UNIVERSIDADE NOVA DE LISBOA

THE INFLUENCE OF EXORIBONUCLEASES IN THE REGULATION OF STRESS RELATED SMALL RNAs

AFONSO MARTINS BRAVO

DISSERTATION PRESENTED TO OBTAIN THE MASTER DEGREE IN MEDICAL MICROBIOLOGY

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Abstract

Escherichia coli must be able to withstand anaerobic conditions and pH as low as 2 for several hours when it colonises a human. As a consequence of this selective pressure, and in order to be able to survive in such rapid changing environments, complex networks of genetic regulation have emerged.

Post-transcriptional regulatory mechanisms are crucial in bacterial adaptation. Based on the concerted actions of both the ribonucleases and the sRNAs, these regulatory networks allow the cell to quickly and efficiently change their genetic programs.

In this work, we determined how the *E. coli* exoribonucleases (RNase II, RNase R and PNPase) and the Hfq RNA chaperone influence the cellular anaerobic and acidic response pathways. We discovered that RNase II appears to be essential in the general acid shock response mechanism, with RNase II deficient cells indefinitely stopping their growth after acid shock. Moreover, we report that both RNase II and Hfq are required for the expression of the acid related sRNA ArrS. Surprisingly, we also discovered that in the RNase II and, to a lesser extent, in the RNase R mutant strains, the anaerobically induced sRNA FnrS is highly expressed in aerobic conditions. Until now, the expression of this sRNA had only been observed in an anaerobic environment.

In this work, we demonstrate that RNase II is more versatile than previous thought, being a crucial post-transcriptional regulator in the adaptation to low pH environments. Furthermore, we also demonstrated that RNase II and RNase R are involved in the regulation of the respiratory pathways. Curiously, we also show that PNPase, considered the main exoribonuclease involved in sRNA degradation, does not influence either the ArrS or FnrS sRNAs. With this work, we improved our understanding on the regulatory mechanism that contribute to stress adaptation in *E. coli*.

Resumo

De modo a poder colonizar um ser humano, a bactéria *Escherichia coli* tem de ser capaz de resistir tanto a condições de anaerobiose como a um pH de 2. Como resultado directo desta pressão selectiva, e de modo a permitir uma adaptação eficiente em ambientes instáveis, várias redes de regulação genética emergiram. Entre estas, a regulação pós-transcricional é crucial. Baseada nas acções concertadas de tanto as ribonucleases como dos sRNAs esta rede de regulação permite que a célula altere o seu programa genético de forma rápida e eficiente.

Neste trabalho determinámos como é que as exoribonucleases de *E. coli* (RNase II, RNase R e PNPase) e a proteína Hfq influenciam as vias de resposta a ambientes acídicos e anaeróbios. De facto, descobrirmos que a RNase II parece ser essencial no mecanismo geral de resposta ao choque acídico, com as células deficientes em RNase II a parar o seu crescimento após este. Determinámos ainda que tanto a RNase II como a Hfq são essênciais à expressão do sRNA ArrS, envolvido na adaptação a ambientes acídicos. Surpreendentemente, descobrimos também que tanto no mutante da RNase II como no da RNase R, o sRNA FnrS é expresso em condições aeróbias. Até hoje, este sRNA apenas tinha sido detectado em ambiente anaeróbio.

Neste trabalho mostramos que a RNase II é mais versátil do que previamente pensado, assumindo um lugar de destaque na regulação pós-transcricional da adaptação a ambientes ácidos. Demonstrámos também que tanto a RNase II como a RNase R estão envolvidas na regulação das vias respiratórias celulares. Curiosamente, revelamos ainda que a PNPase, considerada a principal exoribonuclease na degradação de sRNAs, não parece afectar o ArrS e FnrS. Com este trabalho, contribuímos para a compreensão dos mecanismos regulatórios responsaveis pela adaptação a condições de *stress* em *E. coli*.

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1. Introduction

In an existence that spans more than 3.5 billion years on planet Earth [1], bacteria have endured drastic environmental changes and survived several extinction events. As a consequence of this selective pressure, bacteria have evolved into versatile organisms, capable of thriving in almost every known niche [2]. For example, by the means of infecting and proliferating in a host, pathogenic bacteria are able to survive due to the abundant source of nutrients [3]. Nonetheless, in order to evade the hostile background created by the host's immune response, an invading bacteria must possess machinery capable of swiftly adapting to rapid environmental changes. Overall, the pressure to survive and quickly adapt to stress conditions drives forward the selection for complex regulatory mechanisms, such as RNA based regulators [3].

1.1. From RNA to DNA: The Beginning

The RNA World hypothesis is a conceptual idea about the origin of life. In this hypothesis, RNA was the first "life form" to appear, capable of both processing information and catalysing chemical reactions [4]. Evidence for the latter was established with the discovery of ribozymes, such as self-splicing introns [5], the ribonuclease P RNA [6] or even the ubiquitous ribosomal RNA [7]. These RNA catalysts could be RNA "fossils" from a simpler RNA based metabolism, the first version of the far more complex DNA/RNA/protein system. Furthermore, the realisation that RNA can evolve in an acellular environment in response to selective pressure [8] and the discovery of both RNA riboswitches [9] and a self-catalytic system based on RNA ligases [10] have all contributed to the plausibility of the RNA World hypothesis.

Considering this hypothesis, DNA would have been originated prior to the existence of the first cellular common ancestor in an already established RNA/protein world [11]. It is speculated that uracilated DNA would first have appeared in viruses, where it was protected from ribonucleases (RNases) and RNA based proteins. Ultimately, the incorporation of thymine instead of uracil and the increased stability and repair efficiency achieved by the double helix conformation would have given DNA the advantage needed to overcome RNA as the holder of the genetic material. DNA had thus

opened the door for the stable formation of larger genomes and, consequently, to the emergence of cells [11].

1.2. From DNA to RNA and Protein: Transcription and Translation

Transcription and translation are the fundamental processes associated with the phenomenon of genetic expression, the process by which the information contained in a DNA sequence - a gene - is used to modulate the synthesis of a functional product [12, 13].

Gene expression initiates with gene transcription, characterised as the mechanism by which an RNA polymerase transcribes the DNA sequence of a gene into an RNA molecule. Ultimately, this phenomenon yields an RNA sequence complementary to that of the strand of DNA used as a template. The swift release of the nascent RNA from the coding DNA strand results in a highly efficient process in which several RNA copies can be transcribed simultaneously from the same gene in a short amount of time, effectively increasing the cell's proficiency when a change in genetic program is required. The newly synthesised RNAs often fold into specific tri-dimensional structures. This is due to both the single stranded nature of RNA and the existence of complementary base pairing within the same RNA molecule [12, 13].

Cells produce several functionally distinct types of RNA. For example, RNAs transcribed from genes that encode amino acid sequences, and that ultimately direct the synthesis of a protein, are denominated as messenger RNAs (mRNAs). In prokaryotic cells the majority of the genes are of this type. However, there are also genes whose final product is the RNA itself, and not the protein [14]. These RNAs that do not encode proteins (non-coding) often operate as enzymatic, structural or regulatory components in numerous cellular processes. Among the non-coding RNAs we find the ribosomal RNAs (rRNAs), which constitute approximately 80% of the cellular RNA pool. rRNAs are part of the ribosome, a complex ribozyme responsible for protein synthesis [12, 13]. In prokaryotes, the ribosome is composed of the 5S, 16S and 23S rRNAs, amid several other proteins. Of special importance is the 23S rRNA, which accounts for the ribosome's peptidyl transferase activity [7].

The process of translation represents the conversion of the information found in the nucleotide sequence of an mRNA into the amino acid sequence that, ultimately, constitutes a protein. Translation begins with a ribosome binding to the Shine-Dalgarno sequence of an mRNA. The nucleotide information encoded in the mRNA is then read in the 5´ to 3´ direction in consecutive groups of three nucleotides, the codons. Depending on the genetic code of the organism in question, each codon is either associated with a specific amino acid or a stop in translation. The codons in the mRNA are recognised by the transfer RNAs (tRNAs), another type of non-coding RNA. tRNAs act as adaptor molecules, recognizing the codon and associating it with the correspondent amino acid. When a correct match is obtained, the amino acid is added to the nascent protein. Ribosomes are extremely efficient, with bacterial ribosomes adding about 20 amino acids per second to a growing polypeptide chain [12, 13].

The end of the mRNA protein coding region is signalled by the presence of a stop codon, which isn't recognised by a tRNA. The binding of a release factor to the empty site created by the lack of a tRNA signals the ribosome to stop translation, thus freeing the polypeptide chain and promoting the dissociation of the ribosome/mRNA pair. Lastly, the polypeptide undergoes folding and becomes an integral part of the protein pool [12, 13].

1.3. Quality Control

Despite the existence of several proofreading mechanisms, the process of transcription and translation, like all biological processes, is not errorproof. Incorrectly processed or transcribed RNAs may, inadvertently, commence translation or, in the case of non-coding RNAs, function improperly. The danger of producing both aberrant proteins and RNAs has led to the emergence of quality control mechanisms. One such mechanism is the *trans*-translation process, which operates when a ribosome stalls due to an encounter with a broken/incompletely synthesised RNA. In this case, the lack of a STOP codon in the mRNA blocks translation termination and, consequently, impedes

ribosome/mRNA dissociation. This particular situation is resolved by the transfer-messenger RNA (tmRNA), which acts both as a tRNA and an mRNA. In order to be functional, tmRNA requires the presence of the SmpB protein, which mimics the anticodon structure naturally found in tRNAs and that is lacking in the tmRNA. This process of *trans*-translational is initiated by the formation of the tmRNA-SmpB complex, which then binds to the ribosome in the tRNA binding site and ejects the defective mRNA. The tmRNA is then translated as a normal mRNA would be, tagging the nascent defective protein with an amino acid tag that directs the polypeptide chain to degradation. The existence of a STOP codon in the tmRNA promotes ribosome dissociation, allowing the complex to be recycled [12, 13]. Finally, the defective mRNA is degraded [15].

1.4. RNA Degradation

The RNA degradation pathway is a major component of the cellular metabolism, being required for ribonucleotide turnover and RNA quality control mechanisms. Moreover, by modulating the kinetic rate of RNA decay, RNA degradation is able to influence the intracellular level of a RNA species, thereby facilitating the continuous adjustment of the RNA population to the needs of the cell [16, 17].

Several factors dictate RNA stability in prokaryotes. Among these, the best studied are the secondary structures found in the 5´ and 3´ UTRs [18]. Considerable differences in stability control have been observed in both these regions, with specific structures conferring stabilizing properties and others promoting instability.

The ribonucleases are the main effectors of the RNA degradation pathway. Due to the existence of functional overlaps between these enzymes, it is now clear that several RNases can simultaneously participate, or even substitute each other, in the degradation of a given RNA molecule [16, 19]. Indeed, some RNases are known to form RNA-degrading multiprotein complexes in order to degrade extensively structured RNAs. These complexes are believed to expedite and facilitate RNA turnover by promoting the cooperation of different enzymes in RNA degradation [17, 20, 21].

In *E. coli*, mRNA degradation usually begins with an endoribonucleolytic cleavage, followed by 3´ to 5´ exoribonucleolytic decay (Fig. 1). The need for this specific

order is explained by the frequent presence of secondary structures in the 3´end of the transcript [22]. These structural conformations usually impair an exoribonucleolytic attack, thereby requiring an initial endoribonucleolytic cleavage to generate unprotected 3´ extremities. Alternatively, or simultaneously, the transcript can undergo polyadenylation. In this case, the presence of an unstructured poly(A) tail facilitates exoribonuclease binding and promotes RNA destruction through the exoribonucleolytic pathway [16, 19, 23, 24]. Typically, multiple rounds of polyadenylation and endoribonucleolytic/exoribonucleolytic cleavages are required until an mRNA is completely fragmented (Fig. 1).

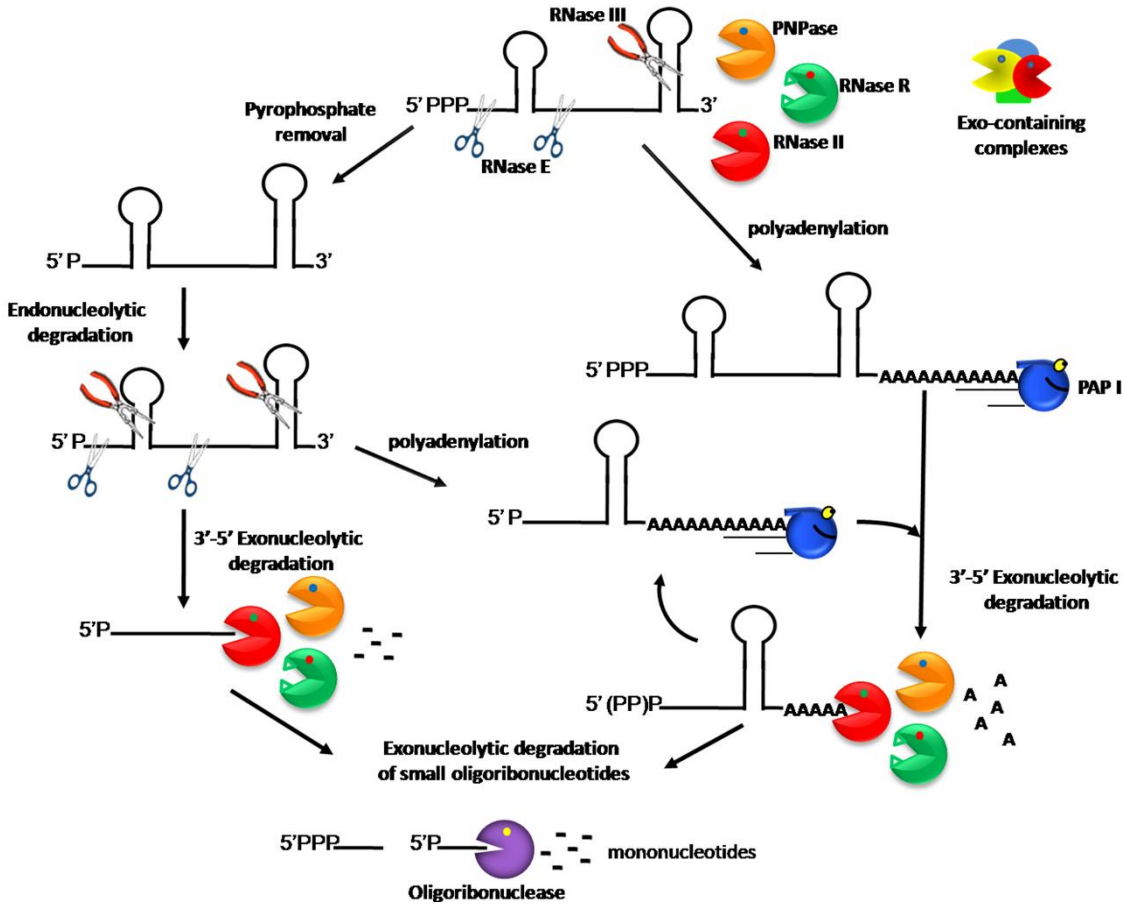

Fig. 1 - Model of RNA degradation pathways in *E. coli*.

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. RNase III is another enzyme responsible for the initial endoribonucleolytic cleavage of structured RNAs. However, unlike RNase E, RNase III cleaves dsRNAs. After endoribonucleolytic cleavages, the linear transcripts are rapidly degraded by the 3´ to 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. Image originally published in "The critical role of RNA processing and degradation in the control of gene expression" [21].

1.5. Ribonucleases

Ribonucleases (RNases) are RNA specific hydrolases, or phosphorylases, that are capable of catalysing the cleavage of the RNA phosphodiester bonds in a reaction that, ultimately, yields monoribonucleotides. Depending on the manner in which they cleave RNA, RNases can be subdivided into two classes: exoribonucleases, which cleave the RNA from its 5´ or 3´ extremity, and endoribonucleases, witch cleave the RNA's internal phosphodiester bonds [21, 25] (Table 1).

Besides intervening in RNA degradation, RNases are also the main effectors of the post-transcriptional regulation network. RNases thus directly intervene in the degradation, processing, maturation and quality control of all RNA molecules. For example, the cellular concentration of an RNA molecule arises from the dynamic balance established between the transcription frequency and the RNA decay rate. By controlling the latter, RNases display a fundamental role in gene expression, quickly adjusting the cellular RNA levels.

RNases also participate in RNA maturation. For instance, a few RNAs, such as tmRNA, tRNA and rRNA, require ribonuclease mediated processing from a longer transcript prior to becoming active [19, 26]. Unexpectedly, RNase action can also inhibit RNA degradation. For example, the enzyme ribonuclease II (RNase II) stabilizes the *rpsO* mRNA by removing its poly(A) tail, which blocks degradation by other exoribonucleases [27].

At the small regulatory RNA (sRNA) level, the global dynamics of RNA processing is still not well understood. Some sRNAs, such as the GadY, apparently occur as multiple size active species [28], while others seem to require processing to a single final active form [26]. Taking into consideration the little that is known about sRNA processing and its implications in bacterial environment adaptation, the study of ribonuclease activity becomes an aspect of utmost importance.

1.5.1. Exoribonucleases

In *E. coli* there are eight characterised exoribonucleases, and they all degrade RNA in the 3´ to 5´ direction [25]. Among these, the three main exoribonucleases involved in RNA decay are RNase II, polynucleotide phosphorylase (PNPase) and ribonuclease R (RNase R) (Table 1).

1.5.1.1. Ribonuclease II

E. coli RNase II, encoded by the *rnb* gene, is a processive 3´- 5´ ssRNA hydrolase [29]. RNase II is sensitive to structured RNAs, easily stalling 7 nucleotides (nt) before a double stranded region.

Regardless of being sequence independent, RNase II displays increased reactivity in the presence of poly(A) tails [30], a RNA degradation marker in bacteria. Paradoxically, this augmented degradation of poly(A) tails by RNase II can hinder the activity of other exoribonucleases, thereby partly protecting some RNAs from the decay process [27, 31]. In fact, it is estimated that around 31% of all cellular mRNAs are protected, to some degree, by RNase II, which is considered to be the major cellular hydrolytic exoribonuclease [21, 32].

Among the antisense RNAs, RNase II has only been found to protect the RNA-OUT from degradation [31]. This RNA regulates Tn10/IS10 transposition, a transposon involved in tetracycline resistance [33]. Furthermore, it has been recently described in a eukaryote, *Plasmodium falciparum*, an enzyme with an RNase II domain, PfRNase II, capable of regulating several non-coding RNAs. This report not only attributes to the enzyme a role in the post transcriptional regulation of several non-coding RNAs, but also suggests a liaison between PfRNase II and virulence related genes [34]. Moreover, both in *Salmonella thyphimurium* and *E. coli*, RNase II was demonstrated to be involved in biofilm formation, albeit with opposite trends [35, 36] .

1.5.1.2. Ribonuclease R

E. coli RNase R, encoded by the *rnr* gene, belongs to the RNase II family and, as such, is a processive 3´- 5´ hydrolase [37]. Nonetheless, unlike, RNase II, RNase R is capable of degrading highly structured RNAs, required that there is a 3´ single-stranded RNA (ssRNA) overhang, such as a poly(A) tail [38, 39]. RNase R thus displays a fundamental role in the degradation of several structured types of RNA, such as tRNAs, rRNAs, sRNAs and mRNAs, especially when stable stem loops are present [15, 40, 41]. This ribonuclease also plays an important role in RNA quality control mechanisms [17, 21]. For example, both RNase R and PNPase intervene in the degradation of aberrant 16S and 23S rRNAs, thereby affecting ribosome maturation and assembly [17, 42]. RNase R further participates in defective tRNA degradation [15, 21]. Regarding the process of *trans*-translation, RNase R was associated with both the maturation of the regulatory RNA, tmRNA, and with the degradation of the defective mRNA ejected by the tmRNA in the *trans*-translation process [15, 43].

Regarding stress adaptation responses, RNase R is a general stress induced enzyme, with its levels being increased in stationary phase and in both cold and heat shock [17, 41, 44]. Indeed, in cold conditions, the RNase R levels increase several fold due to the stabilisation of both the *rnr* operon and of the protein itself. Interestingly, this latter is dependent on the acetylation of a lysine residue in the RNase R protein. In both cold shock and stationary phase cells, the lysine residue is not acetylated and the free form of the RNase R is stabilised. In fact, when acetylated, both tmRNA and SmpB bind to the enzyme, associating it to the ribosome where it participates in *trans*-translation [45, 46]. Ultimately, the modulation of RNase R activity allows the cell to efficiently respond to both the increase in RNA secondary structures that normally arise in lower temperatures and to the higher requirement for RNA quality control mechanisms in the growing phase [44, 47].

RNase R has been implicated in the establishment of virulence in both *Shigella flexneri* and enteroinvasive *E. coli* [48]. RNase R was also shown to be important in the adhesion and invasion of *Campylobacter jejuni* into eukaryotic cells [49]. Moreover, in *Legionella pneumophila* and *Mycoplasma genitalium*, RNase R is essential for both growth and competence, being the only hydrolytic exoribonuclease present [50].

1.5.1.3. Polynucleotide Phosphorylase

E. coli PNPase is an exoribonuclease encoded by the *pnp* gene. PNPase is characterised for displaying processive, sequence independent, phosphorolytic activity, being capable of both synthetizing and degrading ssRNA, depending on the conditions. For instance, at a low nucleotide diphosphate (ndp)/high inorganic phosphate (iP) concentration, PNPase catalyses the 3´- 5´ phosphorolytic degradation of RNA in a reaction that increases the cellular ndp pool. Interestingly, at a high ndp/low iP concentration, the enzyme yields iP by catalysing the polymerisation of heteropolymeric RNAs in a template-free manner [21, 25, 51, 52]. Due to this intriguing action mechanism, PNPase was originally described as a "RNA Polymerase" [53]. Indeed, this erroneous characterisation awarded Severo Ochoa the 1959 Nobel Prize.

The phosphorolytic activity exerted by PNPase requires a 3´ overhang of 7 to 9nt of RNA, frequently staling in the presence of structured RNAs [52]. Nonetheless, folded RNAs, such as sRNAs, can also be targeted by PNPase if a 3´ polyadenylated tail is present. In this case, PNPase typically associates with other enzymes in a multiprotein complex called the degradosome, capable of degrading highly structured RNAs [17]. PNPase can also associate and form complexes with Hfq and poly(A) polymerase I (PAP I). In fact, in an Hfq mutant in stationary phase and in the presence of low concentrations of iP, PNPase becomes the primary polyribonucleotide polymerase, adding heteropolymeric tails to 3´ truncated mRNAs [54].

PNPase activity is required in several stress related responses. For example, PNPase is important in cold conditions, with its levels increasing about 2 fold during cold shock [55]. Moreover, after the acclimatisation phase, PNPase acts as a post-transcriptional regulator by degrading the mRNAs of several cold shock proteins (CSP), thus helping in the transition from the cold shock acclimation phase to the cell growth resumption stage [56]. Additionally, PNPase has likewise been established as a virulence factor in several pathogens, such as *Salmonella enterica* and *Yersinia spp* [50, 57].

PNPase has also been linked to stationary phase associated stress, with the steady-state levels of several sRNAs being increased in *pnp* mutants [58, 59]. Moreover, PNPase has been implicated in the post-transcriptional regulation of several sRNAs, having a

preponderant role in their respective degradation [59, 60]. Indeed, PNPase is the main ribonuclease involved in the decay of hfq-free-sRNAs [61]. These findings shine new light on the importance of the exoribonucleolytic turnover in the RNA networks, opening the door for future work on this yet poorly understood sRNA regulation mechanism.

1.5.2. Endoribonucleases

In *E. coli*, the process of RNA degradation usually begins with an endoribonucleolytic cleavage at one or more internal sites of the RNA molecule. Two endoribonucleases have been associated with these initial cleavage events: ribonuclease III (RNase III) and ribonuclease E (RNase E) [21] (Table 1).

1.5.2.1. Ribonuclease III

Ribonuclease III was the first dsRNA specific endoribonuclease to be discovered [62]. Since then, it has been shown that RNase III is widely distributed among other organisms, being the prototype of the RNase III family [21].

RNase III, like all members of its family, is a hydrolytic enzyme capable of degrading dsRNAs and yielding both a 5´ monophosphate end and a 2nt overhang at the 3´ hydroxyl terminus [63]. In *E. coli*, RNase III is encoded by the *rnc* gene and plays an essential role not only in rRNA and tRNA maturation, but also in the decay of some mRNAs, such as the *pnp* mRNA [21].

RNase III action is required for a plethora of distinct bacterial stress responses [50], being necessary for both heat and cold shock adaptation [64], cobalt and nickel resistance [65] and osmotic stress adaptation [66]. Furthermore, it is also important for biofilm formation [36], *S. enterica* motility and proliferation inside the host cells [67] and acid adaptation [68].

In similar fashion to the Eukaryotic RNase III orthologues, Dicer and Drosha, the *E. coli* RNase III is also involved in the translational silencing and degradation of sRNA-mRNA complexes. Moreover, like Dicer and Drosha, which play a key part in microRNA

(miRNA) maturation, RNase III is likewise involved in the processing of some described bacterial sRNAs [63, 66, 69, 70].

1.5.2.2. Ribonuclease E

Encoded by the *rne* gene, RNase E is an ssRNA endoribonuclease that primarily cleaves A/U rich sequences [21]. RNase E is essential for cell growth, being primarily involved in RNA decay, 5S and 16S rRNA processing and in the maturation of tmRNAs and several tRNAs.

In *E. coli*, RNase E also functions as the scaffold for the assembly of the degradosome [21]. This multi protein complex is assembled around the C-terminal region of RNase E, which localises the complex to the inner cytoplasmic membrane and acts as a scaffold for protein association [19, 21, 71]. Depending on the growth conditions, and among other minor components [20], the degradosome encompasses the exoribonuclease PNPase [72], the endoribonuclease RNase E [72], the DEAD-box helicase RhlB and the glycolytic enzyme enolase [73]. Furthermore, a recent report by Feng Lu and Aziz Taghbalout associated the exoribonuclease RNase II with the complex [74].

During cold conditions, RNase E further interacts with another DEAD-box helicase, DeaD, which is incorporated in the degradosome and probably assists in the degradation of structured RNAs [47, 75].

Curiously, in *Pseudomonas syringae*, RNase E only interacts with the exoribonuclease RNase R (instead of PNPase) and with another helicase, RhlE [76].

Table 1

Major Ribonucleases involved in RNA degradation in *E. coli* [21].

Ribonuclease	Gene	Notes
RNase II	*rnb*	Exoribonuclease. Stalls in the presence of RNA secondary structures and can protect RNAs from degradation.
RNase R	*rnr*	Exoribonuclease. Efficiently degrades RNA secondary structures. Important in RNA quality control and stress adaptation.
PNPase	*pnp*	Exoribonuclease. Forms multiprotein complexes with other enzymes. Major ribonuclease involved in the degradation of Hfq-free sRNAs.
RNase E	*rne*	Endoribonuclease. Scafold for the assembly of the degradosome. Cleaves ssRNA.
RNase III	*rnc*	Endoribonuclease. Cleaves dsRNA. Associated with stress conditions.

1.6. Regulatory RNAs

RNA based regulators were initially discovered in 1981 as a replication control mechanism for the colE1 plasmid, a process based on the hybridisation of the regulator RNA, RNA I, with the colE1 replication initiation primer [77, 78]. Three years later, in 1984, Mizuno *et al* described the first chromosomally encoded small RNA regulator, *micF*, a 174 bp transcript responsible for the premature translation termination of the *OmpF* gene mRNA [79]. Taken together, these findings have opened the door for the discovery, firstly in bacteria and later in eukaryotes, of a previously unknown RNA based post-transcription regulation network, capable of coordinating a multitude of physiological responses in a variety of changing environments. For example, the OxyS sRNA, which is induced under oxidative stress, controls the expression of as many as 40 genes [80]. Another example is the sRNA FnrS, which helps in the transition from an aerobic to an anaerobic genetic program [81]. Biofilm formation can also be influenced by regulatory RNAs, namely in the integration of external stimuli, such as pH and osmolality, in the curli and cellulose synthesis pathways [82]. Nowadays, approximately

110 sRNAs have been experimentally proven to exist in *Escherichia coli* [83], with several others waiting validation.

When compared with other regulatory effectors, such as proteins, sRNAs display the clear advantage of being more cost-effective. For example, sRNAs do not require translation, thereby both avoiding the need for more regulatory proteins and saving time and energy on the translational process itself. Moreover, sRNAs generally act at the post-transcriptional level, which not only allows a much faster control of gene expression, but a better management of the RNA pool as well. sRNAs are also less stable than regulatory proteins and can be rapidly degraded if needed [18, 84].

In a similar fashion to the functional mechanics of the bacterial sRNAs, the eukaryotic counterparts, the miRNAs and short interfering RNAs (siRNA), can also efficiently regulate their targets, thereby acting as an extra layer of cellular regulation [85]. Nonetheless, striking differences emerge. For example, while miRNAs and siRNAs typically have between 21 and 25 nt in length and require processing from a longer single or double stranded precursor by a RNase III like enzyme, sRNAs tend to be more heterogenic both in size and in structure. For instance, although some sRNAs may require the action of different ribonucleases to be activated, the majority is commonly produced as a highly structured, single, unprocessed primary transcript, with an average length varying from 50 to 250 nt. [19, 86].

sRNA mediated regulation can act by two distinct mechanisms. One based on the direct interaction of a sRNA with a protein, thereby modulating its activity, the other, more frequent, on the base pairing of a sRNA (antisense) with a target RNA (sense), resulting in an alteration of the target stability and/or translation [19, 58].

1.6.1. Protein binding sRNAs

There are only a few known examples of sRNA mediated protein regulation in *E. coli*. Nonetheless, all share a common trait: the sRNA operates as a protein antagonizer; a process possible due to the close resemblance between the protein's ligand and the sRNA's structure and/or sequence. The sRNA thus competes, alongside the ligand (if present), for the protein binding site [19, 84].

The 6S RNA, which was the first sRNA proven to impact transcription, can be considered the perfect example of the previously described mechanism. This sRNA operates by mimicking a promoter, which binds to the holoenzyme RNA polymerase-σ^{70}, thereby impairing its activity. This process ultimately translates in the transcriptional inhibition of several "housekeeping" σ^{70} promoters, especially in the stationary phase, where the 6S RNA is more abundant [87].

Another interesting example is the RNA binding CsrA (carbon storage regulator) protein. CsrA represses several carbon related metabolic pathways by binding to the mRNA of its targets and inhibiting their translation [86, 88]. This activity is counteracted by the CsrB sRNA, which sequesters multiple copies of the CsrA protein by mimicking the shine-dalgarno sequence of the CsrA mRNA targets. [89]. Considering that CsrA activates *csrB*, this mechanism creates a negative feed-back loop in which CsrA modulates its own activity [90].

1.6.2. Antisense sRNAs

Unlike what happens with protein binding sRNAs, most of the known sRNAs act on other RNAs by an antisense mechanism: A process that can be mediated by either *trans*-encoded (*trans*-sRNAs) or *cis*-encoded (*cis*-sRNAs) sRNAs. When considering their genetic expression, almost all known *trans*/*cis*-sRNAs are preferentially expressed under specific growth conditions, such as limiting carbon or oxidative stress [86].

Regarding the base-pairing mechanisms, sRNAs frequently bind stoichiometrically near the mRNA 5´ end. This process can either result in the degradation or stabilisation of the sRNA/mRNA pair [22]. For instance, in the case of the *ryhB* sRNA, which is involved in the regulation of the iron metabolism, the sRNA represses the target expression by binding to the target mRNA and inducing the degradosome degradation pathway [91]. On the other hand, in the less frequent case of transcript stabilisation, the binding of an activator sRNA prompts a conformational change that commonly results in the exposure of the mRNA Shine-Dalgarno sequence [18, 19, 86, 92].

Independently of the sRNA action mechanism, the relative concentrations of the sRNA and mRNA are crucial. In the sRNA regulatory networks, when the sRNA

concentration is higher than that of the mRNA, gene expression if shut off. However, when the sRNA concentration is lower than that of the mRNA, little impact is observed. This critical sRNA threshold phenomenon suggest than sRNAs are more effective at repressing mRNA translation when the sRNA activator signal is persistent and abundant, whereas in the case of weak and transient signals, proteins are more efficient [84].

1.6.3. *Cis* encoded sRNAs

Cis-sRNAs are encoded on the opposite DNA strand from which their RNA target is transcribed. This results in the existence of extended sections of complementarity between the *cis*-sRNA and the respective target, frequently 75 nt or more [84, 93]. Nonetheless, despite being transcribed from the same DNA region, both antisense and sense RNAs act as independent molecules, each subject to individual reaction kinetics in the cellular environment.

Most of the known *cis*-sRNAs regulate the replication of mobile elements such as the colE1 plasmid [78, 93]. Others repress the translation of deadly toxic proteins, thereby acting as antitoxins by inhibiting cell death in the presence of the sRNA antitoxin genetic carrier [84]. A few *cis*-sRNA can also influence the expression of chromosomally encoded genes, such as those in the glutamate acid response (gad) operon [19, 84].

1.6.3.1. *Cis*-RNAs and the gad system: an intricate story

The gad system is the most prominent acid response mechanism in *E. coli*. The gad system main effectors, GadA and GadB, lower the intracellular pH by consuming protons during the decarboxylation of glutamate. This reaction ultimately originates γ-aminobutyric acid, which is then exported out of the cell by GadC [94]. This system's regulatory network is complex and still under debate, however, it has been proven that it is targeted by several layers of post-transcriptional control that include, at least, two *cis*-RNA activators, GadY and ArrS.

GadY is a *cis*-RNA encoded in the 3´ UTR of the *gadX* mRNA. Its expression is increased upon entry to stationary phase by the σ^S factor and it is also up-regulated in low

pH conditions [95, 96]. Despite being *cis* encoded, GadY is able to bind the RNA chaperone Hfq, which is normally associated with *trans*-RNAs, as discussed in the next section [28]. This intriguing sRNA binds to the intergenic region of the *GadX-GadW* mRNA and promotes RNase III mediated processing of the transcript, which results in an increased level of both the *GadX* and *GadW* mRNAs and in a decreased concentration of the longer transcript *GadX-GadW* mRNA [28, 58, 68]. GadX, among others, partly activates the expression of the main gad activator, GadE, and of the GadA, GadB and GadC proteins. Inversely, GadW appears to be primarily involved in negatively regulating the transcription of both *gadX* and GadY [97, 98].

ArrS is a *cis*-RNA encoded in the unusually long 5´ UTR of the T3 *gadE* form. ArrS is normally expressed during stationary phase and is dependent upon the factors gadE and σ^S. ArrS expression is further increased in acidic conditions [99, 100]. ArrS controls the levels of the *gadE* mRNA T3 form, which abruptly decrease and give rise to its smaller T2 active form when the sRNA availability increases [100]. The GadE transcription factor, which is originally transcribed in its apparently inactive T3 form, is a major gad system activator, being required for *gadA*, *gadB* and *gadC* expression (Fig. 2). Curiously, both gadX and gadW are only needed in some circumstances [101]. The existence of a monophosphorilated 5´ extremity (instead of a triphosphorilated one) in the *gadE* T2 form and the lack of T2 expression in RNase III deficient cells suggest a ribonuclease involvement in both the *gadE* mRNA processing and in the cellular adaptation to acidic conditions [100].

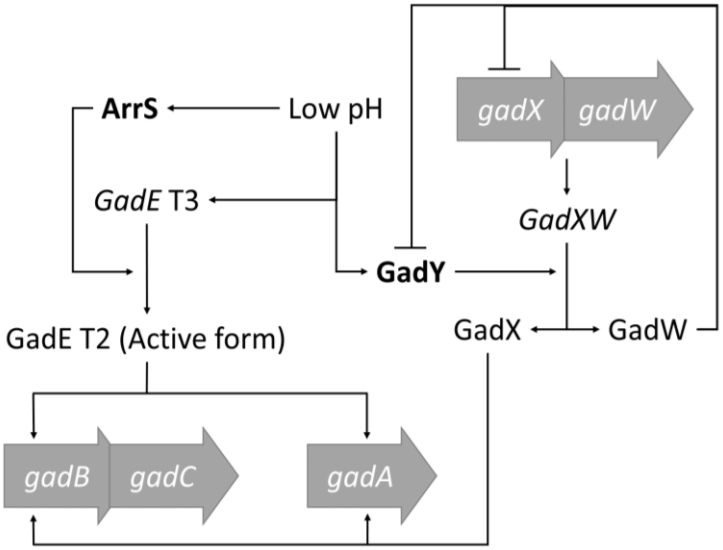

Fig. 2 - Simplified model of the gad system regulatory network.

Lines indicate regulation. The processing from the *gadE* T3 mRNA to the *gadE* T2 mRNA form is catalysed by the sRNA ArrS in an RNase III dependent reaction. The *GadXW* transcript cleavage is catalysed by the sRNA GadY in a reaction that yields both *gadX* and *gadW*.

1.6.4. *Trans* encoded sRNAs

Trans-sRNAs are, in their majority, characterised for being encoded in a distinct chromosomal location than the one the target mRNA is transcribed from. When compared with the *cis*-sRNA, they have a more limited, non-contiguous complementarity between the mRNA-sRNA pair. By having a more discrete base-pair complementation with its targets, a *trans*-sRNA molecule can, therefore, carry more than one mRNA binding site, thus explaining the *trans*-sRNA's capacity to regulate multiple mRNAs [19, 84, 86]. Unfortunately, the mechanisms that determine the specificity between the sRNAs and their target mRNAs are still largely unknown. For this reason, the several sRNA/mRNA interaction predicting algorithms that today exist frequently display erroneous results. In order to increase their accuracy at predicting productive, *in vivo*, sRNA/mRNA interactions, further work on this subject thus needs to be done. This will effectively increase experimental efficiency by avoiding the study of false targets [84].

Trans-sRNAs are the most abundant and well-studied class of sRNAs, representing a promising field of medical research due to their role in virulence and bacterial stress responses [85]. For example, in an anoxic environment like the one found in the human gastrointestinal track, *E. coli* survives by modulating its metabolic pathways

to produce energy from fermentation and/or anaerobic respiration. These changes in gene expression are partly regulated by the *trans*-sRNA FnrS, whose expression is dependent on the transcriptional fumarate and nitrate reductase regulator (FNR), an oxygen availability sensor. When in anoxic conditions, the sRNA FnrS is highly expressed in order to repress the translation of several aerobic metabolic enzymes, hence increasing the efficiency of the anaerobic metabolism [81, 102]. *Trans*-sRNAs thus have the capability of swiftly regulating entire physiological pathways, quickly forwarding large quantities of obsolete mRNAs into degradation and effectively stopping a metabolic program at the RNA level. Furthermore, due to differences in binding affinities, sRNAs help in prioritizing the regulation of different target mRNAs and, consequently, in controlling the various stages of a changing gene expression program [84, 103].

Unlike the Eukaryotic miRNAs and siRNAs, which require complex proteic machinery to operate, the Enterobacteriaceae *trans*-sRNAs's have only been found to require the presence of the Hfq RNA chaperone (Hfq).

1.7. Hfq

Hfq, originally described as HF-1, was firstly discovered in *E.coli* cells infected by the Qβ RNA bacteriophage as a key host factor involved in viral RNA synthesis [104, 105]. Since then, Hfq has been found to be the *E.coli* counterpart of a ubiquitous family of RNA-binding proteins, the Sm and Sm-like protein family. Hfq thereby shares, alongside the rest of the members of the family, what is considered to be the family's main feature, the presence of a multimeric ring-like quaternary conformation in the active protein form. This peculiar organisation facilitates the discontinuous and imperfect interactions that are established between a *trans*-sRNA and its respective target mRNA. Hfq thus acts as a chaperone, mediating the formation of the RNA-RNA pair, expediting its assembly and, consequently, increasing the efficiency of the *trans*-sRNA regulatory network [106-108]. In fact, Hfq seems to be the limiting factor for sRNA mediated regulation, being essential for productive *trans*-sRNA-mRNA base pairing [109, 110]. Moreover, sRNA competition for Hfq may further limit sRNA action in stressful circumstances, where higher levels of sRNA are normally detected. In these conditions, a sRNA will compete for Hfq at the expense of another. This situation implies that, for

example, an induced sRNA can affect the action of another, unrelated, sRNA. Indeed, this mechanism was firstly described as an explanation for the *oxyS* dependent negative regulation of the σ^S factor, whose translation is dependent on an activator sRNA [111].

In Hfq mutant cells, pleiotropic effects such as impairment of stress response pathways, metabolic regulation deficiencies and loss of virulence can occur. However, several of this consequences may be amplified by the multitude of interactions that Hfq establishes with other proteins, such as RNase E, PNPase and PAP I [54, 60, 109].

Hfq seems to bind to both A/U and poly(A) rich sequences [112], with its activity being dependent on the tri-dimensional arrangements found in the same RNAs with which it interacts. Theoretically, this RNA structural information renders a conformational change in the Hfq protein, resulting in the formation of different complexes and, thus, in the display of several distinct *modus operandi* [107] (Fig. 3). Hfq action can, thereby, cause different outcomes depending on the RNA-RNA interactions. For example, Hfq is involved not only in mediating the interactions that are established between an mRNA-sRNA pair, but also on its recycling, mostly through the recruitment of ribonucleases such as RNase E [54, 106, 107, 113]. Paradoxically, by binding to poly(A) tails and RNase E cleavage sites, Hfq can likewise help protect a few described RNAs, such as *dsrA, ryhB* and *rpoS*, from ribonuclease activity [61, 110, 112-115] (Fig. 3). Ultimately, the presence/influence of the Hfq protein results in a faster cellular adaptation to hostile environments, such as those encountered in a potential host.

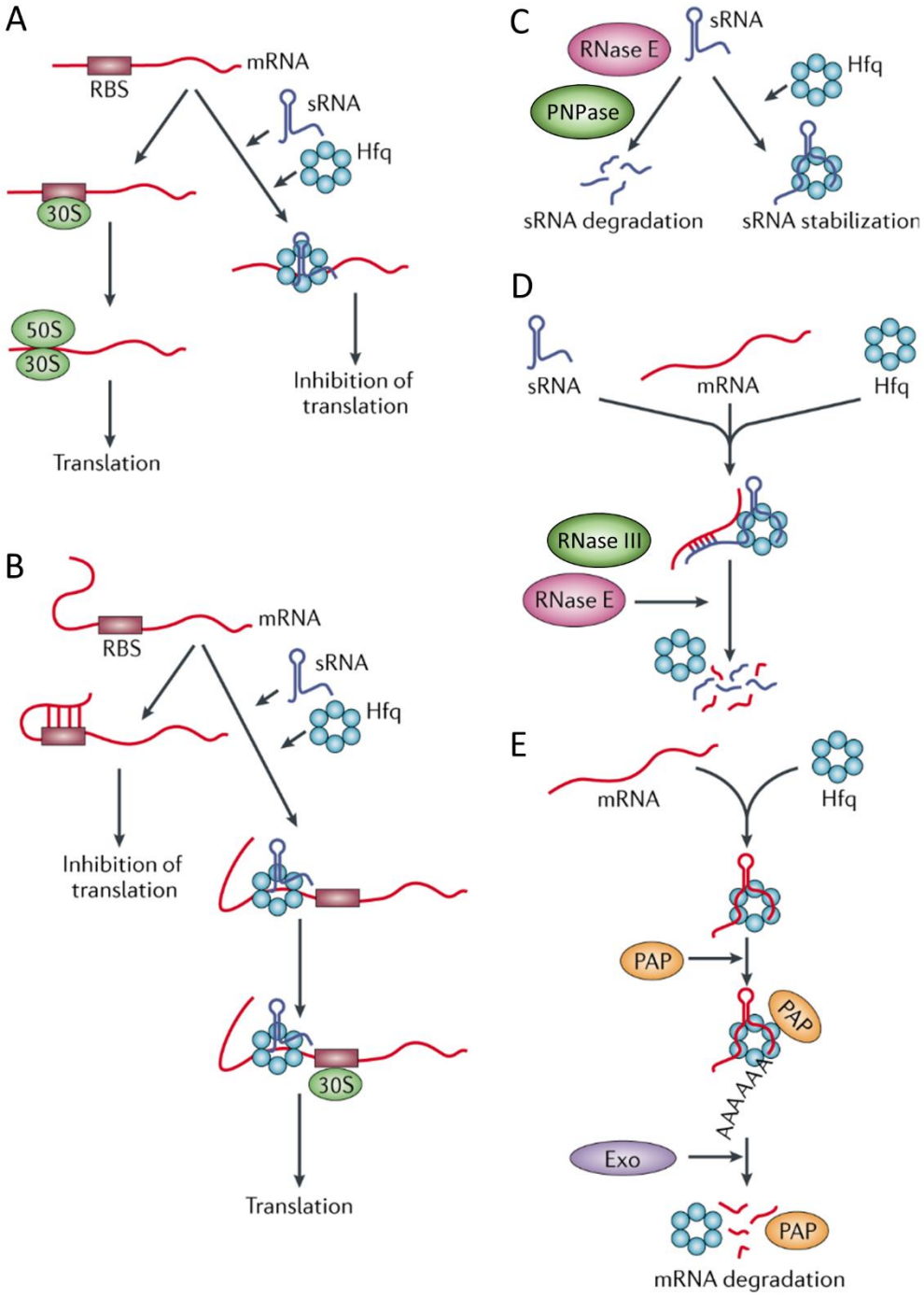

Fig. 3 - Widely accepted modes of Hfq activity.

(A) Hfq in association with a sRNA may sequester the ribosome-binding site (RBS) of a target mRNA, thus blocking binding of the 30S and 50S ribosomal subunits and repressing translation. (B) In some mRNAs, a secondary structure in the 5′ UTR can mask the RBS and inhibit translation. A complex formed by Hfq and a specific sRNA may activate the translation of one of these mRNAs by exposing the translation initiation region for 30S binding. (C) Hfq may protect some sRNAs from ribonuclease cleavage. (D) Hfq may induce the cleavage (often by RNase E) of some sRNAs and their target mRNAs. (E) Hfq may stimulate the polyadenylation of an mRNA by poly(A) polymerase, which in turn triggers 3´ to 5´ degradation by an exoribonuclease (Exo). In *E. coli*, the exoribonuclease can be PNPase, RNase R or RNase II. Image adapted from "Hfq and its constellation of RNA" [107].

1.8. Introductory Remarks

It is now becoming increasingly clear that RNases, and not just regulatory RNAs, play a predominant role in the post-transcriptional regulation of several distinct stress activated pathways. While sRNAs are capable of efficiently regulating entire metabolic pathways, RNases can act on both the sRNAs and their RNA targets, effectively regulating the regulators. Indeed, both are required to effectively control the vast interconnected network that constitutes the cellular RNA pool [19, 36]. Ultimately, post-transcriptional regulators not only allow the cell to swiftly respond to changing environments, but also anticipate the regulatory requirements needed for a change in genetic expression.

1.8.1. Objectives

In this work, we will focus on how exoribonucleases influence the acidic and anaerobic stress response pathways. We will determine the levels of several stress related sRNAs in the RNase II, RNase R and PNPase mutant strains and evaluate how these strains respond to acidic and anaerobic conditions. Moreover, we will determine how these sRNAs respond to the absence of Hfq. Finally, we will also examine how the exoribonucleases influence the levels of several of the sRNAs targets.

2. Materials and Methods

2.1. Strains and Growth Conditions

E. coli K-12 MG1693 and all derivative strains are listed in Table 2. All strains were grown at 37ºC with agitation at 180 rpm in Luria-Broth medium (LB) (Table 3) supplemented with thymine (50µg ml^{-1}). When required, antibiotics where supplemented in the following concentrations: Tetracycline (tet) (20µg ml^{-1}), kanamycin (kan) (50µg ml^{-1}), streptomycin (str) (25µg ml^{-1}), spectinomycin (spc) (25µg ml^{-1}), ampicillin (amp) (100µg ml^{-1}) and chloramphenicol (cam) (20µg ml^{-1}).

All cell cultures inoculated in Luria-Agar medium (LA) (Table 3) were supplemented with thymine (50µg ml^{-1}) and incubated at 37ºC until individualised colonies were visible. When necessary, antibiotics were also added to the medium in the previously referred concentrations.

Overnight cultures were performed by inoculating isolated colonies in LB medium (supplemented as required), followed by incubation with agitation for 16h (overnight) at 37ºC and 180 rpm.

Optical density values were obtained by pippeting 1ml of cell culture/medium to a disposable plastic cuvette of 1.5mL (*Sarstedt*) and measuring the respective absorbance at 600nm (OD_{600}) in a spectrophotometer (BioPhotometer plus, *Eppendorf*).

Exponential phase cells were obtained by inoculating fresh LB medium (supplemented as required) with overnight cultures to an initial OD_{600} of ~ 0.03. The cell cultures were then incubated with agitation at 37ºC and 180 rpm until an OD_{600} of 0.5 (exponential phase).

Stationary phase cells were obtained by inoculating fresh LB medium (supplemented as required) with overnight cultures to an initial OD_{600} of ~ 0.03. The new cultures were then incubated overnight (16h) with agitation at 37ºC and 180 rpm. Stationary phase was confirmed by measuring the OD_{600} of the cell cultures after overnight incubation.

Table 2

Plasmids and *E. coli* strains used in this work

Strains	Relevant Genotype	Reference
MG1693 (WT)	*thyA715 rph-1*	[116]
CMA201 (Δ*rnb*)	*thyA715 rph-1 Δrnb-201::tet*^{R}	[117]
HM104 (Δ*rnr*)	*thyA715 rph-1 Δrnr::kan*^{R}	[44]
SK10019 (Δ*pnp*)	*thyA715 rph-1 pnpΔ683::str*^{R}*/spc*^{R}	[32]
CMA599 (Δ*Hfq*)	*thyA715 rph-1 Δhfq::cam*^{R}	Unpublished
pCMA01	*6.5kb HindIII-XhoI fragment of pDK07 cloned into pBluescript SK*$^+$	[118]

Table 3

Luria-Agar and Luria-Broth Mediums recipe for 1L of medium. H_2O Milli-Q® was added until 1L and the mixture was sealed and autoclaved.

Luria-Agar (LA)	
Reagents	**Quantities**
Tryptone	10g
Yeast Extract	5g
NaCl	10g
Agar	15g

Luria-Broth (LB)	
Reagents	**Quantities**
Tryptone	10g
Yeast Extract	5g
NaCl	10g

2.2. Growth curves

2.2.1. Acid shock growth curves

Overnight cultures for the WT, Δ*rnb*, Δ*rnr* and Δ*pnp* strains were performed as described. Respectively, each cell culture was then used to inoculate to an initial OD_{600} of ~ 0.03, 50ml of fresh LB medium in triplicate (supplemented as required). Following inoculation, all cultures were incubated at 37ºC with agitation at 180 rpm. The culture's OD_{600} was measured hourly and plotted against time of measurement. In each strain, one of the triplicates was used as the control growth curve. In the other two, acid shock was performed by adding 150µl of HCl 4N (pH drop from ~ 7 to ~ 4) at an OD_{600} of either 0.5 (exponential phase) or 1.5 (early stationary phase).

2.2.2. Anaerobic growth curve

Overnight cultures for the WT, Δ*rnb*, Δ*rnr* and Δ*pnp* strains were performed as described. Each cell culture was then used to respectively inoculate 50ml of fresh LB medium (supplemented as required) to an initial OD_{600} of ~ 0.03. All cultures were then transferred to an anaerobic chamber (with a CO_2 and argon atmosphere) and incubated at 37ºC in a dry water bath, without agitation. Every hour, cell OD_{600} was measured as described above. A final OD_{600} value was registered after overnight incubation in the same conditions.

2.3. Acid shock survival assay

Overnight cultures for the WT, Δ*rnb*, Δ*rnr* and Δ*pnp* strains were obtained and used to respectively inoculate to an initial OD_{600} of ~ 0.03 50ml of fresh LB medium, supplemented as required. The newly inoculated cultures were then incubated with agitation at 37ºC and 180 rpm. For each strain, at an OD_{600} of 0.5, and prior to the acid shock, 1ml of cell culture was removed and, in triplicate, serially diluted 1:100 three times. From the last serial dilution, 100µl were used to, also in triplicate, inoculate plaques with LA medium supplemented as needed. Immediately after removing the 1ml from the cell cultures, acid shock was performed by adding 150µl of HCl 4N (pH drop from ~ 7 to ~ 4). All cultures were then incubated as described for another 2h, point at which another 1ml was extracted from the cell cultures and plated as previously depicted. The LA plaques were then incubated at 37ºC and the number of colony forming units (CFU) in each plaque counted after 40h. The relative survival of each strain was determined as the ratio between the average of the number of CFUs before performing the acid shock and the average of the number of CFUs 2h after the acid shock.

2.4. Total RNA extraction

2.4.1. Total RNA extraction for RNA half-life determination

Rifampicin solution (Table 4), a bacterial transcription inhibitor, was prepared immediately before use. NaOH was added to the solution until the rifampicin appeared dissolved. 1.5ml of rifampicin solution per 50ml of LB medium was then added to either stationary or exponential phase cultures. 30s after adding the rifampicin solution, the first time point was taken by decanting either 10 or 20ml (see below) of cell culture to either 10 or 20ml of ice-cold TM STOP buffer (

Table 6). The culture conditions were maintained at 37ºC with agitation at 180 rpm for the duration of the experiment and at the required time points another 10 or 20ml of culture were decanted to 50bml falcons with ice-cold TM STOP buffer. RNA was then extracted as described below.

Table 4

Rifampicin solution recipe for 50ml of cell culture.

Reagents	Quantities
Rifampicin	0,05g
Nalidixic acid solution (Table 5)	500µl
Methanol	1ml

Table 5

Nalidixic acid solution recipe for 2ml.

Reagents	Quantities
Nalidixic acid	0,0010g per 50ml of cell culture
NaOH 10N	20µl
H_2O Milli-Q®	1980ml

Table 6

TM STOP buffer recipe for 100ml.

Reagents	Quantities
Tris 1M at pH 7.2	1 ml
$MgCl_2$ 1M	0.5 ml
NaN_3 1M	2.5 ml
Chloramphenicol (4mg ml^{-1})	12.5 ml
H_2O Milli-Q®	83.5 ml

2.4.2. Total RNA extraction of steady-state RNAs

Total RNA was either extracted from cells in exponential phase (OD_{600} ~ 0.5) or in stationary phase (after ~ 16h of growth). RNA from stationary and exponential phase cultures was extracted by respectively decanting 10 or 20ml of culture to a 50ml falcon tube with 10 or 20ml of ice-cold TM STOP buffer.

2.4.3. Determination of the degradation rate of FnrS sRNA under induction of anaerobic conditions.

Exponential phase WT, Δ*rnb*, Δ*rnr* and Δ*pnp* cell cultures were obtained as described. At an OD_{600} of 0.5, all cultures were transferred to an anaerobic chamber (*Plas labs*) with a CO_2, N_2 and H_2 atmosphere at 37ºC in order to induce the expression of the FnrS sRNA (anaerobic shock). After 2h, the cultures were removed from the anaerobic chamber and 10ml of cell culture decanted to ice-cold TM STOP buffer (time 0). All cell cultures were then incubated at 37ºC with agitation at 180rpm in an aerobic environment. The remaining time points (3, 6, 10 and 20 minutes) were extracted as in time 0.

2.4.4. Total RNA extraction: Phenol/chloroform method

The 50ml falcon tubes with cell culture and cold TM STOP buffer were centrifuged for 20 minutes at 4ºC and 3600 rpm. The supernatants were decanted and the cell pellets ressuspended in 800µl of freshly prepared lysis buffer (Table 7). The cell extracts were then transferred, respectively, into glass COREX tubes and incubated for 5 minutes at 42ºC. Next, three cycles of freezing in liquid nitrogen and thawing at 42ºC were performed. 140µl of acetic acid (20 nM) and 90µl of sodium dodecyl sulphate (SDS) 10% were added in the third cycle and the freezing/thawing cycles continued until the cell solutions appeared lysated (transparent). After the lysis procedure, an enzymatic digestion with DNase (TURBO™ DNase, *Ambion*®) (2U/µl) was performed for 1h at 37ºC. Either 5µl or 10µl of DNase were added to the cell extracts, depending on whether the cells were obtained from exponential phase or stationary phase cultures, respectively. After DNase digestion, the cell extracts were transferred into 2ml tubes and phenol:chloroform RNA extraction was performed. To this effect, 1ml of phenol at pH 5.2 was added, respectively, to the cell extracts, which were then vortexed for 2 minutes and centrifuged for 10 minutes at 4ºC and 14000 rpm. The supernatants were carefully collected to new 2ml tubes and the previous process repeated. In the third step, 500µl of phenol at pH 5.2 and 500µl of chloroform/alcohol isoamylic (24:1) solution were added to the collected supernatants, which were then vortexed for 1 minute and centrifuged for 5 minutes at 4ºC and 14000 rpm. The supernatants were carefully collected to new 2ml tubes and the previous process repeated one more time. Finally, 1ml of chloroform/alcohol isoamylic (24:1) solution was added to the collected supernatants, which were then vortexed for 1 minute and centrifuged for 5 minutes at 4ºC and 14000 rpm. The supernatants were carefully collected to new 2ml tubes and sodium acetate 3M pH 5.2 and ice-cold ethanol 100% added in a volume corresponding to 0.1x and 2.5x that of the collected supernatants, respectively. The supernatants were left to precipitate overnight at -20ºC.

The precipitated RNA samples were centrifuged for 45 minutes at 4ºC and 14000 rpm and the resulting supernatant discarded. The remaining RNA pellets were washed by adding 1ml of ice-cold ethanol 75%, after which another centrifugation for 30 minutes at 4ºC and 14000rpm was performed. The obtained supernatant was discarded and the pellets left in the fume hood do dry. When fully dehydrated, an appropriate volume of H_2O Milli-Q® was added to ressuspend the pellet.

RNA integrity and DNA contamination were assessed by examining 1µl of the RNA samples in an agarose-gel electrophoresis. In case of DNA contamination, a new DNase digestion was performed by adding to the RNA samples 2.5µl of DNase (TURBO™ DNase, *Ambion*®), 20µl of DNase buffer and H_2O Milli-Q® to a final volume of 200µl. After incubating the samples at 37ºC for, at least, 1h, a second phenol:chloroform RNA extraction was performed. To this effect, H_2O Milli-Q® was added to a final volume of 400µl. 200µl of phenol at pH 5.2 and 200µl of chloroform/alcohol isoamylic (24:1) solution were then added to the RNA samples and vortexed for 1 minute, after which a centrifugation for 5 minutes at 4ºC and 14000 rpm was performed. The supernatant was collected and transferred into a new 2ml tube. 400µl of chloroform/alcohol isoamylic (24:1) solution were added to the supernatants and vortexed and centrifuged as before. The supernatants were extracted to new 2ml tubes and sodium acetate 3M pH 5.2 and ice-cold ethanol 100% added as previously described. Precipitation and verification of RNA integrity and DNA contents were also performed as stated. Finally, the RNA samples concentration was quantified using a Nanodrop Spectrophotometer (Nanodrop ND1000, *Alfagene*). All RNA samples were stored at -20ºC.

Table 7

Lysis buffer recipe for 20ml.

Reagents	Quantities
Tris 1M at pH 7.2	200µl
$MgCl_2$ 1M	100µl
Turbo DNase (2U/µl) (*Ambion*)	20µl
Lysozyme from chicken egg white (*Sigma-Aldrich*)	0.020g
H_2O Milli-Q®	Until 20ml

2.5. Genomic DNA extraction

Overnight cultures (in stationary phase) for the WT, Δ*rnb*, Δ*rnr* and Δ*pnp* strains were performed as described and used to extract DNA using the Citogene® DNA Cell & Tissue Kit (*Citogene*®). DNA samples were ressuspended in H_2O Milli-Q®. DNA integrity was verified by agarose-gel electrophoresis and quantified as previously described. All DNA samples were stored at 4ºC.

2.6. Electrophoresis

12µl of loading solution (constituted, unless stated otherwise, by 2µl of Orange G loading buffer, 9µl of H_2O Milli-Q® and either 1µl of RNA or DNA) was loaded in an electrophoretic agarose-gel (Table 8). An electrophoresis was then performed at 100V for 30 minutes in 1x TBE buffer. The agarose-gel was then visualised under UV light in a Gel Doc XR+ system (*Bio-rad*®).

Table 8

Electrophoretic Agarose-gel (1.5%) recipe for 100ml.
Agarose was dissolved in boiling 1x TBE buffer.

Reagents	Quantities
Agarose (*SeaKem® LE AGAROSE*)	1.5g
Ethidium bromide (1mg ml^{-1})	50µl
1x TBE (Table 11)	100ml

2.7. Northern Blot

2.7.1. Northern Blot sample preparation

Northern blot samples were prepared by pipetting the volume corresponding to 40µg of RNA into a new 1.5ml tube and evaporating the sample to a final volume of 5µl (SpeedVac SVC 100, *Savant*). 15µl of PAA loading buffer (Table 9) were then added to all samples.

Table 9

PAA loading buffer recipe.

Reagents	Quantities
Deionized Formamine	5ml
EDTA 0.5M (pH 8)	100µl
Xylene Cyanol	0.005g
Bromo Phenol Blue	0.005g

2.7.2. Northern Blot

RNase free 1.5mm spacers were assembled in top of a Northern blot glass plate. A notched Northern blot glass plate was then placed in top of the spacers and the sides were sealed with agarose-gel.

In all performed Northern blots, 50ml of 10% polyacrylamide gel solution (Table 10) were prepared and mixed with 500µl of APS (10%) and 50µl of TEMED. The obtained solution was poured into the space between the two Northern blot glass plates and a comb was inserted at the top. After overnight polymerisation, the comb and the bottom spacer were removed and the glass plates with the polyacrylamide gel placed in the Northern blot apparatus. 1x TBE buffer (Table 11) was then added to the Northern blot apparatus´ reservoirs and any air bubbles removed by rinsing both the gel wells and the bottom of the gel with a syringe loaded with 1x TBE. The gel wells were further washed until all residual urea was removed.

A pre-run of the gel (without sample) was then performed for ~ 1h at 420V with wattage limited to either 24 or 48W (PowerPac^{TM} HV, *Bio-Rad*), depending on whether one or two Northern blots were being simultaneously performed, respectively.

The Northern blot RNA samples were denatured at 80ºC for 10 minutes and then incubated in ice for another 2 minutes.

After terminating the pre-run, all polyacrylamide gel wells were rinsed with 1x TBE using a syringe and the full volume of the Northern blot RNA samples was applied. The polyacrylamide gel loaded with the RNA samples ran for 2h at 420V, with wattage limited to either 24 or 48W, depending on whether one or two Northern blots were being performed, respectively.

Four litters of 1x TAE buffer (Table 12) was prepared and a positive charged nylon membrane with 0.45µm pores (Amersham Hybond-N+, *GE Healthcare Life Sciences*) was cut together with 10 pieces of blot absorbent filter paper (3MM paper,

Whatman®). The nylon membrane was activated by submerging it for at least 5 minutes in Milli-Q® water.

In order to perform the RNA transfer from the polyacrylamide gel to the membrane, two sponges and blot filter papers were saturated with 1x TAE in a RNase free recipient. The transfer apparatus was then assembled in the following order: negative side of transfer cassette, sponge, blot filter papers, polyacrylamide gel, nylon membrane, blot filter papers, sponge and positive side of gel cassette. Air bubbles were removed by manually pressing the saturated blot filter papers with a glass tube. The assembled apparatus was inserted in the transfer chamber (Owl™ VEP-3 Large Tank Electroblotting System, *Thermo Scientific*™), which was then filled to the top with 1x TAE. The transfer was then performed at 24V (PS200-HC, *Hoefer*) for 1h and 45 minutes at 4ºC. When the transfer had finished, the transfer cassette was disassembled and the transferred RNAs fixed to the nylon membrane by UV crosslinking at 1200µj cm^{-2} for 3 minutes (UVC 500 Crosslinker, *Amersham Biosciences*). Transfer efficiency was ascertained by incubating overnight the polyacrylamide gel in a recipient with bidistilled water and ethidium bromide and examining it under UV light in a Gel Doc™ XR+ system (*Bio-rad*®).

Table 10

10% polyacrylamide gel solution recipe for 500ml. Urea was dissolved at 42ºC and the solution filtered with a 0.45µm filter.

Reagents	Quantities
Urea	210g
1x TBE (Table 12)	50ml
Polyacrylamide 40% 19:1 (RNA)	125ml
Autoclaved H_2O Milli-Q®	Until 500ml

Table 11

10x TBE buffer recipe for 1L.

Reagents	Quantities
Tris Base	108g
Boric Acid	55g
EDTA	9.3g
Autoclaved H_2O Milli-Q®	Until 1L

Table 12

20x TAE buffer recipe for 1L.

Reagents	Quantities
Tris Base	48.4g
Acetic Acid (100%)	11.4ml
EDTA 0.5M (pH 8)	20ml
Autoclaved H_2O Milli-Q®	Until 1L

2.8. Synthesis and labelling of probes for Northern Blot analysis

2.8.1. Primer labelling

The ArrS and GadY DNA Northern blot probes were obtained by primer labelling. To this effect, specific DNA oligonucleotides (design in the Clone Manager software, version 9) complementary to the ArrS and GadY genes internal sequences were synthesised (STAB VIDA) (ArrS probe and GadY probe, Appendix, Table I). The obtained DNA oligonucleotides were, respectively, mixed with the reagents depicted in Table 13 and incubated at 37ºC for, at least, 1h. The resulting 5´end [γ-^{32}P] labelled DNA Northern blot probes were purified with a G-25 MicroSpin column (*GE Healthcare Life Sciences*). Labelling was confirmed by measuring radioactivity with a Geiger counter (Mini900 Ratemeter, *Thermo Electron Corporation*) and the probes stored at -20ºC in a lead container.

Table 13

Primer labelling mix used for labeling with [γ-^{32}P] ATP the ArrS and GadY DNA oligonucleotides.

Reagents	Quantities
DNA oligo (10nM)	0.5µl
10x T4 PNK Reaction Buffer (*Thermo Scientific*)	3µl
H_2O Milli-Q®	23.5µl
[γ-^{32}P] ATP (*PerkinElmer*)	2µl
T4 Polynucleotide Kinase (10U/µl) (*Thermo Scientific*)	1µl

2.8.2. Synthesis of the FnrS probe

2.8.2.1. FnrS gene PCR and purification

In order to obtain a probe for the sRNA FnrS, the FnrS gene was firstly amplified using the primers FnrS_*FW* and FnrS_*T7* (designed in the Clone Manager software and synthesised by STAB VIDA) (Appendix, Table I) by polymerase chain reaction (PCR). The FnrS_*T7* primer contains a T7 RNA polymerase promoter, which allows the amplified sequence to be transcribed by this enzyme. The PCR reaction was performed (Table 14) using the DreamTaq™ kit (Thermo Scientific™) and the MyCycler™ thermal cycler (*Bio-Rad*). The PCR program used is depicted in Table 15.

In order to determine whether the PCR was specific, an electrophoresis was performed with 10µl of PCR product. Only one band corresponding to the FnrS amplicon was observed, indicating that the PCR was specific. The PCR product was then purified using the kit "NucleoSpin® Gel and PCR Clean-up" (MACHEREY-NAGEL). To confirm that the PCR product was not lost in the purification step, an agarose-gel electrophoresis with 1µl of the purified PCR product was, then again, performed.

Table 14

PCR mix used for FnrS amplification.

Reagents	Quantities
Genomic DNA	1µl
FnrS_*FW* (1pM)	2µl
FnrS_*T7* (1pM)	2µl
dNTPs (10nM)	1µl
10x DreamTaq Buffer	5µl
DreamTaq DNA polymerase (5U/µl)	0.25µl
H_2O Milli-Q®	38.75µl

Table 15

Thermocycler program used for FnrS amplification.

Cycle Step	Temperature	Time	Number of cycles
Initial Denaturation	95ºC	5 minutes	1
Denaturation	95ºC	30 s	30
Annealing	60ºC	30 s	
Extension	72ºC	30 s	
Final Extension	72ºC	10 minutes	1

2.8.2.2. *In vitro* transcription

In order to obtain a [α-^{32}P] UTP (*PerkinElmer*) labelled RNA Northern blot probe from the amplified FnrS product with a T7 polymerase promoter sequence, an *in vitro* transcription reaction was performed. To this end, the reaction reagents depicted in Table 16 were mixed in a 1.5ml eppendorf tube and incubated for 1h at 37ºC, after which another 0.5µl of T7 RNA Polymerase (Riboprobe® *in vitro* transcription systems, *Promega*) was added. The mix was further incubated for another 2h at 37ºC. When finished, 2µl of DNase (TURBO™ DNase, *Ambion*®) (2U/µl) was added to the mix and incubated for 15 minutes at 37ºC, point at which H_2O Milli-Q® was added to a final volume of 50µl. The [α-^{32}P] UTP RNA FnrS probe was purified from the mix by using a G-25 MicroSpin column (*GE Healthcare Life Sciences*). The probe was then stored at -20ºC inside a lead container.

Table 16

In vitro transcription mix used for the synthesis of RNA from the FnrS PCR product using the Riboprobe® *in vitro* transcription systems (*Promega*)

Reagents	Quantities
Purified FnrS PCR product (FnrS PCR, Appendix, Table I)	2µl
5x Transcription buffer	2.5µl
DTT (10mM)	1µl
rNTP mix (except rUTP) (2.5mM/each)	2µl
rUTP (0.1mM)	1.2µl
RNasin™ Ribonuclease Inhibitor (40U/µl)	0.4µl
[α-^{32}P] rUTP	2.5µl
T7 RNA Polymerase (20U/µl)	1µl

2.9. Northern blot membrane hybridisation and exposure

Northern blot nylon membranes were placed inside a hybridisation bottle (*Stuart*) with 15ml of hybridisation solution (PerfectHyb™ Plus Hybridisation buffer, *Sigma-Aldrich*) and incubated for, at least, 30 minutes at either 68ºC (for the FnrS probe) or 42ºC (for the ArrS and GadY probes) in a hybridisation oven (Hybridisation oven/shaker, *Stuart*) with rotation (4 rpm).

Prior to application, all Northern blot probes were denatured in boiling water for 5 minutes and then chilled in ice for 2 minutes. 10µl of probe were then added to the hybridisation bottles with the hybridisation solution and incubated overnight at either 68 or 42ºC.

After overnight incubation, all radioactive liquid was poured from the hybridisation bottles into a recipient and stored for subsequent disposal. 50ml of wash solution I (Table 17) was added to the hybridisation bottles, which were then incubated at room temperature, with rotation (10rpm), in a hybridisation oven. After 10 minutes, the liquid waste was once again stored and the background radioactive signal of the membrane was measured. If the observed background radioactive intensity was either similar to that of the probe's specific signal or too intense, other washing steps were performed. Increasingly stringent solutions (wash solution II and III) (Table 17) and increasing temperatures were used until either a difference between the background and the probe signal was observable or until the background signal was around 3 or 4 scintillations per second (cps). At this point, the membranes were dried in blot filter papers and encased inside a plastic folder, which was then exposed overnight to a phosphor screen (*GE Healthcare Life Sciences*). The radioactive signal was detected by phosphorimaging using the STORM™ 860 molecular imager (*Molecular Dynamics*). All Northern blot signal quantifications were done using the *ImageQuant TL 8.1*® software (*GE Healthcare Life Sciences*).

Table 17

Wash solution (wash sol.) I, II and III recipe for 1L.

	Wash sol. I	Wash sol. II	Wash sol. III
Reagents	**Quantities**	**Quantities**	**Quantities**
20x SSC (Table 18)	100ml	50ml	25ml
SDS 10%	10ml	10ml	10ml
Bidistilled Water	Until volume	Until volume	Until volume

Table 18

20X SSC buffer recipe for 1L.
pH was adjusted for 7.0 with HCl.

Reagents	Quantities
NaCl	175.3g
Sodium Citrate	88.2g
Autoclaved H_2O Milli-Q®	Until volume

2.10. Quantitative PCR

2.10.1. cDNA synthesis

In order to obtain cDNA without genomic DNA contamination, a DNase (TURBO™ DNase, *Ambion*®) digestion was performed (Table 19) with RNA samples isolated from the WT, Δ*rnb*, Δ*rnr* and Δ*pnp* strains. To this effect, all samples were incubated at 37ºC for 1h, after which another 1µl of DNase was added. Following incubation at 37ºC for another hour, the reaction was stopped by adding inactivation buffer (TURBO™ DNase Kit) and incubating the samples at room temperature for 5 minutes. Finally, the samples were centrifuged and the supernatant collected and stored. Next, in order to further ensure an absence of genomic DNA (gDNA) in the RNA samples, a gDNA wipeout step was performed as described in Table 20 (Quantitect™ reverse transcription Kit, *Qiagen*). A test PCR was then performed (Table 14 and Table 15) in order to verify whether the samples were contaminated with gDNA or not. When negative for gDNA, cDNA was synthesised by adding to the gDNA depleted mix the reagents described in Table 21 and incubating the solution in a thermocycler

(MyCycler™ thermal cycler, *Bio-Rad*) at 42ºC for 30 minutes and then at 95ºC for 3 minutes. The cDNA was stored at -20ºC.

Table 19

TURBO™ DNase digestion for cDNA synthesis protocol

Reagents	Quantities
RNA sample	RNA volume for 5µg
H_2O Milli-Q®	H_2O Milli-Q® volume for 22µl
10x TURBO™ DNase buffer	2.5µl
TURBO™ DNase (2U/µl)	1µl

Table 20

gDNA wipeout step (Quantitect™ reverse transcription Kit, *Qiagen*). The samples were incubated at 42ºC for 2 minutes.

Reagents	Quantities
RNA sample	RNA volume for 1µg
H_2O Milli-Q®	H_2O Milli-Q® volume for 12µl
gDNA Wipeout Buffer	2µl

Table 21

cDNA synthesis mix (Quantitect™ reverse transcription Kit, *Qiagen*).

Reagents	Quantities
Quantiscript Reverse Transcriptase	1µl
Quantiscript RT Buffer	4µl
RT Primer Mix	1µl

2.10.2. qPCR Reaction

Quantitative PCR (qPCR) primers were ordered from STAB VIDA and designed using the QuantPrime software (http://www.quantprime.de/) (Appendix, table I).

WT cDNA was diluted in H_2O Milli-Q® to a final concentration of 10 (1:5), 5 (1:10), 2.5 (1:20), 1 (1:50) and 0.67ng μl^{-1} (1:75). The calibration curves were performed by respectively pipetting, in triplicate, 1.25µl of the previously referred dilutions (1:5, 1:10, 1:20, 1:50 and 1:75) to a new qPCR tube (Strip tubes and caps 0.1ml, *Qiagen*). qPCR mix for the different genes was then prepared, in ice, as described in Table 22.

8.75µl of qPCR mix was then added to all qPCR tubes and a qPCR (Rotor Gene RG-3000, *Corbett*) performed by following the program depicted in Table 23. A no template control (NTC) with H_2O Milli-Q® instead of cDNA was also used. The results were analysed in the Virtual Rotor Gene RG-3000 software (Corbett). Several genes were tested to choose a housekeeping gene and since the *ihfB* gene displayed the lowest inter-strain variance in all examined strains it was chosen as the control gene. Calibration curves were performed as previously described for all the analysed transcripts.

Next, to determine the relative abundance of a given transcript in the mutant strains, we diluted 1:10 the previously obtained Δ*rnb*, Δ*rnr* and Δ*pnp* cDNA in H_2O Milli-Q®. 1.25µl of the dilutions were then pipetted, in triplicate for each strain, to new qPCR tubes. 8.75µl of gene specific qPCR mix was then added to the qPCR tubes and a qPCR performed as previously depicted.

Relative transcript abundance was determined by the ΔΔCt method [119]. *ihfB* was used as the reference gene and the WT as the reference strain.

Table 22

qPCR mix recipe for 1 reaction (8.75µl)
(SensiFAST™ SYBR no-ROX kit, *Bioline*).

Reagents	Quantities
SensiFAST SYBR® No-ROX mix	5µl
primer_FW (1pM) (Appendix, Table I)	0.25µl
primer_RV (1pM) (Appendix, Table I)	0.25µl
H_2O Milli-Q®	3.25µl

Table 23

Thermocycler (Rotor Gene RG-3000, *Corbett*) program used for the qPCRs.

Cycle Step	Temperature	Time	Number of cycles
Initial Denaturation	95ºC	2 minutes	1
Denaturation	95ºC	10s	40
Annealing	60ºC	15s	
Extension	72ºC	20s	
Melting Curve	72-95ºC (increments of 1ºC)	5s/degreeºC	1

3. Results

3.1. Acid Adaptation

3.1.1. The growth of the RNase II mutant is inhibited after acid shock

Previous work has identified several differentially expressed transcripts in the RNase II, RNase R and PNPase deletion strains [35] (unpublished data). Among these, several acid response transcripts (such as ArrS and *gadE*) were found to be down-regulated in all the exoribonucleases deletion strains (unpublished data). We wanted to evaluate how this differential expression of acid related transcripts could affect the response of different exoribonuclease mutants to acidic conditions. To this effect, we determined the growth profile of the MG1693 (wild-type), Δ*rnb,* Δ*rnr* and Δ*pnp* strains after acid shock (in two distinct growth conditions). Cells where either submitted to acidic shock in mid exponential phase (OD_{600} ~ 0.5) (Fig. 4B) or in early stationary phase (OD_{600} ~ 1.5) (Fig. 4C). A control growth curve was performed in parallel (Fig. 4A).

We observed that, when challenged with acid in mid exponential phase, the WT, Δ*pnp* and Δ*rnr* strains recuperated and continued their growth. Nonetheless, Δ*pnp* exhibited a longer lag phase than the one displayed by the WT and the Δ*rnr* strains, which immediately resumed their growth, albeit with a reduced speed when compared with the control curve. Surprisingly, unlike the Δ*rnr* and Δ*pnp* strains, the Δ*rnb* strain never resumed growth after adding HCl in both mid exponential phase and early stationary phase, effectively stabilising at the OD_{600} value in which the acid was added (Fig. 4).

When challenged with acid at OD_{600} ~ 1.5, both WT and Δ*pnp* recovered to values similar to those of the control growth curve (Fig. 4A and 4C). On the other hand, the Δ*rnr* mutant never fully recovered to its control curve OD_{600} values in neither the growth curves (Fig. 4). Furthermore, the final measured OD_{600} was curiously similar in both mid exponential phase and early stationary phase, ~ 4 and ~ 4.3, respectively. When compared to both the WT and the Δ*pnp* strain, a longer lag phase was also observed. Overall, RNase

II seems to have a critical role in acid shock adaptation, with RNase R only displaying a smaller role.

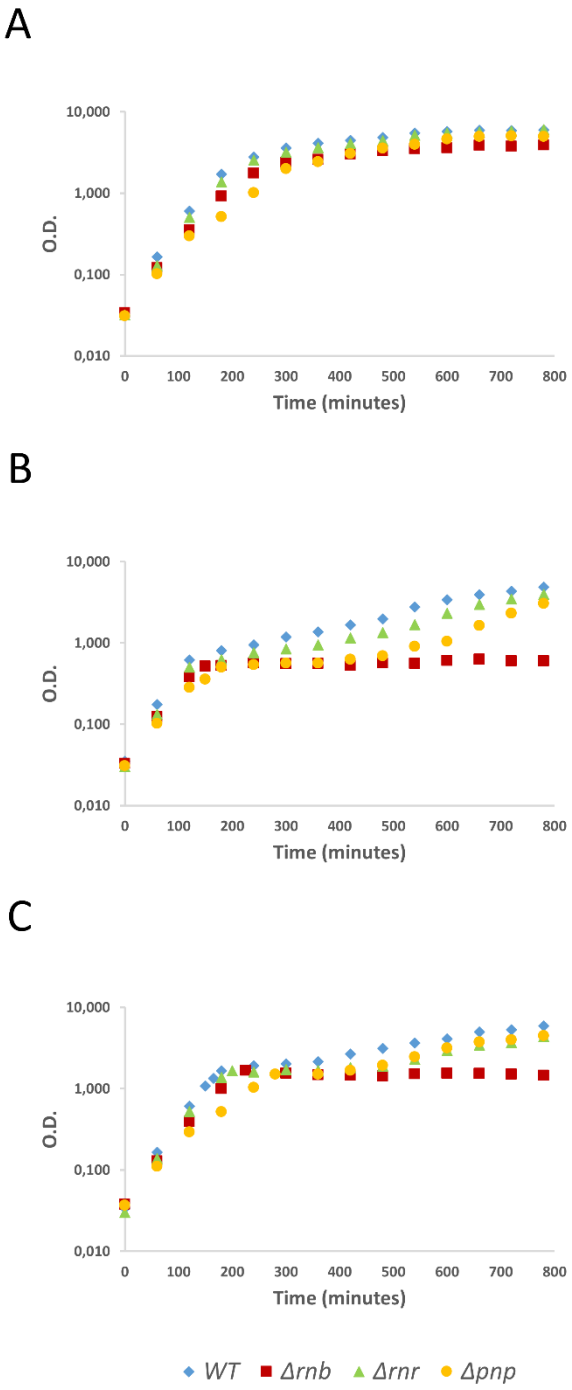

Fig. 4 - Growth curves of the WT, Δ*rnb*, Δ*rnr* and Δ*pnp* strains when submitted to acid shock.

All strains were grown in LB medium supplemented as required (see Methods section). Growth was monitored by measuring the optical density (OD_{600}) at 600nm in time intervals of 60 minutes. Acid shock was performed by adding 150µl of HCl to the medium of the growing cell cultures, changing the culture pH from 7 to 4. (A) Control Growth Curve – All strains were grown in normal conditions, as described in the Methods section. (B) Growth curve with acid shock performed at an OD_{600} of 0.5 – All strains were grown until an OD_{600} of 0.5, point at which acid shock was performed. (C) Growth curve with acid shock performed at an OD_{600} of 1.5 – All strains were grown until an OD_{600} of 1.5, point at which acid shock was performed.

3.1.2. The survival of RNase II mutant is not affected by acid shock

The impaired growth curves observed in Fig. 4 prompted us to hypothesise whether the growth inhibition observed in the Δ*rnb* strain was due to a sharp decrease in overall cell survival following acid shock. In order to test this hypothesis, we assessed and compared the survival of the WT, Δ*rnb,* Δ*rnr* and Δ*pnp* strains before and after acidic shock (Fig. 5).

Unexpectedly the Δ*rnb* strain survival before the acid shock was similar to the survival rate after the acid shock (fold-change of ~ 1). Acid shock thereby appears to influence the Δ*rnb* growth rate, but not the cell survival. On the other hand, in the Δ*rnr* strain, cell survival was in average ~ 2.3 fold lower in the cells submitted to acid shock, when compared with the control. A similar situation was also observed in the WT, although to a lesser extent (in average 1.5 fold). Regarding the Δ*pnp* strain, a slight decrease in cell survival (~ 1.3 fold) following acid shock was observed.

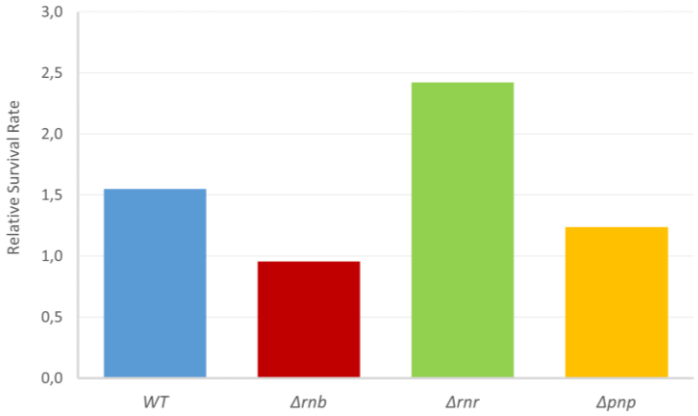

Fig. 5 - Relative Survival of the WT, Δ*rnb*, Δ*rnr* and Δ*pnp* strains after acid shock.

All strains were grown in LB medium supplemented as required (see Methods section). Growth was monitored by measuring the OD_{600} at 600nm and acid shock was performed at an OD_{600} of 0.5. Both before and after acid shock, the cells were inoculated in LA medium supplemented as required and incubated at 37ºC. Colonies were counted after 40h (see Methods section). The results represent the average of two independent experiments. The relative survival represents the fold-change between the survival of cells not submitted to acid shock and the cells submitted to acid shock, in a given strain.

3.1.3. ArrS expression is growth phase dependent, GadY is not

In order to explain the growth changes observed following acidic shock (Fig. 4), we theorised about the possible involvement of the exoribonucleases in the regulation of acid response related sRNAs. For example, by impairing proper sRNA function, RNase II could be hindering cell growth in an acidic environment. To further explore this hypothesis, we examined the levels of the *cis*-sRNAs ArrS and GadY, which were previously determined to be important in the *E. coli* adaptation to acidic stress [28, 100]. To this effect, several Northern blots with RNA extracted in distinct growth conditions were performed, using probes for ArrS and GadY sRNAs.

We started by confirming whether, in our conditions, the expression of both sRNAs was growth phase dependent as previously reported [28, 100]. To this end, two Northern blots were performed with RNA samples extracted from the WT, Δ*rnb*, Δ*rnr* and Δ*pnp* strains in both exponential and stationary phase (Fig. 6A and 6B).

Similarly to what was reported, ArrS expression was confirmed to be induced in stationary phase, but not in exponential phase (Fig. 6A). Moreover, in accordance with what was previously published, multiple ArrS break-down products are also observed [99]. We also observe that, in the Δ*rnb* mutant, the ArrS levels are drastically reduced when compared with the WT.

Regarding the GadY sRNA, no differential expression was detected between the stationary and the exponential phase (Fig. 6B). The GadY levels were similar in all tested strains, although its levels are slightly increased in the Δ*rnb* strain. These results were unexpected as previous reports have stated that GadY is undetectable in exponential phase. Moreover, and unlike what was previously observed (Opdyke and collaborators), only one of the three described forms of GadY sRNA was detected [28]. Despite this apparently contradictory results, it is noteworthy to specify that a different *E. coli* strain was used in the described reports.

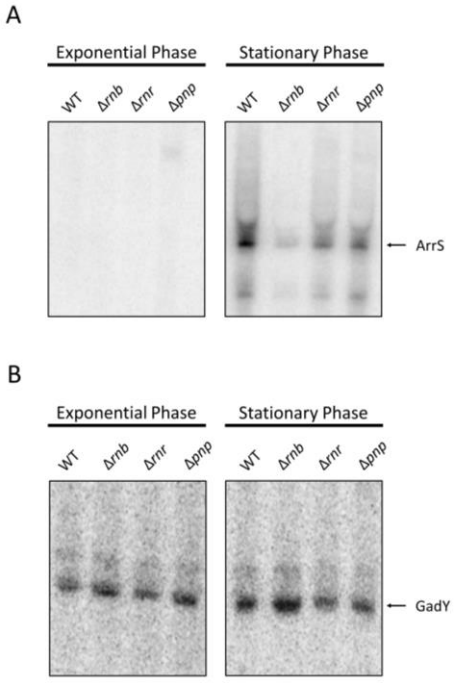

Fig. 6 - Growth phase expression of acid response related sRNAs in the WT, Δ*rnb*, Δ*rnr* and Δ*pnp* strains.

Small RNA expression was analysed by Northern blot. Exponential phase RNAs were isolated from all the referred strains in mid exponential phase (OD_{600} of 0.5) and stationary phase RNAs were isolated after 16h of incubation, as described in the Methods section. A sRNA specific [γ-^{32}P] labelled DNA probe was used to detect the ArrS (A) and GadY (B) sRNAs, respectively.

3.1.4. ArrS levels are dependent on RNase II

As observed in Fig. 6A, ArrS was differentially expressed in all the exoribonuclease mutant strains, especially in the RNase II mutant. This result prompted us to investigate the role of the exoribonucleases in the degradation of the ArrS sRNA. To this end, we determined the ArrS half-life in the WT, Δ*rnb*, Δ*rnr* and Δ*pnp* strains by performing a Northern blot with RNA samples extracted at different time points after inhibiting transcription with rifampicin (see methods section, Fig. 7).

Similarly to what was observed in Fig. 6A, ArrS was not significantly expressed in the Δ*rnb* mutant in levels that could be used to calculate its half-life (Fig. 7). ArrS levels seem to be, therefore, dependent on the presence of the exoribonuclease II. When compared with the WT, the ArrS levels are also reduced in the Δ*rnr* strain. Curiously, this strain displayed the longest ArrS half-life (over 60 minutes). In the Δ*pnp* mutant

strain, the sRNA ArrS seems to be slightly less stable, with a half-life of only 38 minutes, which is inferior to the one determined for the WT (47 minutes).

Until now, PNPase was considered to be the main exoribonuclease associated with sRNA degradation [58]. Nonetheless, our results indicate that ArrS, a major sRNA in acid response regulation, is primarily affected by RNase II and, to a lesser extent, RNase R.

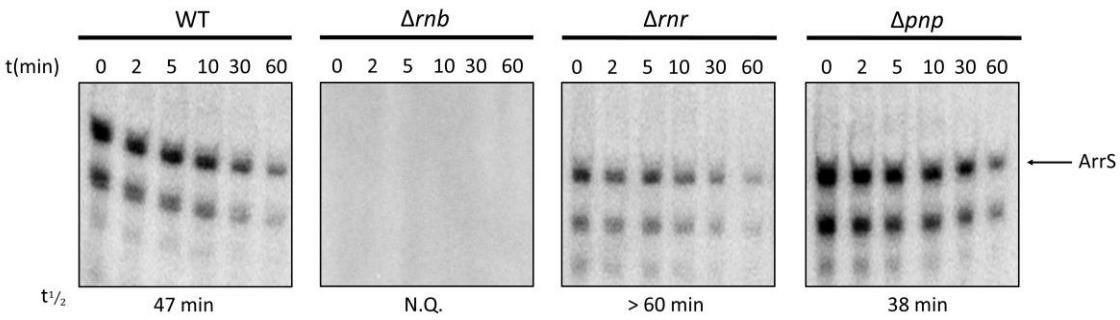

Fig. 7 - Northern blot analysis of the stability of the sRNA ArrS in the WT, Δ*rnb*, Δ*rnr* and Δ*pnp* strains.

RNA was extracted from the MG1693 (WT), Δ*rnb*, Δ*rnr* and Δ*pnp* strains in stationary phase at the specified time periods after adding rifampicin to inhibit transcription (see Methods section). The top bands corresponding to the full length ArrS sRNA were quantified and plotted against the extraction time, thus allowing half-life calculation. N.Q. refers to non-quantifiable due to low band signal.

3.1.4.1. RNase II complement strain rescues ArrS levels

The decreased ArrS sRNA levels obtained in the Δ*rnb* strain prompted us to question whether the inverse situation would be observable in a Δ*rnb* strain complemented with an RNase II expression plasmid. To this end, we performed a Northern blot with RNA extracted from the WT, Δ*rnb* and Δ*rnb* containing a plasmid expressing RNase II (Δ*rnb* + pCMA01) strains (Fig. 8A).

We observe that the ArrS levels are partly restored to their WT values (Fig. 8B) in the Δ*rnb* expressing RNase II strain and that in the Δ*rnb* strain there is a ~90% reduction in ArrS levels. These results seem to confirm our previous hypothesis of the ArrS sRNA levels being directly dependent on the expression of the RNase II enzyme.

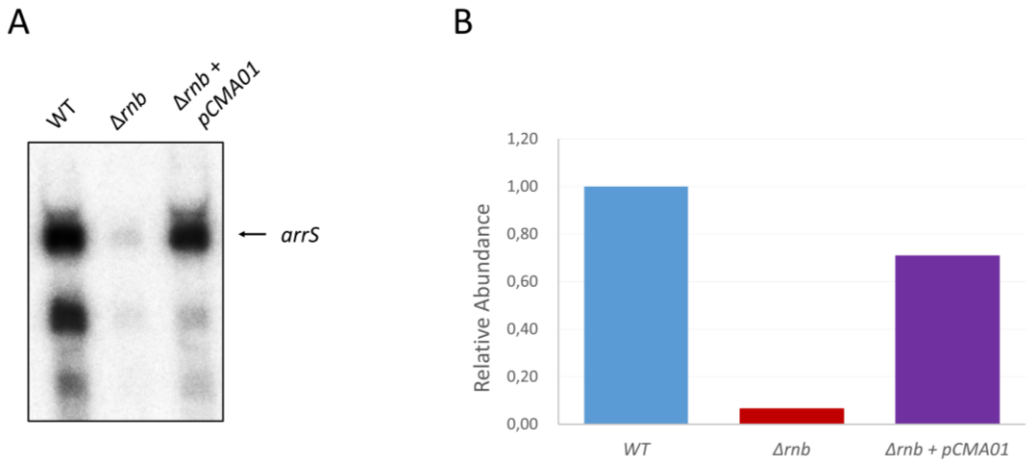

Fig. 8 - Northern blot analysis of the stability of the sRNA ArrS in the WT, Δ*rnb* and Δ*rnb* + pCMA01 strains and strain specific fold-change values.

(A) RNA was extracted from the MG1693 (WT), Δ*rnb*, and Δ*rnb* + pCMA01 strains in stationary phase and a northern blot was performed (see Methods section). (B) The bars represent the strain specific fold change (ratio) in the sRNA ArrS levels when compared with the WT (fold change of 1).

3.1.5. GadY stabilisation is exoribonuclease independent

Despite the identical levels of GadY sRNA in all analysed exoribonuclease mutants (Fig. 6B), we decided, similarly to what was performed for ArrS, to determine whether the half-life of the GadY sRNA differed between the WT and the Δ*rnb*, Δ*rnr* and Δ*pnp* mutants.

Much alike our previous results, no differential expression was observed, with all the strains displaying similar levels of GadY sRNA (Fig. 9). Furthermore, no GadY degradation was observed in the 60 minute time frame that the RNA samples encompass. In fact, its half-life was superior to 60 minutes in all tested strains.

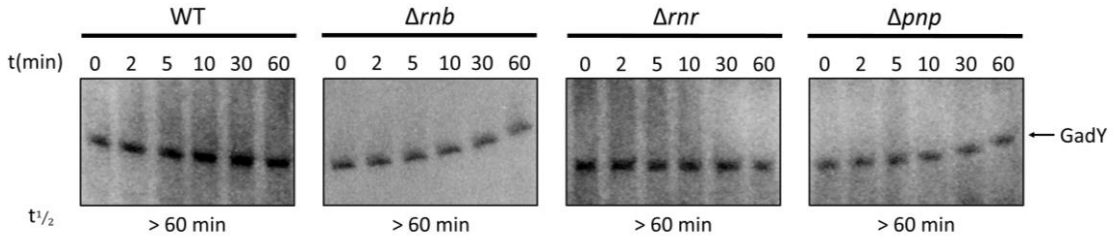

Fig. 9 - Northern blot analysis of the stability of the sRNA GadY in the WT, Δ*rnb*, Δ*rnr* and Δ*pnp* strains.

RNA was extracted from the MG1693 (WT), Δ*rnb*, Δ*rnr* and Δ*pnp* strains in stationary phase at the specified time periods after adding rifampicin to inhibit transcription (see Methods section). The GadY sRNA bands were quantified and plotted against the extraction time, thus allowing half-life calculation.

3.1.6. Hfq influences the levels of the sRNA ArrS

Amidst other functions, the RNA chaperone Hfq primarily facilitates the base-pairing interactions that are established between the *trans*-sRNAs and their targets [107]. Despite this important function, the Hfq role in the establishment of productive *cis*-sRNA/mRNA interactions is still debatable.

Previous work has implicated Hfq in the stabilisation of the *cis*-sRNA GadY [28]. In order to confirm this findings and also examine the stabilisation level of the *cis*-sRNA ArrS, we decided to determine the half-life of both the GadY and ArrS *cis*-sRNAs in a Δ*hfq* strain (Fig. 10).

Curiously, by comparison with the WT, Δ*rnr* and Δ*pnp* strains (Fig. 7), the sRNA ArrS levels were significantly reduced in the Δ*hfq* mutant (Fig. 10A). In fact, its levels were more similar to those of the Δ*rnb* mutant, possibly suggesting an involvement of the Hfq protein in ArrS stabilisation. Regarding the GadY sRNA, and unlike what was previously described [28], the levels in the Δ*hfq* strain were not different from those in the WT. Actually, GadY displayed similar levels in all the tested strain (WT, Δ*hfq*, Δ*rnb*, Δ*rnr* and Δ*pnp*) (Fig. 9 and 10B).

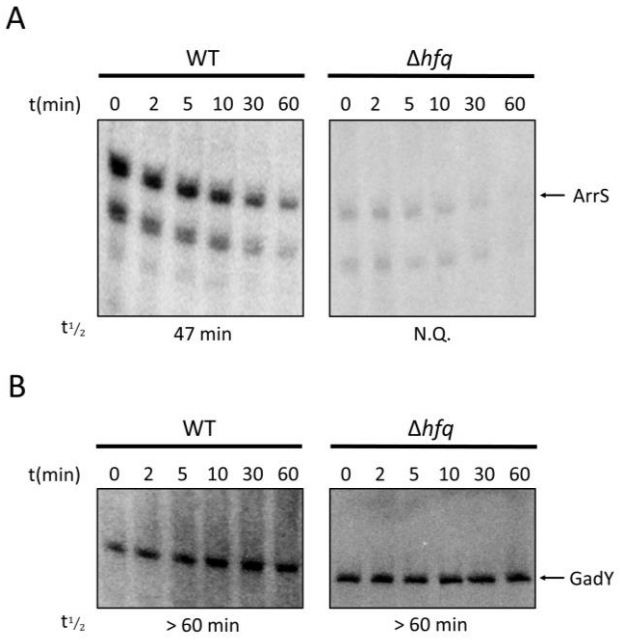

Fig. 10 – Northern blot analysis of the ArrS and GadY sRNA in the WT and Δ*hfq* strain.

RNA was extracted from cells in stationary phase at the specified time periods after inhibiting transcription. A Northern blot with the obtained RNA samples was performed as previously described. Due to low signal, no half-life was determined in the Δ*hfq* strain hybridised with the ArrS probe.

3.1.7. Exoribonucleases influence the levels of mRNAs related with acid response mechanisms

The lack of the acid response activator ArrS in the RNase II mutant strain (Fig. 7) seems to correlate with the growth arrest observed in the referred strain when submitted to acid shock (Fig. 4C). On the other hand, the GadY sRNA, which is also an acid response activator, is not down-regulated in any tested strain. This result apparently discards GadY as the preponderant responsible for the RNase II mutant growth arrest. Nonetheless, as both the ArrS and GadY sRNAs influence the expression of mRNAs, we decided to test if the exoribonucleases also affected the levels of the respective target mRNAs. To this effect, we decided to assess the levels of four acid-related mRNAs: *gadE*, *gadX*, *gadW* and *hdeA*. The *gadE* mRNA is an ArrS target, the levels of the *gadX* and *gadW* mRNAs are regulated by GadY and the *hdeA* mRNA is regulated by *gadE*, *gadX* and *gadW* [120, 121]. *hdeA* is required for chaperone function in low pH conditions [122].

The levels of the mRNAs were determined by quantitative real-time PCR (qPCR) and are relative to the expression values of the WT strain (fold-change = 1.00).

In order to compare the abundance of a transcript in the WT strain with the respective abundance in the Δ*rnb*, Δ*rnr* and Δ*pnp* mutants, a control gene with similar expression values in all the tested strains was required. By using this control gene as a comparison point, it is possible to ascertain if a transcript is down or up regulated in regards to the WT. To this effect, a calibration curve (see Method section) was performed for the *pepA*, *ybbN* and *ihfB* genes, which, according to our unpublished RNA-seq data, exhibited a low inter-strain expression variance. Due to displaying the lowest qPCR inter-strain variance, the *ihfB* gene was chosen as the control gene.

As expected, in the Δ*rnb*, Δ*rnr* and Δ*pnp* strains, the mRNA *gadE* was down regulated, having similar values in both Δ*rnb* and Δ*rnr* (Table 24). This results were foreseeable as the ArrS sRNA is down-regulated, to different extents, in all the examined exoribonuclease mutant strains (Fig. 7 and Fig. 6). Nonetheless, despite the absence of the activator ArrS sRNA in the Δ*rnb* mutant, the *gadE* transcript was still detected by qPCR, possibly due to the different *gadE* mRNA forms. Regarding the *gadX* and *gadW* mRNAs, the Δ*pnp* strain displayed the highest fold change (2.78 and 3.41, respectively), with the Δ*rnb* strain also having a fold change superior to 1, although not as expressive. In the Δ*rnr* mutant the expression values of both mRNAs seem to be closer to the levels found in the WT, possibly discarding RNase R as a *gadX* and *gadW* regulator. Regarding the *hdeA* mRNA, despite being regulated by the GadX, GadW and GadE proteins, we observe that its levels are more similar to the ones of the *gadE* mRNA than to those of the *gadX* and *gadW*. In fact, the *hdeA* mRNA was also down-regulated in all the tested strains, especially in the Δ*rnb*, where it was ~ 7 fold lower than in the WT. Indeed, the combined effects of the reduced levels of both the *gadE* and *hdeA* mRNAs might partly explain the growth arrest observed after performing acid shock in the RNase II mutant.

Overall, these results indicate that the exoribonucleases have a role on the regulation of acid induced RNAs in both normal and acidic pH conditions. This is surprising, as it implies the existence of a yet unknown exoribonuclease layer of regulation in the *E. coli* acid adaptation system.

Table 24

Fold-change values of the *gadE*, *gadX*, *gadW* and *hdeA* mRNAs in the Δ*rnb*, Δ*rnr* and Δ*pnp* strains.

Transcript	Strain	Fold-change*
gadE	Δ*rnb*	0.17
	Δ*rnr*	0.10
	Δ*pnp*	0.50
gadX	Δ*rnb*	1.69
	Δ*rnr*	1.58
	Δ*pnp*	2.78
gadW	Δ*rnb*	2.08
	Δ*rnr*	0.81
	Δ*pnp*	3.41
hdeA	Δ*rnb*	0.14
	Δ*rnr*	0.63
	Δ*pnp*	0.87

* Fold-changes were calculated as the ratio between the mutant and the WT expression values. Values superior/inferior to 1.00 correspond to up-regulated/down-regulated transcripts, respectively.

3.2. Anaerobic Adaptation

3.2.1. RNase II stabilises the sRNA FnrS in aerobic conditions

Recently published RNA-Seq data regarding the roles of the exoribonucleases in *E. coli* metabolism suggest that FnrS might be expressed in aerobic conditions in the RNase II and RNase R mutants [35]. A surprising result, considering that almost no FnrS expression had been previously detected in aerobic conditions [81]. In fact, FnrS is induced in anaerobic conditions and acts as repressor of several aerobic related mRNAs.

To further explore the influence of the exoribonuclease II on the regulation of stress related sRNAs, we decided to both examine the levels of the FnrS sRNA and determine its half-life. To this effect, Northern blots were performed with RNA samples extracted at specific time points after inhibiting transcription (Fig. 11)

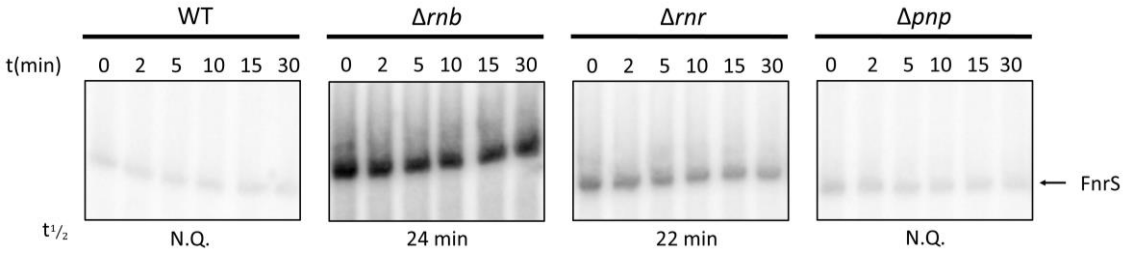

Fig. 11 - Northern blot analysis of the stability of the sRNA FnrS in the WT, Δ*rnb*, Δ*rnr* and Δ*pnp* strains incubated in aerobic conditions.

RNA was extracted from cells in exponential phase at the specified time periods after inhibiting transcription. A Northern blot with the obtained RNA samples was performed as described. The FnrS sRNA bands were quantified and plotted against the extraction time, thus allowing half-life calculation. N.Q. refers to non-quantifiable due to low band signal. An FnrS sRNA specific [α-^{32}P] labelled RNA probe was used to detect the FnrS sRNA.

As expected, in both the WT and Δ*pnp* strains, almost no FnrS was detected in aerobic conditions, thus preventing half-life calculation. In the Δ*rnb* strain however, we determined, at time 0, an increase of ~ 27 fold in the FnrS levels when compared with the WT (Fig. 12A). The Δ*rnr* mutant also exhibited an increased FnrS expression, although of only 5 fold. Despite this difference, the FnrS half-life in both the Δ*rnb* and Δ*rnr* strains is strikingly similar, 24 and 22 min, respectively (Fig. 11).

Interestingly, in the Δ*rnb* strain, the FnrS levels are dramatically increased, being similar to those found in anaerobic conditions (Fig. 12A and 12B). This situation is novel and reveals that RNase II, by controlling the levels of the FnrS sRNA, displays a key role in the anaerobic/aerobic metabolism.

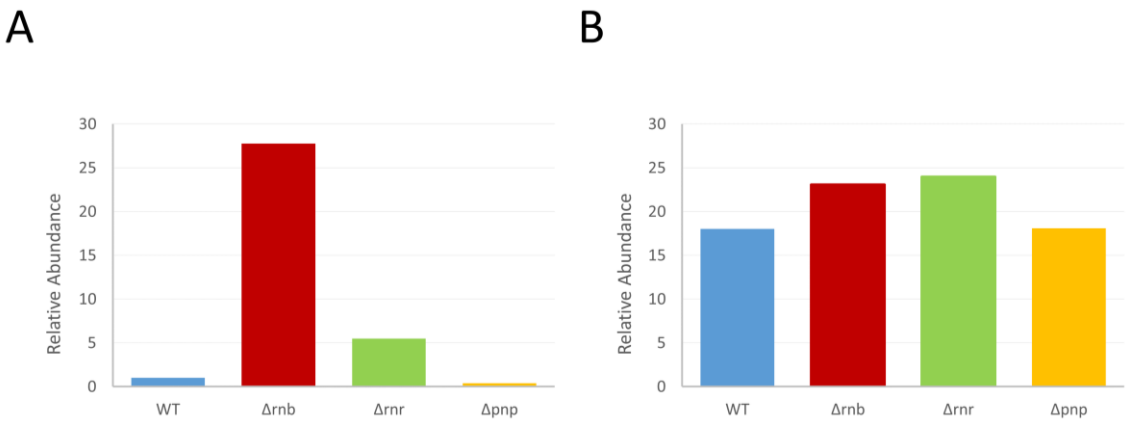

Fig. 12 – Strain specific sRNA FnrS fold-change values in aerobic and anaerobic conditions.

(A) The bars represent the strain specific fold change (ratio) in the sRNA FnrS levels at time 0 in aerobic conditions (**Fig.** 11), when compared with the WT (fold change of 1). (B) The bars represent the strain specific fold change obtained when the FnrS levels at time 0 in cells submitted to an anaerobic environment (**Fig.** 13) are compared to those of the WT in aerobic conditions (**Fig.** 11). A higher relative abundance level represents a higher level of detected FnrS in the compared strain. The quantifications of the Northern blot bands were performed as previously described.

3.2.2. The sRNA FnrS levels are differentially degraded before and after anaerobic induction

Next, we analysed the FnrS levels in an anaerobic environment and determined how it is degraded following aerobic shift. To this effect, we firstly induced the expression of the sRNA FnrS by incubating the cells in anaerobic conditions. We then shifted the cells into an aerobic environment and extracted RNA samples at different time points after the anaerobic shock. Next, we determined by Northern blot the levels of the sRNA FnrS (Fig. 13).

Similarly to what occurred in aerobic conditions (Fig. 12A), in an anaerobic environment the FnrS levels are superior in both the Δ*rnb* and Δ*rnr* strains (Fig. 12B).

In this conditions, the WT exhibited the lowest FnrS half-life (5.82 min) and Δ*rnb* the highest (11 min) (Fig. 13). In the Δ*pnp* strain, the half-life value was similar to the

one obtained for the WT. Curiously, the FnrS half-life in the Δ*rnb* and Δ*rnr* strains was similar, 11 and 9.63 min, respectively. An analogous situation was observed in aerobic conditions (Fig. 11), with the half-life in both strains diverging no more than 2 minutes.

When comparing the FnrS levels in aerobic and anaerobic conditions, we observe that the sRNA levels are significantly increased in the WT, Δ*rnr* and Δ*pnp* strains under an anaerobic environment. In the Δ*rnb* strain, however, we determined a fold change decrease from ~ 28 to ~ 23 (Fig. 12). Both the WT and Δ*pnp* strains displayed similar levels, having a ~ 18 fold increase in anaerobic conditions. Curiously, both the half-life and the FnrS levels in the Δ*rnr* strain were similar to those of the Δ*rnb* strain after anaerobic shift, a situation that, although present, was less evident in aerobic conditions (Fig. 11 and 13). Both RNase II and RNase R thus appear to destabilise the sRNA FnrS, suggesting an involvement of these RNases in anaerobic regulation.

Interestingly, unlike what was observed in aerobic conditions, the FnrS sRNA is rapidly degraded in all the strains subjected to anaerobic shock (11 min. in the Δ*rnb* mutant subjected to anaerobic shift Vs. 24 min. in the Δ*rnb* mutant grown in aerobic conditions). Indeed, after 20 minutes, the FnrS levels are similar in all the tested strains, a situation that was not observed in aerobic conditions. This is surprising as, according to the results obtained for the Δ*rnb* mutant in aerobic conditions, the levels of the sRNA FnrS after anaerobic induction (with degradation monitored in aerobic conditions) should not be lower than those found in the RNase II mutant in aerobic conditions. This discrepancy is especially visible at 20 minutes after anaerobic shock.

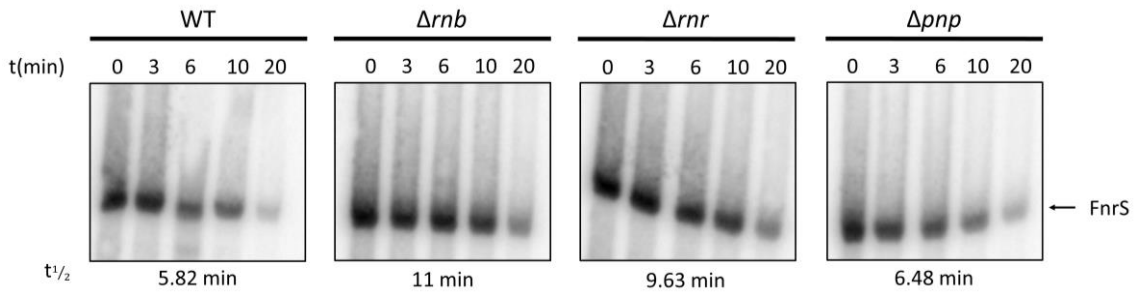

Fig. 13 - Northern blot analysis of the stability of the sRNA FnrS **in the WT, Δ*rnb*, Δ*rnr* and Δ*pnp* strains after anaerobic induction.**

The sRNA FnrS expression was induced by incubating exponential phase cells (OD_{600} of 0.5) for 2h in an anaerobic chamber. RNA was extracted at the specified time periods immediately following the shift to aerobic conditions. A Northern blot was performed with the purified RNAs and the half-life determined, as previously described.

3.2.3. RNase II influences the levels of *gpmA*, an FnrS target

Based on the results obtained for the FnrS sRNA in aerobic conditions, we decided to examine if the increased levels of FnrS in the RNase II mutant resulted in a reduced level of an FnrS target. To this effect, a qPCR of the *gpmA* mRNA was performed (Table 25). *gpmA* encodes the enzyme 2,3-bisphosphoglyerate-dependent phosphoglycerate mutase, which is involved in the glycolytic pathway [123]. By blocking the *gpmA* mRNA translation, among others, FnrS can fine tune the cell adaptation to anaerobic conditions.

As expected, *gpmA* down-regulation was more pronounced in the Δ*rnb* strain (0.43), followed by the Δ*rnr* mutant. The Δ*pnp* mutant displayed values similar to those in the WT (0.93), in accordance with the results obtained for FnrS expression.

Table 25

Fold-change values of the *gpmA* mRNA in the Δ*rnb*, Δ*rnr* and Δ*pnp* strains.

Transcript	Strain	Fold-change*
gpmA	Δ*rnb*	0.43
	Δ*rnr*	0.79
	Δ*pnp*	0.93

*Fold-changes were calculated as previously referred. Values superior/inferior to 1.00 correspond to up-regulated/down-regulated transcripts, respectively. The values represent the mean and standard deviation of three independent experiments.

3.2.4. Anaerobic growth rate is not affected by exoribonucleases

Next, we tested if the increased stability of the FnrS sRNA in the RNase II and RNase R deletion strains would result in a faster adaptation to anaerobic conditions in the same mutants. To this effect, the WT, Δ*rnr*, Δ*rnb* and Δ*pnp* strains were grown in an anaerobic atmosphere and their respective OD_{600} regularly measured. None of the strains passed the OD_{600} mark of 1 (Fig. 14), with the WT maxing out at an OD_{600} of 0.92 and the Δ*rnb* only achieving an OD_{600} of 0.26, the lowest of the 4 tested strains. Despite this difference, when compared with the control growth curve (Fig. 4A) similar discrepancies are observed in all the tested strains, with the Δ*rnb* strain also displaying the lowest OD_{600} at the end of the experiment.

We hypothesised that the RNase II mutant would adapt faster to the anaerobic conditions due to the increased levels of FnrS in this strain. Despite not being able to confirm this hypothesis, the anaerobic growth curve results are still expected. Being a strictly anaerobic growth curve and with all the strains displaying similar values of the FnrS sRNA in anaerobic conditions (Fig. 13), it was predictable that all the growth curves would be analogous to the control (Fig. 4A), albeit in an anaerobic environment. Indeed, this was what was observed.

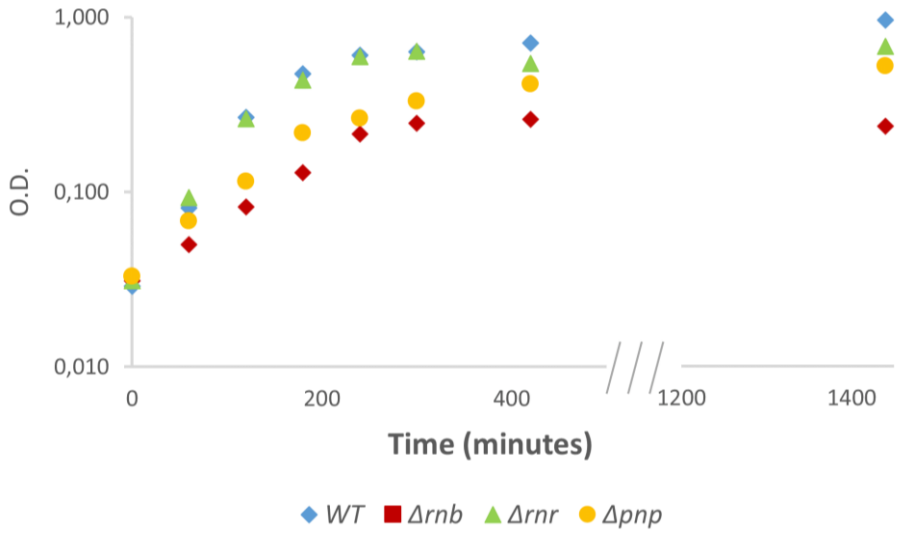

Fig. 14 – Anaerobic growth curve of the WT, Δ*rnb*, Δ*rnr* and Δ*pnp* strains.

All strains were grown in anaerobic conditions in LB medium supplemented as required (see Methods section). Growth was monitored by measuring the OD_{600} in time intervals of 60 minutes.

4. Discussion and Conclusions

In this work we have shown that the cellular role of the RNase II enzyme is more versatile than previously thought. Indeed, by controlling the levels of the acid induced ArrS sRNA and being essential for cell growth resumption following acid shock, we have demonstrated that RNAse II is a crucial post-transcriptional regulator in acid stress adaptation. Moreover, we have also shown that both RNase II and, to a lesser extent, RNase R, seem to have an important role in the early phases of anaerobic adaptation and in the regulation of the FnrS sRNA in aerobic conditions. In this latter case, we suspect that the expression of the FnrS sRNA is the result of complex and indirect RNase mediated effects.

4.1. Acid Adaptation

The general stress response is induced in the presence of stresses, such as low pH, and is accompanied by a reduction or cessation of growth [124]. This general response is σ^S dependent and acts by rearranging the genetic program of the cell. In the growth curves obtained for the WT, RNase R and PNPase mutant strains this situation is clearly noticeable, with all the referred strains displaying, to different extends, a lag phase immediately after acid shock (Fig. 4B and 4C). These results correlate with the survival assay data, where the referred strains displayed, although with different extents, an impaired survival capacity 2h after acid shock, which was still in the lag phase (Fig. 5 and 4B). Curiously, the RNase R mutant strain exhibited the lowest acid survival capacity. This was unexpected as this strain growth was similar to that of the WT, which prompted us to anticipate that a similar survival capability would be observable. The PNPase mutant strain displayed the longest adaptation phase and only a slightly impaired survival (Fig. 4B, 4C and 5), possibly due to its effects on the regulation of general stress responses [58].

In the RNase II mutant strain we observed that, unlike what occurred for the other strains, the culture did not resume growth following acid shock. In fact, after adding HCl, the cell culture OD_{600} values neither decreased nor increased for the remaining duration of the experiment (Fig. 4B and 4C). RNase II deficient cells seem, therefore, to shift into

a dormant state in low pH conditions. We firstly considered that this growth arrest could be originating from an improperly regulated acid-related sRNA. In fact, after analysing our unpublished RNA-seq data, we discovered that RNase II was significantly affecting the levels of the *cis*-sRNA ArrS. This sRNA is expressed in both stationary phase and low pH conditions and controls the change between the T3 and T2 forms of the *gadE* mRNA, whose protein product is a master regulator in the gad system [99, 100]. This system facilitates *E. coli* survival in environments with pH as low as 2 and requires the presence of glutamate. The system *modus operandi* is based on the action of its two main effectors, GadA and GadB, which lower the intracellular pH by consuming protons during the decarboxylation of glutamate. This reaction ultimately originates γ-aminobutyric acid, which is exported out of the cell by GadC [94].

We found that ArrS is severely down-regulated in the absence of RNase II and is restored to its WT levels in the RNase II mutant strain complemented with RNase II (Fig. 7, 8A and 8B). These results suggest that RNase II somehow protects the ArrS sRNA from degradation. This is not surprising, as similar situations have been previously reported for this enzyme [32]. For example, the *rpsO* mRNA and the antisense RNA-OUT, involved in Tn10/IS10 transposition, are destabilised in cells lacking RNase II [27, 31]. This protection mechanism is an indirect result of RNase II activity, which removes poly(A) tails but is incapable of proceeding through secondary structures, leaving the bulk RNA intact [27, 31]. The lack of poly(A) tails in the RNase II substrates hinders both PNPase and RNase R activity, which require, although to different extents, a ssRNA overhang. The consequent decrease in the degradation rate eventually results in an accumulation of the protected RNase II targets [39, 59, 60]. Therefore, RNase II could be contributing to the stability of the ArrS sRNA by removing its poly(A) tail. However, another hypothesis is also plausible. RNase II could be affecting ArrS transcription by destabilising an ArrS transcriptional activator.

PNPase is the main exoribonuclease involved in the degradation of Hfq-free sRNAs, however, in mRNAs, RNase R has been demonstrated to surpass PNPase activity when in the presence of short poly(A) tails [39, 59]. We thus propose that a similar process is at play for the ArrS sRNA, with RNase R being the main responsible for the degradation of the ArrS sRNA in the presence of RNase II. Indeed, we observe that the

ArrS levels are stabilised in the RNase R mutant strain, indicating an involvement of this enzyme in the degradation of this sRNA (Fig. 7). Therefore, RNase R could be surpassing the activity of PNPase due to the existence of short 3´ ssRNA segments, to which PNPase difficultly binds to [39]. Interestingly, in the PNPase mutant strain we observe that the ArrS sRNA is destabilised when compared with the WT, possibly indicating that, to a small degree, the levels of this sRNA are also protected by PNPase.

Our results further display that in the absence of Hfq, and similarly to what is observed in the RNase II mutant strain, the ArrS sRNA is also drastically reduced (Fig. 10). Likewise RNase II, Hfq could be protecting the ArrS sRNA from the action of other ribonucleases. Indeed, this situation is not novel, being well documented [61, 113, 114]. However, considering our results, it seems that both RNase II and Hfq are simultaneously required to maintain an ArrS level similar to that of the WT. We therefore suspect that other effects, more complex than simple Hfq/RNase II simultaneous RNA protection, may be in action. Interestingly, a similar situation has been previously reported for RNase E and Hfq [125]. In this case, the authors attributed the condition to both a direct RNase effect on RNA processing and to an Hfq mediated indirect effect in various transcriptional activators. Considering that ArrS expression is possibly regulated by σ^S and σ^{70}, a parallelism with the previous report could be established. Encoded by the *rpoS* gene, the σ^S is a master stress regulator in *E. coli*. It controls, among others, the expression of genes involved in both the acid response system and in the entry to stationary phase [126]. We thus hypothesise that, like previously stated, RNase II could be either directly protecting the ArrS sRNA from degradation or indirectly affecting its transcription through the regulation of an ArrS transcriptional activator, such as RpoD (σ^{70}) or RpoS. In fact, according to our unpublished RNA-seq data, RpoD is down regulated in the RNase II mutant strain. Regarding the *rpoS* mRNA, it requires the action of a sRNA activator, *dsrA*. This sRNA binds to the 5´ region of the mRNA and releases an inhibitory stem-loop, allowing *rpoS* to be transcribed. It is noteworthy to specify that this reaction is mediated by Hfq, being severely hindered in the absence of this latter [127]. Considering that Hfq is crucial for the proper function of *trans*-sRNAs, not being considered fundamental to *cis*-sRNAs action [107], it would be plausible to suggest that the role of Hfq on ArrS regulation is, most likely, indirect. Taken together, the described mechanisms could explain why both Hfq and RNase II need to be simultaneously present

in order for the ArrS sRNA to maintain its WT-like levels. The lack of RNase II would cause the ArrS sRNA to be rapidly degraded (or not transcribed) and the absence of Hfq would hinder the transcription of ArrS by affecting a transcriptional activator, such as RpoS. Overall, the absence of either one of the referred proteins affects ArrS action and jeopardises the expression of the gad system.

It is plausible to suggest that ArrS could be responsible for the growth inhibition observed after acid shock in the RNase II mutant strain (Fig. 4B and 4C). The low levels of ArrS detected in this strain could be affecting the activation of the *gadE* mRNA. Being GadE a major activator in both the gad system and in the general acid adaptation mechanism, it is expected that a deficiency in the processing of this transcript results in a major disruption of this adaptive pathway [98, 101].

Besides ArrS, we also examined the acid related GadY sRNA. GadY is also a *cis*-sRNA whose expression is increased in entry to stationary phase and in low pH conditions. [95, 96]. The GadY sRNA promotes the cleavage of the *gadX-gadW* transcript, which originates both the *gadX* and *gadW* mRNAs. Overall, the results obtained for GadY were not as expected as they significantly diverged from what was previously described [28]. For example, Opdyke and collaborators detected three GadY forms while we only observed one (Fig. 9). Our data for the GadY expression in the Hfq mutant strain was also not as expected since it was previously reported that GadY expression was dependent on Hfq and our results indicate that Hfq does not affect GadY (Fig. 10) [28]. Moreover, once again contrary to what was previously reported, we also observed GadY expression in exponential phase (Fig. 6). In all this cases we believe that the obtained divergent results are a by-product of the different strains used in the performed experiments.

Both ArrS and GadY displayed unusually long half-life values in the presence of rifampicin, a transcriptional inhibitor (Fig. 7, 9 and 10). This situation is not uncommon in antisense sRNAs [128, 129]. In fact, according to Massé and collaborators, this condition occurs due to the great abundance of a sRNA when a specific stress signal, such as stationary phase, induces its expression [110]. When transcription stops due to the presence of rifampicin, the accumulated sRNA reacts stoichiometrically with the remaining target until this latter is eliminated, point at which the residual sRNA is

indefinitely maintained in the cell. This situation explains, to different extents, the time dependent steady decrease of the ArrS sRNA levels in the WT, PNPase and RNase R mutant strains. In both the RNase II and Hfq mutant strains this situation is not observable because the ArrS sRNA is not present.

The GadY sRNA does not seem to decay over time in any of the tested strains. This result possibly suggests that the GadY sRNA target was already almost absent when transcription was inhibited. Considering that GadY function is self-destructive, a lack of targets would cause an indefinite accumulation of this sRNA in the cell. Indeed, previous reports stated that the *gadX-gadW* mRNA, which is the target of the GadY sRNA, is nearly undetectable, possibly being processed as it is transcribed [68]. Overall, the constant expression of GadY in all the examined strains prompted us to discard this sRNA as an explanation to the growth arrest observed in the RNase II mutant strain after being submitted to acid shock.

Despite possibly explaining the growth arrest in the RNase II mutant strain, the lack of ArrS does not seem to explain the existence, in the same strain, of similar survival values in both acid and normal pH conditions (Fig. 5). Considering this and the apparent dormant state found in the RNase II mutant strain after acidic shock, we theorise that there is a possible involvement of a toxin/antitoxin system. Toxin/antitoxin systems are widespread in bacteria and typically encode both a stable toxin and an unstable, antagonist antitoxin [130]. Although no pH related toxin/antitoxin system has been, to day, described in *E. coli*, there are examples in which a toxin/antitoxin system has severely inhibited cell growth while maintaining cellular viability [131]. The absence of RNase II could, theoretically, result in the expression of a low pH induced toxin from a toxin/antitoxin system. This toxin, which would normally be regulated by RNase II, would then cause the observable growth arrest without viability loss (Fig. 4B, 4C and 5).

GadE is a major transcriptional activator in the gad system of both *E. coli* and *Shigella flexneri* [132]. It induces the expression of the effector proteins GadA, GadB and GadC, which are also controlled by the GadX and GadW transcription factors. Interestingly, both GadX and GadW seem to be only primarily needed in the early processes of acid adaptation by forecasting the need for GadE and overall acid adaptation mechanisms [133]. When a low pH situation is, indeed, present, the levels of GadE

increase and the need for the transcriptional regulators GadX and GadW decreases [98, 101]. This is in agreement with our qPCR results for the *gadE*, *gadX* and *gadW* transcripts, were, when compared with the WT, low *gadE* transcript levels and high *gadX* and *gadW* transcript levels were obtained in all the exoribonuclease mutant strains. Moreover, in all the exoribonuclease mutant strains, we observed that a lower level of *gadE* corresponded to a lower level of both the *gadX* and *gadW* transcripts. (Table 24). Taken together, this results further our hypothesis that exoribonucleases are involved in the regulation of the gad system. Nonetheless, this low *gadE* transcript level in all the exoribonuclease mutant strains was surprising since the *gadE* activator ArrS sRNA was only significantly reduced in the RNase II mutant strain (Table 24 and Fig. 7). Considering that ArrS activates the change from the *gadE* T3 form to the active T2 form and that this, in turn, increases the expression of the other active T1 form [100], we only expected a significantly reduced *gadE* level in the RNase II and, to a lesser extent, RNase R mutant strains.

The HdeA protein displays chaperone activity by maintaining the integrity of the periplasmatic proteins in low pH conditions [122]. HdeA is, therefore, an essential factor in acid adaptation. Interestingly, its expression is induced by several gad related effectors, such as GadX, GadW and GadE [97, 121].

Overall, these results encourage us to suggest the existence of several yet unknown exoribonuclease mediated post-transcriptional networks in the gad system.

4.2. Anaerobic Adaptation

When the cells are submitted to anaerobic conditions, a change in genetic program occurs and the expression of numerous mRNAs involved in the aerobic and energy metabolism are repressed. By targeting for destruction several of these mRNAs, the anaerobic induced FnrS sRNA helps in the initial phases of anaerobic adaptation [81]. Eventually, due to the constitutive expression of this sRNA and the imminent shortage of targets, FnrS accumulates in the cell. When an aerobic environment is again present, the FnrS expression is repressed and its targets induced.

FnrS was previously described as being strongly anaerobically dependent, with only basal expression levels being detected in aerobic conditions [81]. Despite confirming this results for the WT and PNPase mutant strain, in the RNase II mutant strain the sRNA FnrS levels in aerobic conditions appear as strongly induced as the WT after anaerobic induction (Fig. 12A and 12B). Curiously, in the RNase R mutant strain, the FnrS sRNA displays levels that are intermediary to those found in between the WT/PNPase and the RNase II mutant strain. (Fig. 11, 12A, 12B and 13). Moreover, in anaerobic conditions, we also observe that both RNase R and RNase II mutant strains display a greater fold change in their FnrS levels than the ones in the WT and PNPase mutant strain (18 fold for the WT and Δ*pnp* strain and ~ 23 fold for the Δ*rnb*, Δ*rnr* strains) (Fig. 12B). RNase R thus appears to partly share with RNase II a yet unknown FnrS regulatory mechanism in, possibly, both aerobic and anaerobic conditions.

Interestingly, in the RNase II and RNase R mutant strains, the FnrS levels at 20 minutes after inhibiting transcription in aerobic conditions (Fig. 11) are significantly higher than those found in the same conditions, albeit without rifampicin and after anaerobic induction (Fig. 13). These results are puzzling as the FnrS levels in both RNase II and RNase R after aerobic shift (Fig. 13) should be similar to the levels found in the same time period in aerobic conditions (Fig. 11). Either rifampicin is affecting FnrS expression through RNase II and RNase R mediated pathways or the anaerobic induction somehow reverts the RNase II and RNase R mediated FnrS destabilising capability. In both cases, RNase II and RNase R seem to influence FnrS expression through indirect mechanisms.

In the WT and PNPase mutant strain, which exhibit similar FnrS levels, we theorise that during the anaerobic to aerobic adaptation phase the recently aerobically induced FnrS targets are rapidly degraded due to the much more abundant FnrS sRNA. This situation would occur until basal FnrS levels homologous to the ones in the WT (Fig. 11) are achieved, point at which the FnrS targets would begin to accumulate. Thus, the FnrS half-life value would represent the time this transition phenomenon occupies. In the RNase II and RNase R mutant strains, the FnrS half-life seems to indicate, especially in the first mutant, that this reaction happens slower in this mutants than in both the WT and PNPase mutant strain. In fact, this trend seems to correlate with what is also observed in aerobic conditions (Fig. 11). Thus, both RNase II and, to a lesser extent, RNase R, seem to also display a role in the regulation of the transition from aerobic to anaerobic conditions. Curiously, PNPase, a major exoribonuclease and the main responsible for the degradation of Hfq free sRNAs [61], does not seem to display any FnrS related activity.

The *gpmA* mRNA was previously described as an FnrS target [81]. *GpmA* encodes the glycolytic pathway enzyme 2,3-bisphosphoglyerate-dependent phosphoglycerate mutase (dPGM). We determined by qPCR that a higher amount of the FnrS sRNA in the RNase II mutant strain corresponds to a decrease in *gpmA* (Table 25). This situation is also observed for the RNase R mutant strain, although to a lesser extent. Moreover, in the PNPase mutant strain, the *gpmA* values are similar to the ones found in the WT. This is expected as both the WT and PNPase mutant strain displayed analogous FnrS levels. Moreover, the increased FnrS levels in the RNase II and RNase R mutant strains correlates with a decrease in FnrS targets (Table 25 and Fig. 11). We thus confirm that the role of the exoribonucleases in the FnrS sRNA is directly correlated with the levels of its regulatory targets.

We suspected that the increased levels of FnrS in the RNase II mutant would translate in a better adaptation to an anaerobic environment. This was not verified (Fig. 14). However, these results were still as expected since, in anaerobic conditions, the sRNA FnrS exhibited similar expression values in all the tested strains (Fig. 13). This suggested that all the strains would have adapted in a similar manner to the anaerobic conditions. In fact, this was what we observed, with the growth curves being parallel to those obtained in the control (Fig. 4A). Curiously, similar to what was obtained in the

aerobic control growth curve, the RNase II mutant strain displayed the slowest growth (Fig. 4A and 14). In this case, the slower growth could be the result of a higher FnrS expression, which, by hindering the expression of several important aerobic and energy metabolic enzymes, could cause a fitness disadvantage in aerobic conditions.

4.3. Final Remarks

E. coli is a major cause of foodborne diseases. It is estimated that around 2,800,000 people annually develop an acute *E.coli* illness and that 230 die from it [134]. *E. coli* success in colonising the host's intestines is partly due to not only its ability to withstand the pH of the acidic stomach with relative ease but also to its capacity of surviving in the intestine's anaerobic environment. In order to better cure and prevent *E. coli* infections, the study of the mechanisms involved in the *E. coli* acidic and anaerobic adaptation assume, therefore, paramount importance.

With this work we intended to clarify the influences of the exoribonucleases in the adaptation to acidic and anaerobic stresses. Indeed, we highlighted and broadened the role of the RNase II enzyme in both this pathways. In the future, we hope to continue pursuing explanatory hypothesis for the many questions that arose with our work. For example, we would like to perform binding assays with the RNase II enzyme on the ArrS sRNA. This would allow us to determine whether the enzyme binds to this sRNA or not. Moreover, it would also be noteworthy to determine whether the growth arrest in the RNase II mutant strain (Fig. 4B and 4C) was reversible or not by complementing RNase II expression in the RNase II mutant strain. Similarly exciting would also be the analysis of the growth profile of an *hfq* mutant after being submitted to acid shock. Overall, although we haven't yet clarified the abrupt growth arrest observed in the RNase II mutant when submitted to low pH conditions, we intend to continue exploring other hypothesis that can explain this curious phenotype. For example, we also intend to perform a qPCR for the *rpoD* and *rpoS* mRNAs. This will allow us to assess if these mRNAs are down-regulated in an RNase II mutant, possibly explaining the lack of ArrS.

Regarding the FnrS sRNA, to further perceive the obtained results, we would like to perform binding and activity assays between all the tested exoribonucleases and the

FnrS sRNA. It would also be interesting to continue verifying by qPCR whether the remaining FnrS targets are down regulated in the RNase II mutant strain or not. We also intent to perform, for all the exoribonuclease mutant strains, a new growth curve in which an anaerobic shock is performed. This will allow us to assess whether the increased FnrS values in the RNase II mutant influence or not the anaerobic adaptation process.

In conclusion, with this work we have discovered that both RNase II and, to a lesser extent, RNase R, have crucial roles in sRNA metabolism and bacterial adaptation to stress conditions.

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Appendix

Table I
Primer and probe sequences used in this work.

Primer/probe	Sequence (5´ - 3´)
FnrS_FW	GGGCGTTGCGCTCCATATTG
FnrS_T7	GACTCATCAAAGTCGGCGTCG
FnrS PCR	GACTCATCAAAGTCGGCGTCGTACGAATCAATTGTGCTATGCAGTA ATTCAAAAAAGGAAGTAAGACAATATGGAGCGCAACGCCC
FnrS probe	GACUCAUCAAAGUCGGCGUCGUACGAAUCAAUUGUGCUAUGCAG UAAUUCAAAAAAGGAAGUAAGACAAUAUGGAGCGCAACGCCC
ArrS probe	GCCAGCTTAAGTCGAAACAAGGAGAC
GadY probe	TCTGGAGACGGCAGACTATCCTCTTC
*ihfB*_FW	AGCATATGGCCTCGACTCTTGC
*ihfB*_RV	GCGCGGTAGTGCAAAGAGAAAC
*gadE*_FW	GACGCTCAATATTTCGCAACAAAC
*gadE*_RV	GGTGACGATGTCGCTCATAC
*gadW*_FW	ATCGATGAGCTGACAGTTTGCC
*gadW*_RV	CCTAAATTGCGTGGTAGCTGACG
*gadX*_FW	TTATGGGATGACGCCCACAGAG
*gadX*_RV	ACAATACTTGCCGCCGAGTCAC
*hdeA*_FW	GTTGTGAGCAATGCAGCGGATG
*hdeA*_RV	TCTTCACAGGTCCAGGAGTTGAC
*gpmA*_FW	TCTGTGCTGAAACGCGCTATCC
*gpmA*_RV	ATTTCTCAACGGGCAGCCATGC