical barrier against exoribonucleases. PNPase may be favored in this action and progress to degradation of the sRNA body, while other RNases may be inhibited, and therefore could only degrade a few nucleotides before releasing the sRNA. In the presence of Hfq, the shorter sRNAs are barely detected, probably because Hfq protects the 3′-ends of the small RNAs.

Our results also indicate that small RNAs are subject to different degradation pathways, depending on growth (Fig. 6). In the stationary phase, PNPase is shown to be the main enzyme in the degradation of small RNAs (Andrade and Arraiano 2008; this work). On the other hand, it has been proposed that in exponential phase, PNPase can actually protect small RNAs from rapid degradation by other ribonucleases, namely, from RNase E activity (De Lay and Gottesman 2011). The growth-phase regulation of sRNA turnover pathways may help to explain why RNase E was shown to affect sRNA decay in previous studies in which the exponential phase of growth was analyzed (Massé et al. 2003), whereas it is not found to be the predominant degradative enzyme in the stationary phase (this work). PNPase responds to environmental stimuli and has been suggested to be responsible for the addition of heteropolymeric tails to the 3′-end of RNAs in the stationary phase of growth (Cao and Sarkar 1997; Mohanty and Kushner 2000). PNPase could then use those tails to initiate RNA degradation. Accordingly, the growth-phase regulation of PNPase activities may thus help in explaining the growth-phase regulation of small RNAs driven by PNPase.

Variations in the levels of Hfq can most probably influence the degradation pathways of the small RNA. Interestingly, Hfq was reported to vary along the growth, and decreased levels of this protein were found in the entry to stationary phase (Ali Azam et al. 1999). Not only changes in the Hfq expression level, but also variations in the pool of free Hfq can result in low amounts of this protein and, consequently, affect the sRNA-based regulatory pathways. Hfq binds the RNA molecules very tightly and this can result in the sequestration of Hfq. A model in which an increasing concentration of a competitor RNA promotes the dissociation of the Hfq–RNA complexes has recently been proposed to explain how it is possible to cycle the Hfq pool within the cell (Fender et al. 2010). In agreement, it was shown that induction of a sRNA without the concomitant overexpression of its target mRNA (or vice versa) can sequester Hfq and abolish the function of unrelated sRNAs (Hussein and Lim 2011). Hence, Hfq depletion is likely to occur if transcription of sRNA and its target mRNAs is not coordinated. The rapid degradation of sRNA in the absence of interaction with Hfq may thus recycle any small RNAs that are produced in excess over Hfq. This reinforces the importance of studying the degradation of small RNAs when they are not associated with Hfq. Most of our work was performed in stationary-phase cells deleted for Hfq. However, we have shown that PNPase-mediated degradation of small RNAs is also an active regulatory pathway in cells expressing Hfq. This fact may reflect the action of PNPase against small RNAs that do not have their 3′-ends protected by Hfq. Our results are in agreement with in vitro data showing that Hfq can protect an mRNA from the exonucleolytic activity of PNPase (Folichon et al. 2003).

A similar phylogenetic distribution may reflect functionally linked proteins (Pellegrini et al. 1999). A large number of bacteria encode both Hfq and PNPase in their genomes (Zuo and Deutscher 2001; Sun et al. 2002), while the presence of *E. coli* RNase E homologs is far more restricted (Condon and Putzer 2002; Danchin 2009). Interestingly, eukaryotes lack an

TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype	Reference
MC4100*hfq*	*hfq*	Soshy Altuvia
MG1693	*thyA715*	Arraiano et al. 1988
HM104	*thyA715 rnr*	Andrade et al. 2006
SK5665	*thyA715 rne-1*	Arraiano et al. 1988
SK5671	*thyA715 rne-1 pnp7*	Arraiano et al. 1988
SK5691	*thyA715 pnp7*	Arraiano et al. 1988
SK7988	*thyA715* Δ*pcnB*	O'Hara et al. 1995
SK7622	*thyA715* Δ*rnc38*	Babitzke et al. 1993
CMA201	*thyA715* Δ*rnb*	Andrade et al. 2006
CMA413	*thyA715* Δ*micA*	Andrade and Arraiano 2008
CMA428	MG1693 *hfq*	This study
CMA429	MG1693 *hfq rnr*	This study
CMA430	MG1693 *hfq* Δ*rnb*	This study
CMA431	MG1693 *hfq pnp7*	This study
CMA436	MG1693 *rne*-1 *hfq*	This study
CMA441	MG1693 *hfq* Δ*rnc38*	This study
CMA448	MG1693 *hfq rnc105*	This study
CMA449	MG1693 *hfq* Δ*pcnB*	This study
CMA450	MG1693 *hfq pcnB80*	This study
CMA513	MG1693 *hfq* + pHFQ	This study

RNase E, but possess functional homologs of both PNPase and Hfq. The eukaryotic exosome adopts an PNPase-like conformation and is implicated in the processing and degradation of several RNAs, namely, the small nucleolar RNAs (snoRNAs) and the small nuclear RNAs (snRNAs) (Houseley et al. 2006). The exosome activity is suggested to be modulated by the Lsm1–7 complex (whose subunits are homologous to bacterial Hfq), although this interplay is still unclear (Wilusz and Wilusz 2008). The fact that Hfq and PNPase are more widespread than RNase E supports the interesting hypothesis that Hfq protection of sRNA against degradation by PNPase is far more common than was previously envisioned.

MATERIALS AND METHODS

Growth conditions, strains, and plasmids

Bacteria were grown at 37°C unless stated otherwise, with shaking at 180 rpm in Luria-Bertani (LB) medium supplemented with thymine (50 μg mL^{-1}). SOC medium was used to recover cells after heat shock in plasmid transformation steps. When required, antibiotics were present at the following concentrations: chloramphenicol, 50 μg mL^{-1}, kanamycin, 50 μg mL^{-1}; tetracycline, 20 μg mL^{-1}; ampicillin, 100 μg mL^{-1}. The *E. coli* strains used in this work are listed in Table 1. Strain MC4100 *hfq*::cat (kindly provided by S. Altuvia) was used as donor to move the mutant *hfq* allele into MG1693 (wild type) and its derivative isogenic strains. Introduction of mutant alleles to different genetic backgrounds was done by P1 transduction, and positive colonies were checked by PCR. A DNA sequence of Hfq was PCR-amplified with primers *hfq*–EcoRI (5′-GTGACGAAGaATTcCAGGTTGTTG-3′) and *hfq*–HindIII (5′-CGGTCAAACAAGCtTATAACCC-3′), and following enzyme restriction it was cloned into pBAD24, yielding the overexpression pHFQ plasmid. Hfq expression is obtained even without addition of the arabinose inducer, as the cloned DNA retains *hfq*'s own promoters. For plasmid pMicA, primers MicA–PstI (5′-TTTTCGCCACCCGAACTGCAGGC-3′) and MicA–HindIII (5′-GGCTGGAAAAACAaGCtTGACAGAAAAGAAAAAGG-3′) were used to amplify the *micA* gene. Following enzyme restriction, the insert was ligated into pWSK29 in sites PstI and HindIII. DNA polymerases and restriction enzymes were obtained from Fermentas, and T4 DNA Ligase from Roche. All primers were obtained from StabVida (Portugal).

RNA extraction and Northern blot analysis

Overnight cultures from isolated colonies were diluted in fresh medium to an initial $OD_{600} \sim 0.03$ and grown to exponential ($OD_{600} \sim 0.3$) or stationary phase ($OD_{600} \sim 5.5$ to wild-type or $OD_{600} \sim 2.3$ to *hfq* mutants). The growth curves for the wild-type strain and the *hfq* mutant are provided in Supplemental Figure S2. For decay experiments, blocking of transcription was obtained by adding rifampicin to a final concentration of 500 μg mL^{-1}. Culture samples were withdrawn at defined timepoints and mixed with an equal volume of RNA stop buffer (10 mM Tris at pH 7.2, 5 mM $MgCl_2$, 25 mM NaN_3, and 500 μg mL^{-1} chloramphenicol). RNA was isolated following cell lysis and phenol:chloroform extraction. After a precipitation step in ethanol and 300 mM sodium acetate, RNA was resuspended in MilliQ-water. The integrity of RNA samples was evaluated by agarose gel electrophoresis. When necessary, DNase RQ (Promega) treatment following a new phenol:chloroform step was used to remove contaminant DNA. Next, 10–40 μg of total RNA was used to analyze small RNA expression on 6%–12% polyacrylamide/7 M urea gels in TBE 1x. RNA was transferred onto Hybond-N+ membrane (Amersham Biosciences) using TAE 1x as transfer buffer. RNAs were UV cross-linked to the membrane with a UVC 500 apparatus (Amersham Biosciences). DNA templates carrying a T7 promoter sequence for in vitro transcription were generated by PCR using genomic DNA of MG1693 and primers listed in Table 2. GlmY was detected by 5′-end labeling of an antisense primer (Table 2). Radiolabeled probes

TABLE 2. Oligonucleotides used in radiolabeling reactions

Probe	Sequence (5′–3′)
MicA-T7	TAATACGACTCACTATAGGAAGGCCACTCGTGAGTGGCCAA
MicA-F	GAAAGACGCGCATTTGTTATC
SgrS-T7	TAATACGACTCACTATAGGCCAGCAGGTATAATCTGC
SgrS-F	GATGAAGCAAGGGGGTGCCC
RyhB-T7	TAATACGACTCACTATAGGAAAAGCCAGCACCCGGCTGGCTAA
RyhB-F	GCGATCAGGAAGACCCTC
5S-RNA-T7	TAATACGACTCACTATAGGATGCCTGGCAGTTCCCTACTCTCGC
5S-RNA-F	AAACAGAATTTGCCTGGCGGCAGTAG
GlmY	GCACGTCCCGAAGGGGCTGACATAAG

The T7 promoter sequence in the oligos is underlined.

were purified on G25 Microspin columns (GE Healthcare). Hybridizations were carried out overnight at 42°C–68°C with the PerfectHyb Plus Hybridization Buffer (Sigma). RNA Decade markers (Ambion) or the 10-bp Step Ladder (Promega) were used for detection of small RNAs up to 150 nt; for longer transcripts, the 100–1000 bp Ladder (Biotools) was used. T7 RNA polymerase and T4 polynucleotide kinase were from Promega. All radiochemicals were purchased from Perkin-Elmer.

RNA half-life determination

Northern blot signals were visualized on PhosphorImager STORM 860, and bands intensities were quantified using the IMAGEQUANT software (Molecular Dynamics). Half-lives of RNA were determined by linear regression using the logarithm of the percentage of RNA remaining versus time, considering the amount of RNA at 0 min as 100%. A minimum of two independent RNA extractions from each strain were tested and half-lives correspond to average of at least three experiments.

Primer extension analysis

The MicA RNA was analyzed by primer extension analysis using the MicA-PE primer (5′-CGTGAGTGGCCAAAATTTCATCT CTG-3′). A total of 10 μg of each RNA sample was incubated with 1 pmol of 5′-end [γ-32P]ATP-labeled primer. Sample denaturation was done for 5 min at 80°C, immediately followed by the annealing step (30 min at 65°C and 30 min at 48°C). cDNA synthesis was obtained using 200 units of SuperScript III Reverse Transcriptase, following the manufacturer's instructions (Invitrogen). Incubation proceeded for 60 min at 55°C and was terminated by heat inactivation of the samples for 15 min at 70°C. The cDNA products were then ethanol precipitated with the addition of glycogen for 15 min in a −80°C freezer. The cDNA pellet was dissolved in 2 μL of 0.1 M NaOH/1 mM EDTA and 4 μL of formamide loading buffer. Prior to loading, samples were denatured for 5 min at 80°C, and then fractionated on 6% polyacrylamide/7 M urea gels. Plasmid pMicA was used in sequencing reactions with primer MicA-PE following the instructions of the Sequenase Version 2.0 DNA Sequencing Kit (USB). The gel was exposed on a PhosphorImager screen and the signal was detected on a PhosphorImager STORM 860.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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REFERENCES

Afonyushkin T, Vecerek B, Moll I, Blasi U, Kaberdin VR. 2005. Both RNase E and RNase III control the stability of *sodB* mRNA upon translational inhibition by the small regulatory RNA RyhB. *Nucleic Acids Res* **33:** 1678–1689.

Aiba H. 2007. Mechanism of RNA silencing by Hfq-binding small RNAs. *Curr Opin Microbiol* **10:** 134–139.

Ali Azam T, Iwata A, Nishimura A, Ueda S, Ishihama A. 1999. Growth phase-dependent variation in protein composition of the *Escherichia coli* nucleoid. *J Bacteriol* **181:** 6361–6370.

Andrade JM, Arraiano CM. 2008. PNPase is a key player in the regulation of small RNAs that control the expression of outer membrane proteins. *RNA* **14:** 543–551.

Andrade JM, Cairrão F, Arraiano CM. 2006. RNase R affects gene expression in stationary-phase: regulation of *ompA*. *Mol Microbiol* **60:** 219–228.

Andrade JM, Hajnsdorf E, Régnier P, Arraiano CM. 2009a. The poly(A)-dependent degradation pathway of *rpsO* mRNA is primarily mediated by RNase R. *RNA* **15:** 316–326.

Andrade JM, Pobre V, Silva IJ, Domingues S, Arraiano CM. 2009b. The role of 3′-5′ exoribonucleases in RNA degradation. *Prog Mol Biol Transl Sci* **85:** 187–229.

Arluison V, Hohng S, Roy R, Pellegrini O, Régnier P, Ha T. 2007. Spectroscopic observation of RNA chaperone activities of Hfq in post-transcriptional regulation by a small non-coding RNA. *Nucleic Acids Res* **35:** 999–1006.

Arraiano CM, Yancey SD, Kushner SR. 1988. Stabilization of discrete mRNA breakdown products in *ams pnp rnb* multiple mutants of *Escherichia coli* K-12. *J Bacteriol* **170:** 4625–4633.

Arraiano CM, Andrade JM, Domingues S, Guinote IB, Malecki M, Matos RG, Moreira RN, Pobre V, Reis FP, Saramago M, et al. 2010. The critical role of RNA processing and degradation in the control of gene expression. *FEMS Microbiol Rev* **34:** 883–923.

Babitzke P, Granger L, Olszewski J, Kushner SR. 1993. Analysis of mRNA decay and rRNA processing in *Escherichia coli* multiple mutants carrying a deletion in RNase III. *J Bacteriol* **175:** 229–239.

Basineni SR, Madhugiri R, Kolmsee T, Hengge R, Klug G. 2009. The influence of Hfq and ribonucleases on the stability of the small non-coding RNA OxyS and its target *rpoS* in *E. coli* is growth phase dependent. *RNA Biol* **6:** 584–594.

Bossi L, Figueroa-Bossi N. 2007. A small RNA downregulates LamB maltoporin in *Salmonella*. *Mol Microbiol* **65:** 799–810.

Bouvier M, Sharma CM, Mika F, Nierhaus KH, Vogel J. 2008. Small RNA binding to 5′ mRNA coding region inhibits translational initiation. *Mol Cell* **32:** 827–837.

Brennan RG, Link TM. 2007. Hfq structure, function and ligand binding. *Curr Opin Microbiol* **10:** 125–133.

Cao GJ, Sarkar N. 1997. Stationary-phase-specific mRNAs in *Escherichia coli* are polyadenylated. *Biochem Biophys Res Commun* **239:** 46–50.

Chao Y, Vogel J. 2010. The role of Hfq in bacterial pathogens. *Curr Opin Microbiol* **13:** 24–33.

Condon C, Putzer H. 2002. The phylogenetic distribution of bacterial ribonucleases. *Nucleic Acids Res* **30:** 5339–5346.

Coornaert A, Lu A, Mandin P, Springer M, Gottesman S, Guillier M. 2010. MicA sRNA links the PhoP regulon to cell envelope stress. *Mol Microbiol* **76:** 467–479.

Danchin A. 2009. A phylogenetic view of bacterial ribonucleases. *Prog Mol Biol Transl Sci* **85:** 1–41.

De Lay N, Gottesman S. 2011. Role of polynucleotide phosphorylase in sRNA function in *Escherichia coli*. *RNA* **17:** 1172–1189.

Del Favero M, Mazzantini E, Briani F, Zangrossi S, Tortora P, Dehò G. 2008. Regulation of *Escherichia coli* polynucleotide phosphorylase by ATP. *J Biol Chem* **283:** 27355–27359.

Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, Eckert MR, Vogel J, Charpentier E. 2011. CRISPR RNA maturation by *trans*-encoded small RNA and host factor RNase III. *Nature* **471:** 602–607.

Fender A, Elf J, Hampel K, Zimmermann B, Wagner EG. 2010. RNAs actively cycle on the Sm-like protein Hfq. *Genes Dev* **24:** 2621–2626.
Folichon M, Arluison V, Pellegrini O, Huntzinger E, Régnier P, Hajnsdorf E. 2003. The poly(A) binding protein Hfq protects RNA from RNase E and exoribonucleolytic degradation. *Nucleic Acids Res* **31:** 7302–7310.
Folichon M, Allemand F, Régnier P, Hajnsdorf E. 2005. Stimulation of poly(A) synthesis by *Escherichia coli* poly(A) polymerase I is correlated with Hfq binding to poly(A) tails. *FEBS J* **272:** 454–463.
Geissmann TA, Touati D. 2004. Hfq, a new chaperoning role: binding to messenger RNA determines access for small RNA regulator. *EMBO J* **23:** 396–405.
Grunberg-Manago M, Oritz PJ, Ochoa S. 1955. Enzymatic synthesis of nucleic acidlike polynucleotides. *Science* **122:** 907–910.
Hajnsdorf E, Régnier P. 2000. Host factor Hfq of *Escherichia coli* stimulates elongation of poly(A) tails by poly(A) polymerase I. *Proc Natl Acad Sci* **97:** 1501–1505.
Hajnsdorf E, Braun F, Haugel-Nielsen J, Régnier P. 1995. Polyadenylylation destabilizes the *rpsO* mRNA of *Escherichia coli*. *Proc Natl Acad Sci* **92:** 3973–3977.
Houseley J, LaCava J, Tollervey D. 2006. RNA-quality control by the exosome. *Nat Rev Mol Cell Biol* **7:** 529–539.
Hussein R, Lim HN. 2011. Disruption of small RNA signaling caused by competition for Hfq. *Proc Natl Acad Sci* **108:** 1110–1115.
Le Derout J, Folichon M, Briani F, Dehò G, Régnier P, Hajnsdorf E. 2003. Hfq affects the length and the frequency of short oligo(A) tails at the 3′ end of *Escherichia coli rpsO* mRNAs. *Nucleic Acids Res* **31:** 4017–4023.
Lease RA, Woodson SA. 2004. Cycling of the Sm-like protein Hfq on the DsrA small regulatory RNA. *J Mol Biol* **344:** 1211–1223.
Link TM, Valentin-Hansen P, Brennan RG. 2009. Structure of *Escherichia coli* Hfq bound to polyriboadenylate RNA. *Proc Natl Acad Sci* **106:** 19292–19297.
Marujo PE, Hajnsdorf E, Le Derout J, Andrade R, Arraiano CM, Régnier P. 2000. RNase II removes the oligo(A) tails that destabilize the *rpsO* mRNA of *Escherichia coli*. *RNA* **6:** 1185–1193.
Massé E, Escorcia FE, Gottesman S. 2003. Coupled degradation of a small regulatory RNA and its mRNA targets in *Escherichia coli*. *Genes Dev* **17:** 2374–2383.
Mikulecky PJ, Kaw MK, Brescia CC, Takach JC, Sledjeski DD, Feig AL. 2004. *Escherichia coli* Hfq has distinct interaction surfaces for DsrA, *rpoS* and poly(A) RNAs. *Nat Struct Mol Biol* **11:** 1206–1214.
Mohanty BK, Kushner SR. 2000. Polynucleotide phosphorylase functions both as a 3′ right-arrow 5′ exonuclease and a poly(A) polymerase in *Escherichia coli*. *Proc Natl Acad Sci* **97:** 11966–11971.
Mohanty BK, Maples VF, Kushner SR. 2004. The Sm-like protein Hfq regulates polyadenylation dependent mRNA decay in *Escherichia coli*. *Mol Microbiol* **54:** 905–920.
Moll I, Afonyushkin T, Vytvytska O, Kaberdin VR, Blasi U. 2003a. Coincident Hfq binding and RNase E cleavage sites on mRNA and small regulatory RNAs. *RNA* **9:** 1308–1314.
Moll I, Leitsch D, Steinhauser T, Blasi U. 2003b. RNA chaperone activity of the Sm-like Hfq protein. *EMBO Rep* **4:** 284–289.
Morita T, Maki K, Aiba H. 2005. RNase E-based ribonucleoprotein complexes: Mechanical basis of mRNA destabilization mediated by bacterial noncoding RNAs. *Genes Dev* **19:** 2176–2186.
Morita T, Mochizuki Y, Aiba H. 2006. Translational repression is sufficient for gene silencing by bacterial small noncoding RNAs in the absence of mRNA destruction. *Proc Natl Acad Sci* **103:** 4858–4863.
Nurmohamed S, Vincent HA, Titman CM, Chandran V, Pears MR, Du D, Griffin JL, Callaghan AJ, Luisi BF. 2011. Polynucleotide phosphorylase activity may be modulated by metabolites in *Escherichia coli*. *J Biol Chem* **286:** 14315–14323.
O'Hara EB, Chekanova JA, Ingle CA, Kushner ZR, Peters E, Kushner SR. 1995. Polyadenylylation helps regulate mRNA decay in *Escherichia coli*. *Proc Natl Acad Sci* **92:** 1807–1811.
Otaka H, Ishikawa H, Morita T, Alba H. 2011. PolyU tail of rho-independent terminator of bacterial small RNAs is essential for Hfq action. *Proc Natl Acad Sci* **108:** 13059–13064.
Pellegrini M, Marcotte EM, Thompson MJ, Eisenberg D, Yeates TO. 1999. Assigning protein functions by comparative genome analysis: protein phylogenetic profiles. *Proc Natl Acad Sci* **96:** 4285–4288.
Prévost K, Desnoyers G, Jacques JF, Lavoie F, Massé E. 2011. Small RNA-induced mRNA degradation achieved through both translation block and activated cleavage. *Genes Dev* **25:** 385–396.
Rasmussen AA, Eriksen M, Gilany K, Udesen C, Franch T, Petersen C, Valentin-Hansen P. 2005. Regulation of *ompA* mRNA stability: the role of a small regulatory RNA in growth phase-dependent control. *Mol Microbiol* **58:** 1421–1429.
Régnier P, Hajnsdorf E. 2009. Poly(A)-assisted RNA decay and modulators of RNA stability. *Prog Mol Biol Transl Sci* **85:** 137–185.
Reichenbach B, Maes A, Kalamorz F, Hajnsdorf E, Gorke B. 2008. The small RNA GlmY acts upstream of the sRNA GlmZ in the activation of *glmS* expression and is subject to regulation by polyadenylation in *Escherichia coli*. *Nucleic Acids Res* **36:** 2570–2580.
Sauer E, Weichenrieder O. 2011. Structural basis for RNA 3′-end recognition by Hfq. *Proc Natl Acad Sci* **108:** 13065–13070.
Schumacher MA, Pearson RF, Moller T, Valentin-Hansen P, Brennan RG. 2002. Structures of the pleiotropic translational regulator Hfq and an Hfq-RNA complex: a bacterial Sm-like protein. *EMBO J* **21:** 3546–3556.
Slomovic S, Portnoy V, Yehudai-Resheff S, Bronshtein E, Schuster G. 2008. Polynucleotide phosphorylase and the archaeal exosome as poly(A)-polymerases. *Biochim Biophys Acta* **1779:** 247–255.
Soper T, Mandin P, Majdalani N, Gottesman S, Woodson SA. 2010. Positive regulation by small RNAs and the role of Hfq. *Proc Natl Acad Sci* **107:** 9602–9607.
Sun X, Zhulin I, Wartell RM. 2002. Predicted structure and phyletic distribution of the RNA-binding protein Hfq. *Nucleic Acids Res* **30:** 3662–3671.
Suzuki K, Babitzke P, Kushner SR, Romeo T. 2006. Identification of a novel regulatory protein (CsrD) that targets the global regulatory RNAs CsrB and CsrC for degradation by RNase E. *Genes Dev* **20:** 2605–2617.
Tsui HC, Leung HC, Winkler ME. 1994. Characterization of broadly pleiotropic phenotypes caused by an *hfq* insertion mutation in *Escherichia coli* K-12. *Mol Microbiol* **13:** 35–49.
Tuckerman JR, Gonzalez G, Gilles-Gonzalez MA. 2011. Cyclic di-GMP activation of polynucleotide phosphorylase signal-dependent RNA processing. *J Mol Biol* **407:** 633–639.
Udekwu KI, Darfeuille F, Vogel J, Reimegard J, Holmqvist E, Wagner EG. 2005. Hfq-dependent regulation of OmpA synthesis is mediated by an antisense RNA. *Genes Dev* **19:** 2355–2366.
Updegrove TB, Correia JJ, Chen Y, Terry C, Wartell RM. 2011. The stoichiometry of the *Escherichia coli* Hfq protein bound to RNA. *RNA* **17:** 489–500.
Urban JH, Vogel J. 2008. Two seemingly homologous noncoding RNAs act hierarchically to activate *glmS* mRNA translation. *PLoS Biol* **6:** e64. doi: 10.1371/journal.pbio.0060064.
Valentin-Hansen P, Eriksen M, Udesen C. 2004. The bacterial Sm-like protein Hfq: a key player in RNA transactions. *Mol Microbiol* **51:** 1525–1533.
Vecerek B, Moll I, Blasi U. 2007. Control of Fur synthesis by the non-coding RNA RyhB and iron-responsive decoding. *EMBO J* **26:** 965–975.
Viegas SC, Pfeiffer V, Sittka A, Silva IJ, Vogel J, Arraiano CM. 2007. Characterization of the role of ribonucleases in *Salmonella* small RNA decay. *Nucleic Acids Res* **35:** 7651–7664.
Viegas SC, Silva IJ, Saramago M, Domingues S, Arraiano CM. 2011. Regulation of the small regulatory RNA MicA by ribonuclease III: a target-dependent pathway. *Nucleic Acids Res* **39:** 2918–2930.
Waters LS, Storz G. 2009. Regulatory RNAs in bacteria. *Cell* **136:** 615–628.
Wilusz CJ, Wilusz J. 2005. Eukaryotic Lsm proteins: lessons from bacteria. *Nat Struct Mol Biol* **12:** 1031–1036.
Wilusz CJ, Wilusz J. 2008. New ways to meet your (3′) end oligouridylation as a step on the path to destruction. *Genes Dev* **22:** 1–7.
Zuo Y, Deutscher MP. 2001. Exoribonuclease superfamilies: structural analysis and phylogenetic distribution. *Nucleic Acids Res* **29:** 1017–1026.

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