UNIVERSIDADE NOVA DE LISBOA

THE ROLE OF RIBONUCLEASE R IN BACTERIAL ADAPTATION TO COLD SHOCK

CÁTIA BÁRRIA DA SILVA

DISSERTATION PRESENTED TO OBTAIN THE
MASTER DEGREE IN MEDICAL MICROBIOLOGY
FROM FACULDADE DE CIÊNCIAS MÉDICAS
UNIVERSIDADE NOVA DE LISBOA

OCTOBER 2011

UNIVERSIDADE NOVA DE LISBOA

THE ROLE OF RIBONUCLEASE R IN BACTERIAL ADAPTATION TO COLD SHOCK

CÁTIA BÁRRIA DA SILVA

DISSERTATION PRESENTED TO OBTAIN THE
MASTER DEGREE IN MEDICAL MICROBIOLOGY
FROM FACULDADE DE CIÊNCIAS MÉDICAS
UNIVERSIDADE NOVA DE LISBOA

Supervisor: Professora Doutora Cecília Maria Arraiano

Control of Gene Expression Laboratory
Instituto de Tecnologia Química e Biológica

OCTOBER 2011

List of Publications

Publications:

Bária C, Malecki M, Arraiano CM (2011). Bacterial adaptation to cold shock. Microbiology. (Accepted)

Comunications:

Bária, C., Malecki, M., Arraiano, C. M. "Identification of RNase R interactions by Tandem Affinity Purification method" Congress " XXXVI Jornadas Portuguesas de Genética" 30 May – 1 June of 2011, Coimbra, Portugal. Poster.

Acknowledgements

I would like to thank to the Comissão Científica e Organizadora da quarta Edição do Mestrado em Microbiologia Médica for its excellence and for providing the conditions to work.

First of all I would like to tank particularly to Professora Doutora Maria Teresa Marques for the organization of this Master and for the opportunity of developing my work in such a good conditions.

I would like to acknowledge all that supported me in this journey of my life. I could stay here describing all the important persons with whom I shared incredible moments but, I will try to express my gratitude briefly.

I would like to express my gratitude to Professor Cecilia Arraiano for supervising me and, for the opportunity to join her team and grow as a professional and as a person.

To my co-supervisor Michal Malecki for sharing his experience and support me with is knowledge as a student but also as a friend.

To all my Lab team: Andreia Aires, Rute Matos, Clementine Dressaire, Michal Malecki, Margarida Saramago, Inês Guinote, Inês Silva, Sandra Viegas, Susana Domingues, Ricardo Moreira, José Andrade, Vânia Pobre, Joana Pissarra e Filipa Reis. I apologize if I do not discriminate each one of you, my friends, but I suppose you know that you have a special place in my hearth!

To my wonderful family: Mum and Fernando for all the opportunities that you provided me throughout life! João, Bruno and Nadine my little and lovely brothers! To Mário, my best friend and also my husband! Thank you for support me every day of my life and for making me smile even when it's so hard to do it!

And finally to my unconditional friends Eunice, Joana, Gabi, Rita e Pedro for being there even when I am not with a good mud…

To all of you and to the ones I did not mentioned…
Thank you!!!

Abstract

Microorganisms react to the rapid temperature downshift with a specific adaptative response that ensures their survival in unfavorable conditions. Adaptation includes changes in membrane composition, in translation and transcription machinery. Cold shock response leads to overall repression of translation. However, temperature downshift induces production of a set of specific proteins that help to tune cell metabolism and readjust it to the new environmental conditions. For *Escherichia coli* the adaptation process takes only about four hours with a relatively small set of specifically induced proteins involved. After this time, protein production resumes, although at a slower rate.

One of the cold inducible proteins is RNase R, one of the main *E. coli* ribonucleases involved in RNA degradation. RNase R is an exoribonuclease that digest double stranded RNA, serves important functions in RNA maturation and turnover, release of stalled ribosomes by trans-translation, and RNA and protein quality control. The level of this enzyme increases about ten-fold after cold induction, and it is also stabilised in cells growing in stationary phase. The RNase R ability to digest structured RNA is important at low temperatures where RNA structures are stabilized but the exact role of this mechanism remains unclear.

Although specific bacterial cold shock response was discovered over two decades ago and the number of proteins involved suggests that this adaptation is fast and simple, we are still far from understanding this process.

In our work we aimed to discover the proteins interacting with RNase R in different environmental conditions using TAP tag method and mass spectrometry analysis. The information obtained can be used to deduce some of the new functions of RNase R during adaptation of bacteria to cold and in stationary growth phase. Most importantly RNase R can be recruited into a high molecular mass complex of protein in cold shock.

Resumo

Os microrganismos reagem à súbita descida de temperatura através de uma resposta adaptativa específica que assegura a sua sobrevivência em condições desfavoráveis. Esta adaptação inclui alterações na composição da membrana, na maquinaria de tradução e transcrição. A resposta ao choque térmico pelo frio induz uma repressão da transcrição. No entanto, a descida de temperatura induz a produção de um grupo de proteínas específicas que ajudam a ajustar/re-ajustar o metabolismo celular às novas condições ambientais. Em *E. coli* o processo de adaptação demora apenas quatro horas, no qual um grupo de proteínas específicas são induzidas. Depois desde período recomeça lentamente a produção de proteínas.

A ribonuclease R, uma das proteínas induzidas durante o choque térmico pelo frio, é uma das principais ribonucleases em *E. coli* envolvidas na degradação do RNA. É uma exoribonuclease que degrada RNA de cadeia dupla, possui funções importantes na maturação e "turnover" do RNA, libertação de ribossomas e controlo de qualidade de proteínas e RNAs. O nível celular desta enzima aumenta até dez vezes após exposição ao frio e estabiliza em células na fase estacionária. A capacidade de degradar RNA de dupla cadeia é importante a baixas temperaturas quando as estruturas de RNA estão mais estáveis. No entanto, este mecanismo é desconhecido.

Embora a resposta específica ao "cold shock" tenha sido descoberta há mais de duas décadas e o número de proteínas envolvidas sugerirem que esta adaptação é rápida e simples, continuamos longe de compreender este processo.

No nosso trabalho pretendemos descobrir proteínas que interactuem com a RNase R em condições ambientais diferentes através do método "TAP-tag" e espectrometria de massa. A informação obtida pode ser utilizada para deduzir algumas das novas funções da RNase R durante a adaptação bacteriana ao frio e durante a fase estacionária. Mais importante ainda, RNase R poderá ser recrutada para um complexo de proteínas de elevado peso molecular durante o "cold-shock".

Table of Contents

List of Figures

List of Tables

Abbreviations

aa	Aminoacid
Amp	Ampicillin
APS	Ammonium persulfate
ATP	Adenosine triphosphate
Bp	Base pairs
BSA	Bovine Serum Albumin
º C	Degree Celsius
Cam	Chloramphenicol
CBB	Calmodulin binding buffer
CDS	Cold Shock Domain
CELUT	Calmodulin elution buffer
CFU	Colony forming units
Csp	Cold shock protein
ddH_20	Bidistilled water
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
ds	Double Stranded
dsRNA	Double-stranded RNA
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylene Diamine Tetracetic Acid
h	Hour
IPTG	Isopropyl-beta-D-thiogalactopyranoside
Kan	Kanamycin
kb	Kilobase
kDa	Kilodalton
L	Liter

LA	Luria-Bertani Agar
LB	Luria-Bertani Broth
LPS	Lipopolysaccharides
M	molar/molarity (ml/L)
Mg	Miligram
µg	microgram
µl	microliter
min	minute
miRNA	microRNA
ml	milliliter
mmol	millimolar
mol	mole
MQ	Milli-Q water
mRNA	messanger RNA
ncRNA	non coding RNA
NEB	New England Biolabs
ng	Nanogram
Ni-NTA	Ni-nitrilotriacetic acid
nm	Nanometer
nts	Nucleotides
OD	Optical Density
PAGE	Polyacrilamide gel electrophoresis
PCR	Polymerase Chain Reaction
PMSF	Phenyl-Methyl-Sulfonyl Fluoride
PNPase	Polynucleotide Phosphorylase
RNA	Ribonucleic Acid
RNAP	RNA polymerase
RNase II	Ribonuclease II
RNase III	Ribonuclease III
RNase E	Ribonuclease E
RNase R	Ribonuclease R
RNases	Ribonucleases

rnb	Ribonuclease II
rnr	Ribonuclease R
rpm	Rotations per minute
RPOB	RNA polymerase β subunit
RPOC	RNA polymerase β prime subunit
rRNA	Ribossomal RNA
SDS	Sodium dodecyl sulphate
sRNAs	Small RNAs
ss	Single stranded
TBE	Tris-Borate-EDTA
TEMED	Tetramethylethylenediamine
tmRNA	Transfer-messenger RNA
Tris	Trishydroxymethyl aminomethane
tRNA	transfer RNA
X-Gal	bromo-chloro-indolyl-galactopyranoside
wt	Wild type

1. Introduction

Microorganisms have to constantly adapt to different environmental changes. These changes include nutrient and oxygen availability, changes in temperature and osmotic stress. To survive and adapt to these changes, bacteria induce or repress the expression of certain genes leading to changes in cell physiology. Capacity of adaptation is one of the reasons why bacteria can survive under extreme conditions. Understanding mechanisms of bacterial adaptation can help developing strategies to avoid bacterial proliferation in unfavorable conditions like cold, with important applications in food and pharmaceutical industry.

1.1 Cold shock response

One of the environment changes that bacteria have to face is the temperature change. With a downshift of the temperature, bacteria react with a specific response called the cold shock. This mechanism is triggered by an abrupt shift of a culture growing exponentially from its optimum growth temperature (37°C) to a lower temperature (15°C). The cold shock response allows the cell to react and adapt to these changes.

After temperature decrease a number of changes occur in the cellular physiology. These effects include a decrease in the membrane fluidity, stabilization of secondary structures of nucleic acids, which leads to a reduced efficiency of mRNA translation and transcription, inefficient folding of some proteins and inhibition of ribosomal translation (68, 116, 117, 137).

Upon temperature downshift there is a transient arrest of cell growth. This period is termed acclimation phase. Translation of most genes stops and protein synthesis is

blocked (82) except for a group of cold inducible proteins (CIP) that are even induced. During this adaptation period the expression of the cold shock proteins increases. After acclimation phase cells become adapted to low temperature and resume growth at a rate that is slower than the one before the cold shock induction. The expression of the cold inducible proteins declines and the bulk protein synthesis restarts, adapted to the cold (Fig.1).

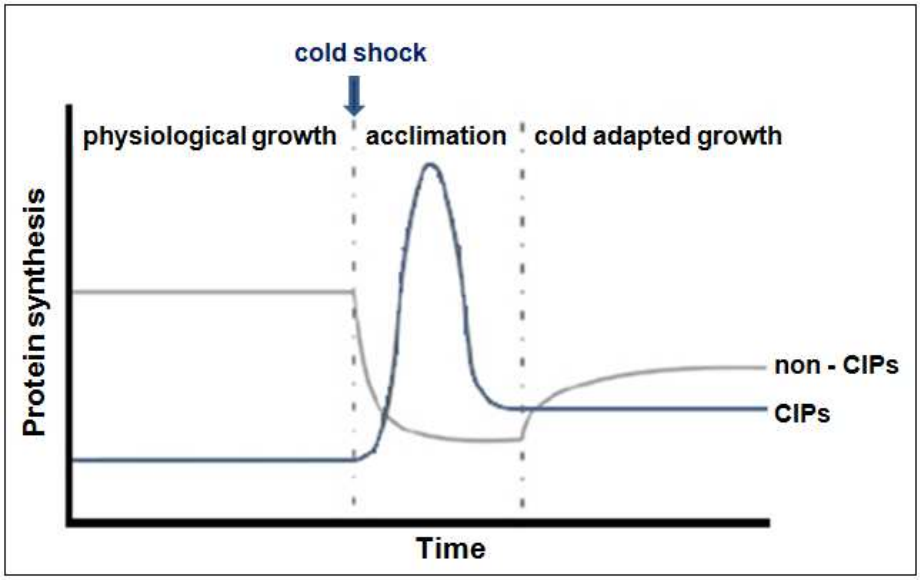

Figure 1 – Representation of the protein expression pattern after cold shock induction (Adapted from Kalbitzer et al. (73)).

Bacteria sense the change in temperature mainly at the level of cell membrane, nucleic acid and ribosomes (116).

Different environmental conditions can change the extend of DNA supercoiling, which affects the expression of several genes. The supercoiling of DNA presumably can act as a thermosensor and its regulation is important to maintain the DNA related functions. These functions include replication, transcription and recombination (53, 72). After temperature downshift the usual negative supercoiling state of DNA transiently increases (108). Consequently, the arrangement between the -10 and -35 region of some

promoters is modified affecting the recognition of some σ^{70} promoters such as the cold inducible *Escherichia coli rec*A promoter (145).

Bacteria also sense the temperature decrease at the level of ribosomes (141). Artificially inducing high levels of the guanosine 5' triphosphate-3' diphosphate and guanosine 5' diphosphate-3' diphosphate the expression of cold shock proteins decrease. However, at low concentration increases their production affecting the magnitude of the cold shock response (77).

The membrane composition is also affected by the temperature downshift. There is a decrease in the membrane fluidity which leads cells to lose viability. In *Escherichia coli* a rapid temperature downshift can induce phase separation of phospholipids. This causes an increase in membrane permeability and possibly death (30).

The membrane of Gram negative cells is composed of lipopolysaccharides (LPS), which consist of a distal polysaccharide (O-antigen), a core polysaccharide and lipid A. *E. coli* Lipid A is required for growth (125, 126), and consists of two glucosamine with attached acyl chains (fatty acids) that normally contain one phosphate group on each carbohydrate. Laurate (glyceryl laurate) is the fatty acyl chain of lipid A, usually detected in cells growing at 37°C. However, at low temperatures, there is a decrease in laurate counterbalanced by the appearance of palmitoleate (palmitoleate acid) (35). In contrast with laurate, that is a saturated fatty acid, palmitoleate is an unsaturated fatty acid. Unsaturated fatty acids increase membrane fluidity and lower its phase transition temperature. Palmitoleate has a double bond ligation, which causes the bending of fatty acid molecules and leads to a lose packing (Fig. 2).

LpxL is an acyltransferase that attaches laurate to the lipid A (134) and is important for lipid A synthesis at normal growth conditions. However, at low temperatures, LpxP (LpxL orthologue) is also expressed. LpxP is a cold inducible protein involved in cold-adapted replication. It is required for the production of lipid A adapted to the cold, because attaches palmitoleate to the Lipid A molecule instead of laurate (134, 143). LpxP was also found in *Shewanella oneidensis* and described to be induced during cold shock (59).

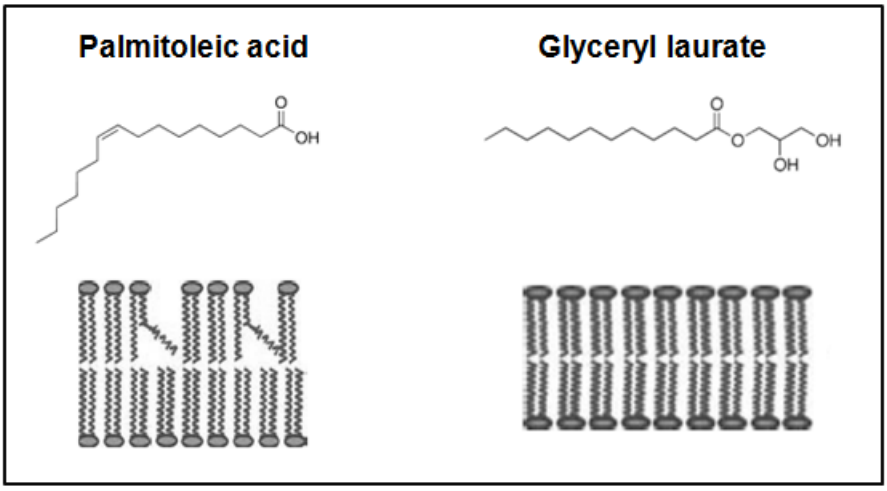

Figure 2 – Representation of membrane fluidity according to the lipid composition. Palmitoleic acid has a double bond ligation that causes the bending of fatty acid molecules, while glyceryl laurate has only a single bond ligation.

In *Bacillus subtilis*, adaptation to the cold shock also triggers changes in the membrane. The adaptation of membrane fluidity to this stress condition involves a rapid desaturation of fatty acids in existing phospholipids. This happens by induction of fatty acid desaturase (Des) due to an increase of fatty acid. The induction of Des is regulated by the sensor kinase DesK and the response regulator DesR (4). The trans-membrane domain of DesK was described as a probable sensor of the membrane fluidity (5). With an abrupt shift to a lower temperature, DesK phosphorylates the transcriptional activator DesR which subsequently binds to the promoter of the *des* gene and activates transcription of the D5-desaturase. This enzyme catalyzes the reaction to introduce a double bound into preexisting fatty acids tails of phospholipids inside the cellular membrane. This will increase the fluidity of the membrane bilayer, as a response to the decrease in temperature (3, 64). In *Legionella pneumophila*, during adaptation to cold shock, bacteria increase the levels of unsaturated fatty acids and lipid A (135).

1.2 Cold induced proteins

After the cold shock induction, cells enter in the acclimation period in which a group of cold inducible proteins (CIP) are expressed. Most of these cold shock proteins are essential for the cell to survive at low temperatures and they are involved in different cellular processes (80, 82, 137). Proteins with described or presumptive functions during cold shock response are listed in table 1.

Gene	Product	Description/Function in cold shock	References
aceE	AceE	Pyruvate dehydrogenase, decarboxylase.	(78)
aceF	AceF	Pyruvate dehydrogenase, dihydrolipoamide acetyltransferase.	(78)
cspA	CspA	Cold-inducible RNA chaperone and anti-terminator; transcriptional enhancer.	(19, 20, 63, 76, 90, 132)
cspB	CspB	Cold shock inducible; Function unknown.	(78, 119)
cspE	CspE	RNA chaperone; transcriptional antitermination.	(71, 119, 149)
cspG	CspG	Cold shock protein homolog, cold-inducible; Function unknown.	(105)
cspI	CspI	Cold shock protein, cold shock inducible; Function unknown.	(146)
deaD	DeaD /CsdA	ATP-dependent RNA helicase, facilitates translation of mRNAs with 5' secondary structures.	(17, 81, 109)
dnaA	DnaA	DNA binding and replication initiator, global transcription regulator.	(11)
gyrA	GyrA	DNA gyrase, subunit A; DNA-binding/cleaving/rejoining subunit of gyrase.	(80)
hns	H-NS	Nucleoid protein, transcriptional repressor, Repressor supercoiling.	(12, 51, 80, 90)
hscA	Hsc66	DnaK-like chaperone	(93)
hscB	HscB	DnaJ-like co-chaperone for HscA.	(93)
hupB	Huβ	Nucleoid protein, DNA supercoiling.	(61, 108)
infA	IF1	Protein chain initiation factor IF1, Translation initiation.	(49, 147)
infB	IF2	Protein chain initiation factor IF2, Translation initiation, fMet-tRNA binding, protein chaperone.	(29, 70, 78)
infC	IF3	Protein chain initiation factor IF3, Translation initiation, stimulates mRNAs translation.	(69)
lpxP	LpxP	Lipid A synthesis; cold temperature inducible.	(35, 134, 143)
nusA	NusA	Transcription termination/antitermination/elongation L factor.	(58, 78)
otsA	OtsA	Trehalose phosphate synthase; cold- and heat- induced, critical for viability at low temperatures.	(83)
otsB	OtsB	Trehalose phosphate phosphatase; cold- and heat- induced, critical for viability at low temperatures.	(83)
pnp	PNPase	3'-5' exoribonuclease; component of RNA degradosome; cold shock protein required for growth at low temperatures.	(52, 78, 154, 156)
rnr	RNase R	3’ -5’exonucleases; increases 10-fold in cold shock.	(9, 14, 28, 41)
rbfA	RbfA	Ribosome binding factor required for efficient processing of 16s rRNA; cold-shock adaptation protein.	(24, 50, 79, 151)
recA	RecA	General recombination and DNA repair; induction of the SOS response.	(78, 144)

tig	Trigger factor	Protein folding chaperone, multiple stress protein, ribosome binding.	(83, 84)
ves	Ves	Cold and stress-inducible protein, function unknown.	(152)
yfiA	pY	Protein Y, associated with 30S ribosomal subunit, Inhibits translation at the elongation stage.	(2)

Table 1 – Representation of the *E. coli* cold shock genes, products and respective functions.

The cold shock proteins can be divided in different groups according to their functions.

Several of these proteins are involved in RNA metabolism. A group of cold shock proteins is involved in transcription: the cold shock proteins (Csp) family, that can work as RNA chaperones but are also involved in transcriptional antitermination (19, 20, 63, 76, 90, 132), DeaD helicase, that works as RNA chaperone and facilitates translation of mRNAs with 5' secondary structures due to its RNA unwinding activity (17, 81, 109, 139) , histone-like protein H-NS, a transcriptional repressor (12, 51, 80, 90), and the transcription factor NusA (58) that is involved in the transcription termination, antitermination and elongation of L factor (58, 78).

During the cold shock response two 3' -5' exonucleases are involved in the RNA degradation. Polynucleotide phosphorylase (PNPase) was shown to be required for growth at low temperature (13, 52, 78, 154, 156), selectively degrades *csp*A mRNA at 15°C and represses the CspA homologues production at the end of the lag phase (114, 154), and the ribonuclease R (RNase R) increases its level about 10-fold in the cold shock (9, 14, 28, 41).

The RNA translation process involves the ribosome-binding factor RbfA that works in maturation of ribosome at low temperature (24, 50, 79, 151), the translation initiation factors IF1(49, 147), IF2 (29, 70, 78) and IF3 (69), and the protein Y (pY) that inhibits translation at the elongation phase (2). pY is a protein with 12.7 kDa shown to stabilize ribosomes against dissociation (1). It is not detected in ribosomes from cells growing at physiological temperature. This protein is only found in ribosomes from cells growing at low temperatures, or in ribosomes from cells that have reached the

stationary phase at a physiological temperature. pY is able to inhibit translation at the elongation stage by blocking the binding of aminoacyl-tRNA to the ribosomal A site and subsequently decline the protein synthesis, for example, during cold shock. When growth is resumed, pY is no longer detected in ribosomes which suggests that it function is to arrest translation in response to an environmental stress such as cold shock (2).

The cold shock proteins are also required for DNA metabolism. These proteins are: a DNA binding and replication initiator (DnaA) (11), a DNA gyrase subunit A (GyrA) (80), the DnaK-like chaperone (Hsc-66) (93) and the DnaJ-like co-chaperone (HscB) (93), the nucleoid protein Huβ involved in the DNA supercoiling (61, 108) and the general recombination and DNA repair protein RecA (78, 144).

The cold shock proteins that are not grouped in the RNA or DNA metabolism are divided in two functional categories according to their targets: lipids or proteins. To the lipid group belong LpxP that is involved in the lipid A synthesis. In the protein group are described the OtsA and OtsB trehalose phosphates and the trigger factor. The OtsA and OtsB contain a cold box characteristic of the cold shock mRNAs, are critical for cell viability at low temperatures (83) and their induction is dependent on RpoS, increasing 8-fold upon temperature downshift (116). The trehalose mRNAs were also shown to enhance stability at low temperature (83). It was suggested that, at low temperatures trehalose acts by preventing denaturation and aggregation of proteins, functioning as a free radical scavenger *in vivo,* protecting against oxidative damage and stabilizing cell membranes (83). The trigger factor is a protein folding chaperone that is induced after a lag period of 2-3 hours upon cold shock and maintains cell viability at low temperatures (83, 84). This protein can associate with ribosomes and has an important role in co-translational protein folding (18, 103). The cell viability at 4°C revealed direct correlation with the intracellular levels of the trigger factor, which is presumably due to its ability to help protein synthesis and folding at low temperature (84). Other cold shock proteins are induced at low temperatures but their functions are not determined yet. They are two pyruvate dehydrogenases, AceE and AceF (78), and Ves a cold and stress inducible protein (152).

The induction levels of the cold shock proteins are different. In *E. coli*, CspA, CspB, CspG, CspI, DeaD, RbfA, NusA, PNPase and RNase R are the most increased proteins comparing to the other CIP.

1.3 RNA metabolism under cold shock

Accordingly to the Central Dogma of Biology, the genetic information is processed from DNA to RNA to Protein. DNA is replicated while cell divides, is transcribed into RNA and subsequently translated into protein. The gene expression is determined by the efficiency of transcription of DNA to mRNA, the stability of mRNA, and the frequency of mRNA translation into proteins. In general the RNA population can be divided in four different categories: messenger RNA (mRNA), ribosomal RNA (rRNA), transfer RNA (tRNA) and small non-coding RNAs. However, these molecules can have different cellular functions, like working as enzymes (ribozymes) or as regulators of genetic expression.

The RNA metabolism at 37°C can be different from the one at low temperatures. With the decrease of the temperature, RNA secondary structures are more stable. Presumably, with RNA stabilization the transcription elongation, the ribosomal movement on RNA and then translation, slows down. Consequently, it requires proteins that destabilize these structures and allow these biologic processes to proceed.

1.3.1 Cold shock proteins – Csp

Csp is a family of small structurally related nucleic acid-binding proteins (115) which are composed of the typical cold shock domain (CSD). These proteins bind preferentially to single-stranded RNA or DNA (76, 101, 157). The Csp in *E. coli* family includes nine members, CspA (63), CspB (92), CspC, CspD, CspE, CspF, CspG (112), CspH and CspI (146). From these, only CspA, CspB, CspE, CspG and CspI are cold inducible (63, 92, 112, 146).

The Csps have been functionally linked to the maintenance of chromosome structure and DNA replication (36) and also affect transcription by acting as transcription antiterminators. The majority of the Csp are able of binding DNA (76, 119, 153). At low temperatures, with the stabilization of RNA structures the protein translation is hampered. The Csp proteins function, as chaperones, is crucial due to mRNA stability in cold conditions. CspA, the major cold induced Csp, function as an RNA chaperone and destabilize the secondary structures (76). The CspE protein melts partially double stranded and hairpin structures (120). CspE was also shown to bind poly-A tails and thereby, stabilizing mRNA by reducing degradation by PNPase and RNaseE (56). CspE and CspC are expressed at both high and low temperatures (155) and have a critical impact on the stabilization of transcripts for a global stress response regulator (rpoS) and the universal stress response protein (uspA) (118).

In *B. subtilis* three proteins homologous to *E. coli* CspA were identified (CspB, CspC and CspD), which are induced at low temperatures (67, 148). In many other bacterial microorganisms this type of cold shock proteins were also described (66, 68).

1.3.2 CspA

CspA was originally identified as the major cold shock protein (63). After the cold shock induction, its level increases up to 13% of the total protein content of the cells (63).

The regulation of CspA and its homologues expression during the cold shock induction occurs at the levels of transcription, mRNA stability and translation.

The induction of *csp*A, at low temperatures, does not require any additional transcription factors (116). The *csp*A, *csp*B, *csp*G and *csp*I have an unusually long 5' untranslated region (5'-UTR). The 5'-UTR contains a highly conserved unique 11-base sequence called the cold box (75, 150). The cold box is a presumed transcriptional pausing site and is involved in the repression of *csp*A expression (116). The *cspA* 5'-UTR is thought to be responsible for the extreme instability of *csp*A mRNA at 37°C, but has a positive effect on mRNA stabilization in the cold shock (107).

The c*sp*A mRNA is dramatically but transiently stabilized immediately following cold shock. Its promoter is active at 37°C. However, CspA is hardly detected at 37°C due to the instability of its mRNA.

The *csp*A mRNA also contains a unique sequence located 14-bases downstream of the initiation codon, the downstream box. It is present in CspB, CspG, CspI, CsdA and RbfA and is presumed to enhance translation initiation in the cold shock mRNAs by facilitating the formation of translation preinitiation complex through binding to 16S rRNA. However, the exact mechanism of the enhancing effect on translation initiation by the downstream box remains unknown (107, 110).

CspA and its homologues have been characterized as RNA chaperones, and this function is thought to facilitate translation at low temperatures (19, 65, 76, 78). These structures of RNA stabilize at low temperatures but CspA and its homologues presumably can destabilize the secondary structures and facilitate transcription and translation by acting as RNA chaperones. At low temperatures, the secondary structures of RNA stabilize, which presumably slows down the transcription elongation and the ribosomal movement on RNA and thus translation. CspA and its homologues presumably can destabilize the RNA secondary structures, and thus facilitate transcription and translation. The increased levels of CspA after cold shock may be important for compensating the higher stability of secondary structures in RNA at low temperatures (116). In *E. coli*, CspA and its homologues binds RNA without apparent sequence specificity and with low binding affinity (76). CspB, CspC and CspE are able to more selectively bind RNA/ssDNA (116). The non-specific and weak binding of CspA homologues to RNA/ssDNA are also important for the chaperone function, as binding of the protein would not hamper ribosome movement on mRNA (116).

CspA was also shown to be a transcription antiterminator by preventing the formation of secondary structures in the nascent RNA (15, 76). At low temperatures, due to the stabilization of RNA structures, the formation of an artificial terminator can occur. Modulation of transcription termination by RNA-binding proteins involves the formation of a new structure that can prevent formation of this artificial terminator, thus leading to transcript elongation.

1.3.3 DeaD

The other group of proteins that can act as RNA chaperones are the DEAD-box-family of helicases (48). It is a family of RNA dependent ATPases able to unwind double-stranded nucleic acids and characterized by 9 conserved motifs (81) (Fig 3).

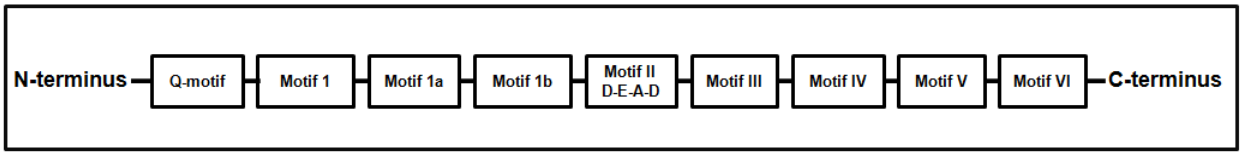

Figure 3 - Overview of the nine conserved motifs (Q to VI) of the DEAD-box family of *E. coli*.

Proteins from the DEAD-box family are involved in different processes of RNA metabolism such as translation initiation, ribosome biogenesis and assembly or RNA degradation (38, 55, 131, 136). *E. coli* has 5 identified DEAD-box proteins, SrmB, DeaD, DbpA, RhlB and RhlE. SrmB and DeaD are essential at low temperature (39), (34) and DeaD is overexpressed in cold shock. DeaD (also named CsdA) is involved in many different processes in the cell. It was described that the deletion of DeaD results in the deficiency in free 50S subunits at low temperature. This results shows that this protein participates in the biogenesis of the 50S ribosomal subunit, probably by binding and changing the RNA structure of a 50S precursor (38). It is also required for initiation of translation of mRNAs with an extensive secondary structure (102), and it interacts with poly(A) polymerase (127). DeaD has the ability to unwind double-stranded RNA in the presence or absence of ATP (81). It is involved in the mRNA degradation of cold shock genes (154). It was proposed that DeaD can interact with RNase E, PNPase and other components of the RNA degradosome under cold shock conditions (123, 124).

DeaD plays a role in mRNA degradation at low temperature but the precise role of this helicase is not completely clear. DeaD helicase was described to be associated

with the RNA chaperone Hfq, but they did not reveal a physical interaction (129). *In vitro* assays showed that it associates directly with RNase E, but not with PNPase (123). However we can not exclude the possibility to interact with other ribonucleases. This hypothesis remains to be solved and more work is required to clarify this question.

1.4 RNA degradation

RNA degradation is the major process that controls the RNA levels in the cell. Degradation is required for elimination of defective RNAs and superfluous transcripts whose expression is no longer required. Ribonucleases (RNases) are enzymes with the strongest role in this process. They can be selective and sensitive to specific elements of the RNA molecule and are involved in the quality control of all types of RNAs. They are involved not only in RNA degradation but also in RNA processing and maturation. There are other enzymes that act synergistically with ribonucleases within these processes, like helicases, polymerases and RNA binding proteins. RNases can act alone or can be part of RNA degradation complexes like the degradosome. Ribonucleases are divided in two main groups, endonucleases and exonucleases.

Endonucleases are the enzymes that cleave RNA internally by digesting phosphodiester bonds of the RNA molecule. In *E. coli,* eight endoribonucleases have been identified, RNase E, RNase G, RNase III, RNase Z, RNase HI, RNase HII, RNase I and RNase P (7, 89). They play an important role in mRNA metabolism and in *E. coli*, the major endoribonucleases are RNase E and RNase III.

Exoribonucleases are the enzymes that degrade RNA by removing terminal nucleotides in the 3'-5' or 5'- 3'direction (9, 158). In prokaryotes, most RNases cleave the RNA in the 3'-5'direction. These proteins act releasing nucleotides that can be reutilized for the synthesis of new RNA molecules. In *E. coli*, seven exoribonucleases were identified, PNPase, RNase II, RNase R, oligorinuclease (Orn), RNase D, RNase PH and RNase T, all 3'-5' exoribonucleases. However, only the first four exoribonucleases appear to accomplish all RNA degradative activity in the cell.

RNase R, RNase II and PNPase are considering the three major 3’ -5’ processing exoribonucleases in *E. coli*. RNase R, together with RNase II belongs to the RNB family (85), while PNPase is a member of the PDX family. RNase R (28) and PNPase (82, 156), are cold shock inducible and were suggested to be responsible for degradation of structured RNA in the cell at low temperatures (44, 95).

1.4.1 PNPase

Polynucleotide phosphorylase (PNPase) is an exoribonuclease encoded by the *pnp* gene (128) and belongs to the PDX family of nucleases. In the genome it is located downstream of the *rpsO* gene (122). Its expression is controlled both at transcriptional and post-transcriptional levels (9, 74). PNPase binds to the 5' end of RNase III-processed *pnp* transcript, resulting in inhibition of translation and leading *pnp* mRNA into the degradation pathway (60, 121). It is not an essential enzyme for the cell at 37°C however, it becomes extremely important at lower temperatures (13) . PNPase can be incorporated into the degradosome, a complex of multiple proteins, which also comprises RNase E (33, 99).

The resolution of the crystal structure revealed that PNPase is a homotrimer. Each subunit is constituted by two RNase PH domains and two RNA binding domains, KH and S1 domains, located at the C-terminal region of the polypeptide (Fig. 4). Both of the phosphorolytic and RNA binding activities are required for autogenous regulation of PNPase (21) and it has been described that the KH and S1 domains of this exoribonuclease are important for its activity at low temperature (106). PNPase PH domains were described to be important for PNPase function in the cold shock, which suggests that the ribonuclease activity is critical for its essential function at low temperatures (13).

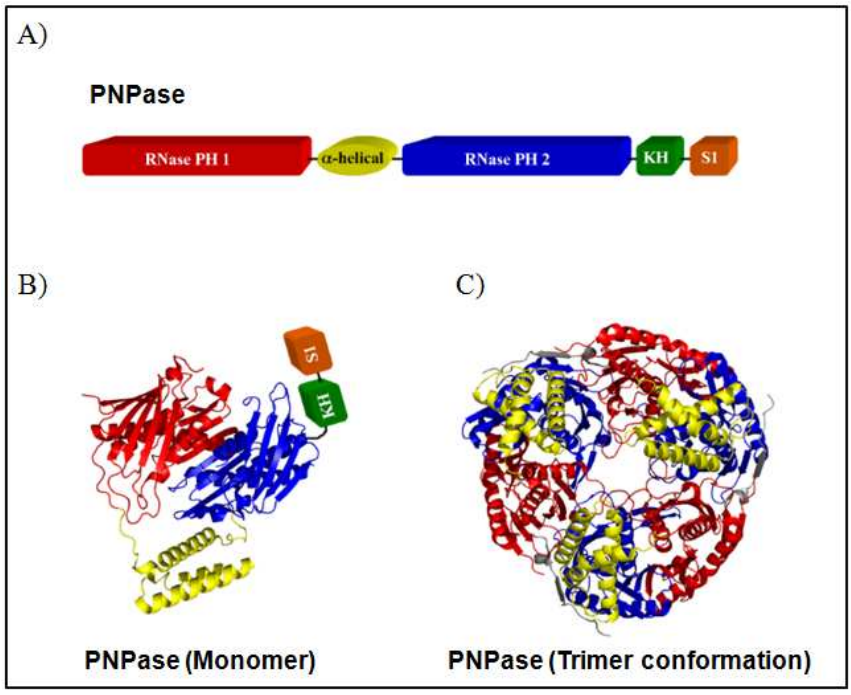

Figure 4 – Representation of *E. coli* PNPase. A) Linear representation of *E. coli* PNPase domains. B) View of PNPase crystal structure in the monomer organization (133). C) PNPase trimer structure.

Most mRNAs that are already present in the cell when cold shock is induced, cannot be efficiently translated until specific cold shock factors like CspA, RbfA and DeaD, are produced (76, 79, 139). However, mRNA from cold shock genes at low temperatures are translatable and, like *csp*A transcripts, can become more stable (19, 62).

It was described that translation of *pnp* mRNA after cold shock may not be very efficient (156). During the acclimation phase PNPase seems to restore intragenic transcription termination and regulate its own expression at the level of transcription elongation (156). With this autogenous regulation, *pnp* mRNA becomes more stable. However, the levels of PNPase protein do not increase accordingly (104, 156).

At the end of the acclimation phase, PNPase is specifically required for the degradation of unnecessary transcripts. During this phase, the level of CspA and its homologues decrease due to the selective degradation of *csp* RNAs by PNPase. PNPase

has been implicated in virulence in several pathogens, namely in *Salmonella*, *Yersinia*, *Campylobacter jejuni* and *Streptococcus pyogenes*. Interestingly, while in some microorganisms PNPase seems to act as a virulence repressor, in others this ribonuclease takes an important role in the establishment of virulence (10).

1.4.2 The Degradosome

The *E. coli* RNA degradosome has been described as an RNA decay "machine" due to its function in mRNA degradation and RNA processing (33). This complex or its individual components can target specific gene products or affect the relative composition of different transcripts through differential decay rates (32).

The degradosome is mainly composed by RNase E, PNPase, RhlB (a DEAD-box RNA helicase) and the glycolytic enzyme enolase (33). The complex was discovered during the purification of RNase E (33), an endoribonuclease that mediates the principal pathway for the mRNA degradation in *E. coli*. This ribonuclease is essential for bacteria. RNase E inactivates polyribosomal mRNA by endoribonucleolytic cleavage, producing mRNA fragments. These are subsequently digested to nucleotides by exonucleases (PNPase), helped by RhlB and poly(A) polymerase. In the presence of ATP, the RhlB helicase unwinds stem-loops to help PNPase digestion (86). The RNA degradosome is built on a region of RNase E that shows high sequence variation among closely related bacteria, the carboxy-terminal half, where PNPase, RhlB and enolase bind (88, 111). Only RNase E, PNPase, and RhlB are essential to reconstitute the activity of the degradosome *in vitro* (47). *E. coli* mutants in which degradosome assembly is disrupted have a slow-growth phenotype and altered metabolic profiles (22). This results from the incapacity to degrade a number of degradosome substrates (88, 111) and reveals that this protein complex is essential in gene regulation.

The degradosome composition changes during the cold shock (123). At low temperatures, DeaD helicase, a cold shock protein, associates with RNase E and other components of the degradosome. This helicase was first proposed to replace RhlB when cells are shift to low temperatures. However, this cold inducible protein binds to a

distinct RNase E binding site (87, 123). To explain the association of DeaD with RNase E two theories were proposed (123). RhlB and DeaD associate with RNase E at the same time but each one bind to a separate site (87) or, cold shocked cells have heterogeneous population of the degradosome, some that can contain both DeaD and RhlB helicase and others with only one of the two helicase proteins (123). Structured mRNA is more difficult to degrade at low temperatures and it may require two RNA helicases (33). These findings showed that the degradosome is capable to adapt depending on the environmental conditions (123).

1.4.3 RNase R

Ribonuclease R (RNase R) belongs to the RNB family of enzymes. The members of RNB family are widely conserved in both prokaryotes and eukaryotes and serve several important functions. In eukaryotes they can be developmentally regulated (25) and mutations in its genes have been related with mitotic control, and cancer (98). In prokaryotes, this family of enzymes is important in stress responses, RNA and protein quality control, and it was shown to be required for virulence in different organisms (8, 28, 40, 44, 45).

Proteins of this family are hydrolytic exonucleases that degrades RNA in the 3' to the 5' direction (9). The best know bacterial protein of this family is RNase II which structure has been solved and mechanism of catalysis was proposed. At the moment, RNase R structure remains unknown and therefore is hard to completely understand its mechanism of action. However, based on the 3D structure of RNase II, a RNase R model was built (16, 57). RNase R follows the typical organization found in RNB family: a RNB catalytic domain flanked by two cold shock domains, CSD1 and CSD2 and a C-terminal S1 domain, the three involved in RNA-binding (57, 105) (Fig. 5).

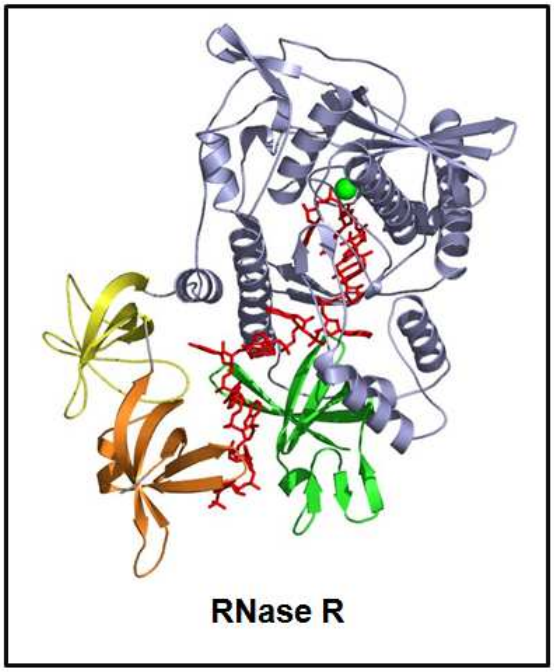

Figure 5 - Representation of the predicted *E. coli* RNase R 3D structure. The structural model of RNase R was constructed based on the RNase II structure (16). It comprises two N-terminal Cold Shock Domains (CSD1 in orange and CSD 2 in yellow), a central RNB domain (in blue) and a C-terminal S1 domain (in green); the RNA molecule is also represented (in red) (57) .

In contrast to RNase II, RNase R is able to digest RNA secondary structures without the help of a helicase. This function can be especially needed at low temperatures when RNA is highly structured. RNase R can also unwind double stranded structures in absence of RNase activity which lead to a suggestion that this protein has "helicase like" activity (14).

In *E. coli*, RNase R is responsible for the degradation of substrates such as defective tRNAs (95), rRNA (44) and small RNAs like the stable SsrA/tmRNA (28). This protein is modulated according to the environmental conditions and its level is upregulated under several conditions, such as stationary growth phase and cold shock (8, 26). Deletion of RNase R gives growth phenotype at low temperatures (28). The phenotype of the *rnr* mutant, under cold shock, is characterized by smaller colonies

compared to the wild-type strain (28). RNase R has also been shown to be involved in virulence mechanisms (54, 91, 138, 140).

After cold shock induction, RNase R levels increase about ten-fold (41). The increased amount of RNase R was shown to be stable for many hours revealing that slow growth conditions at low temperatures leads to RNase R stabilization (42). A recent study revealed that the level of RNase R after addition of chloramphenicol was also elevated when compared with RNase R level from cells grown at 37°C. It was suggested that the regulatory processes that lead to this elevation during cold shock are a consequence of an increase in the amount of *rnr* message, that is substantially stabilized in cold shock (28, 42). Recent results suggests that acetylation regulates RNase R stability (97). The Lys544 interacts with acidic residues within the C-terminal region of the protein. By this interaction, the C-terminal region stays in a position that is poorly accessible to tmRNA-SmpB, whose binding is essential for RNase R instability (96). Addition of the acetyl group would remove the positive charge on lysine, breaking the interaction with tmRNA-SmpB. RNase R in stationary phase cells is not acetylated, position 544 is positively charged so, the C-terminal region would not be accessible. Then tmRNA-SmpB binds weakly, resulting in a stable RNase R (97). These results revealed that the acetylation of one specific lysine residue in RNase R increases binding of the tmRNA-SmpB complex, resulting in protein instability (97). This direct effect of acetylation on the regulatory process of stability of RNase R in the stationary phase can also occur at low temperatures. However, further studies are required to understand it.

RNase R can complement the CsdA cold shock function. It was suggested that it is due to RNase R ability to degrade secondary structures and its presumable helicase activity (14).

It is clear that RNase R plays an important role in RNA degradation at low temperature but further studies are required to clarify how RNase R is involved with other cold shock proteins in the cold shock response.

The cold shock response is a mechanism that bacteria have to adapt and survive at low temperatures. Understand which proteins are involved, how they interact with each other and how this process can be controlled will be highly advantageous to develop strategies to control bacterial surveillance.

1.5 Health and industrial implications of cold shock response

E. coli is a microorganism that is commonly found in the lower intestine of warm-blood organisms. They are usually harmless but some serotypes can cause serious health complications. The presence of these bacteria in food or pharmaceutical products is indicative of fecal contamination.

Although *E. coli* is known as a usual pathogen, this bacterium is still the microorganism of choice for most gene cloning experiments and has been widely used in biotechnology industry to overproduce determinate components.

Understanding cold shock response can be of the extreme importance. Food storage is commonly made using refrigeration to avoid proliferation of bacteria such as *Listeria* or *Campylobacter*. Knowing how to block cold shock protein synthesis will allow us to reduce/inhibit the cold shock response and that can be lethal to bacterial cells at low temperatures. Investigators revealed that *E. coli* growth can be inhibited by the combination of rapid chilling with nisin, a polypeptide produced by lactic acid bacteria which is recognized as natural antimicrobial food preservative (46), reducing more than 6-log of the bacterial population (31). Combining the use of antimicrobial products with bacterial exposition to low temperatures can thus extend the efficiency of drugs to decrease bacterial proliferation.

On the other hand, by controlling the cold shock response, proteins that are not produced efficiently at 37°C can be overproduced at low temperatures using cold-inducible promoters (142).

A common bacteria used in industry is *Lactobacillus* (37). This bacterium also has cold shock proteins that are induced at low temperatures. During the different industrial processes, cells have to face several environmental changes. Bacteria face changes in pH, salt concentration or temperature. By inducing the cold shock response, *Lactobacillus* increases the probability to survive in low temperatures, which can be extremely useful in this industry.

The cold shock response has been widely studied. However, more studies are still required to develop strategies which will allow us to apply its benefits biotechnically.

2. Materials and Methods

All materials and reagents are described on Annex I (table 14).

2.1 Bacterial strains

Bacterial strains used in this work are listed in Annex I (table 15) and were grown in LB medium at 37ºC supplemented, when required, with appropriate antibiotic. Bacterial strains were stored in 10% glycerol at -80ºC.

2.2 Preparation of competent cells

An overnight culture of BL21 (DE3) or DH5α was diluted in LB to a final OD_{600nm} of 0.05. BL21 (DE3) cells are used for protein overexpression and DH5α cells for mutant constructions. Cells were incubated with agitation (180 rpm) at 37ºC.

When the culture reached an OD_{600nm} of 0.45 to 0.55 was kept on ice for 15 minutes and centrifuged at 5000rpm for 10 minutes at 4ºC. The supernatant was discarded and the pellet was gently resuspended in 0,4 volumes of chilled $CaCl_2$ I solution. The suspension was kept on ice for 15 minutes and centrifuged again, in the same conditions. The pellet was resuspended in 0,04 volumes of chilled $CaCl_2$ II, aliquot (200 µl each), frozen immediately in liquid nitrogen and stored at -80ºC. The transformation efficiency was tested with 50 ng of pUC18 or pET28a plasmid DNA following the procedures explained below.

2.3 Transformation

DNA (ligation mixture/plasmid) was added to 100 µL of *E. coli* competent cells. The cells were incubated on ice for 30 minutes, heat shocked at 42ºC for 1 minute and immediately placed back on ice for 1 minute. LB medium was added (1ml) and the tubes were incubated at 37ºC for 45 to 60 minutes. After incubation, cells were plated on LA medium plates with the respective antibiotic and incubated overnight at 37ºC.

2.4 TAP tag purification

The TAP tag purification was performed following these steps:

- Three cultures were started from three single colonies of *E. coli* (BW113) with RNase R protein fused with TAP tag. Colonies were inoculated in LB medium with kanamycin (50 µg/ml), and incubated overnight at 37ºC.
- The three pre-inoculums were diluted in 1L of LB to a final concentration of OD_{600} of 0,05 containing kanamycin (50 µg/ml) and incubated at 37ºC until cells reach exponential growth phase (OD_{600} of approximately 0.5).
- After that, the three cultures were processed in different ways:
    - one of the three cultures was centrifuged at 5000 rpm for 8 minutes at 4ºC and the pellet was stored at -80ºC (exponential growth phase);
    - one of the other cultures was incubated for 3h at 15ºC, centrifuged at 5000 rpm for 8 minutes at 4ºC and stored at -80ºC (cold shock growth phase);
    - the third culture remained at 37ºC for 3h, was centrifuged at 5000 rpm for 8 minutes at 4ºC and stored at -80ºC (stationary growth phase).
- The pellets were unfrozen and each one was resuspended in 8 ml of Lysis buffer.
- Suspensions were lysed by two passages in French press.
- 0.5µl of benzonase (250 U/µl) was added to degrade the nucleic acids in the sample.

- Samples were incubated on ice for 10 minutes and centrifuged at 35000 rpm, for 45 minutes at 4ºC in ultracentrifuge.
- Supernatants were filtered (0.45 µm) and an aliquot (soluble fraction) was taken for further SDS-PAGE gel analysis.
- 200 µL of Rabbit IgG agarose (beads) were added into a column (Poly-Prep Chromatography Columns from Biorad).
- The column was washed with 5ml of IPP150A with Triton X-100.
- Triton X-100 detergent (final 0,1%) was added to each supernatant and the mixture was loaded into the column.
- Columns remained shaking at 4ºC for 1.5h.
- After incubation, columns were washed twice with 10ml of IPP150 and subsequently two times with 10ml of TEVClevBuf.
- 200µL of TEVClevBuf and 35µL of TEV protease (approximately 100 units) were added to the column and incubated for 75 minutes, shaking at room temperature.
- After TEV cleavage, the supernatant was collected into an eppendorf by eluting twice with 250µL of CBB.
- An aliquot (IgG fraction elution) was taken for mass spectrometry analysis.
- $CaCl_2$ (4µl of a 0,2M stock) was added to the mixture.
- The mix was transferred into an eppendorf with 300µL of CBB beads (previously washed 4 times with 500µL of CBB).
- Incubation was performed for 45 minutes shaking at 4 °C.
- After incubation the sample was transferred into a new column and washed twice with 5ml of CBB.
- As a final step, we elute with 600µL of CELUT into an eppendorf.

2.5 Acetone precipitation of proteins

To precipitate the proteins we added into each sample four volumes of cold (-20°C) acetone. The sample was shortly vortexed and incubated for 2 hours at -20°C.

After incubation each sample was centrifuged at 15000 rpm for 15 minutes at 4ºC. Supernatant was discarded and the pellet was washed with 500µL of 80% of cold acetone (-20°C). Supernatant was discarded, pellet was dried and stored.

2.6 DeaD amplification and cloning

The DeaD helicase gene (1,9 kb) from *E. coli* was amplified by PCR using a high fidelity phusion polymerase which produces blunt end PCR products (Finnzymes). We used *E. coli* genomic DNA has a template and the primers described in Annex II, table 17. Forward primers included *Sal*I restriction site on their sequence and the reverse primers contained the *Not*I restriction site. The PCR reaction mixture and the program used are detailed in the table 2 and 3, respectively.

Table 2 – Master mix used in DeaD PCR amplification.

DeaD amplification	
Components	**Volume (µl)**
DNA template (0,3µg/µl)	1 µl
Lprimer (10µM)	1 µl
Rprimer (10µM)	1 µl
dNTPs (10µM)	0,8 µl
5x Phusion buffer HF	8 µl
Phusion DNA polymerase (2U/µl)	0,4 µl
ddH_2O	27,8 µl
Final volume	40 µl

Table 3 - PCR Program used for DeaD gene amplification.

Cycle Step	Temperature	Time	Number of cycles
Initial denaturation	98ºC	120s	1
Denaturation	98ºC	10s	30
Annealing	56ºC	15s	
Extension	72ºC	40s	
Final extension	72ºC	10s	1

After DeaD amplification, the PCR product (1,9 kb) was visualised in the 0,8% agarose gel.

Subsequently, a ligation was performed using the DeaD sequence (directly from the PCR amplification reaction) and the pUC18 (2,7kb) previously digested with *Sma*I *(*Fermentas). This restriction enzyme cleaves the DNA generating blunt ends. The ligation reaction was performed by T4 DNA ligase following the supplier protocol (Fermentas), at 22ºC in a water bath for 20 minutes as described in the following table:

Table 4 - Ligation reaction of the DeaD sequence into the corresponding cloning sites of pUC18.

Ligation	
Components	**Volume (µl)**
PCR product (100 ng/µl)	5 µl
pUC18 (100 ng/µl)	1 µl
10x Buffer T4 DNA ligase	2 µl
50% PEG (PEG 4000)	2 µl
T4 DNA ligase (10U/µl)	0,7 µl
H_2O	9,3 µl
Final volume	20 µl

After incubation, enzymatic inactivation of T4 DNA ligase was performed at 65ºC for 10 minutes.

The ligation product was transformed into DH5α *E. coli* competent cells following the protocol described above (2.3 section).

Transformants were selected on the LB plates supplemented with Ampicillin (100μg/ml), IPTG and X-Gal (See Annex I). The presence of ampicilin allows selecting the colonies with the recombinant DNA by the presence of the resistance gene in the vector. The alpha-complementation allows determining whether a transformed bacterial colony has the ligation product or not. The lac-Z gene product (β-galactosidase) is a tetramer and each monomer is made of two parts - lacZ-alpha, and lacZ-omega. If the alpha fragment is deleted, the omega fragment is non-functional. This alpha fragment functionality can be restored in-trans via plasmid. pUC18 plasmid has the deletion of the lac Z-alpha so, if ligation and transformation works, cell should express non-functional β-galactosidase (lac Z-alpha will be disrupted with the insertion of the gene of interest). In the selection media (with IPTG to induce the lac repressor), the X-gal, a chromogenic substrate that yields a blue product when cleaved by β-galactosidase colonies of interest should appear white. The blue colonies represent the cells that contain the unaltered vector.

The plasmid was then extracted from the positive clones (white colonies) using the ZR Plasmid Miniprep kit. The plasmid concentration was determined using the ND100 Spectrophotometer from NanoDrop.

Subsequently, pUC18 DeaD was digested with *Sal*I and *Not*I restriction enzymes (Fermentas) following the supplier protocol and as described in table 5. This cleavage step was performed to verify the insertion of DeaD gene into the pUC18 plasmid. Digestion reaction was incubated at 37ºC for 2 hours. Enzymatic inactivation was performed at 80ºC for 10 minutes and the cleavage products were visualized in a 0.8% agarose gel (Fig. 10B).

Table 5 - Plasmid digestion of ligation products with *Sal*I and *Not*I restriction enzymes.

Digestion reaction	
Components	**Volume (µl)**
DNA (350 ng/µl)	30 µl
10x Fast digest buffer	6 µl
*Sal*I (10U/µl)	2 µl
*Not*I (10U/µl)	2 µl
H_2O	20 µl
Final volume	60 µl

2.7 SLIC reaction

2.7.1 Insert preparation

DeaD sequence (1.9 kb) from pUC18 DeaD vector was amplified by PCR using a high fidelity enzyme and the primers with 30 bp of overlapping sequence described in Annex III, table 18. The resultant fragment was used to ligate into pET28a using SLIC recombination method.

DeaD amplification was performed in 4 separated tubes each one with 50 uL of mix, following table 6 and 7 indications.

Table 6 – Mix used for DeaD PCR amplification.

DeaD (1890 bps)	
Components	**Volume (µl)**
pUC18 + DeaD (40ng/µl)	0,25 µl
LprimerSLIC (10µM)	1,25 µl
RprimerSLIC (10µM)	1,25 µl
dNTPs (10µM)	1,25 µl
5x Phusion buffer HF	10 µl
Phusion DNA polymerase (2U/µl)	0,5 µl
ddH_2O	35,5 µl
Final volume	50 µl

Table 7 - PCR Program used for DeaD gene amplification.

Cycle Step	**Temperature**	**Time**	**Number of cycles**
Initial denaturation	98ºC	120s	1
Denaturation	98ºC	10s	30
Annealing	56ºC	15s	
Extension	72ºC	40s	
Final extension	72ºC	10s	1

After PCR amplification, the four PCR reactions were joined. We add 2U of *Dpn*I and incubate at 37ºC for 1 hour. *Dpn*I cleaves methylated DNA from *E. coli* (template DNA), whereas unmethylated DNA (*in vitro* synthesized DNA) is not cleaved by these enzyme. By using *Dpn*I after the PCR we are able to eliminate the plasmidic DNA that we used as template.

Enzymatic inactivation was performed at 80ºC for 20 minutes. The DeaD insert was separated in a 0.8% agarose gel and the corresponding band was extracted from the gel. DeaD insert was cleaned using the Zymo's DNA Clean and Concentrator.

2.7.2 Vector preparation

The expression vector, pET28a (5,4 kb), was digested with *Sal*I and *Not*I restriction enzymes (Fermentas). To perform this enzymatic restriction we prepared a digestion reaction as described in table 8.

Digestion reaction was incubated at 37ºC for 2 hours and then, enzymatic inactivation was performed at 80ºC for 10 minutes.

Table 8 - Digestion of pET28a vector with *Sal*I and *Not*I restriction enzymes.

Digestion reaction	
Components	**Volume (µl)**
pET28a (0,1 µg/µl) 10x Fast digest buffer *Sal*I (10U/µl) *Not*I (10U/µl)	50 µl 6 µl 2 µl 2 µl
Final volume	60 µl

Cleavage products were separated in a 0,8% agarose gel and the appropriate size band (5368bps) was cut.

The fragment of interest was extracted from the gel using the kit Zymo's DNA Clean & Concentrator and DNA quantification was performed using NanoDrop.

2.7.3 T4 Treatment

To perform SLIC recombination the prepared products (DeaD insert and pET28a vector digested with *Sal*I and *Not*I) were subjected to T4 polymerase (NEB) treatment. The T4 DNA polymerase catalyzes the synthesis of DNA in 5' to 3' direction but requires the addition of dNTPs. Without dNTPs it can create single stranded regions that will anneal to the prepared insert. The DeaD insert was used directly from PCR amplification, and pET28a was used after digestion and "gel cleaning" as described in 2.7.2 section. T4 Treatment was followed as described in table 9.

Table 9 - T4 DNA polymerase treatment.

.

DeaD (SLIC)		**pET28a**	
Components	**Volume (µl)**	**Components**	**Volume (µl)**
DeaD insert (110 ng/µl)	9 µl	pET28a vector (70,6 ng/µl)	14 µl
T4 buffer 10x (NEB buffer 2)	2 µl	T4 buffer 10x (NEB buffer 2)	2 µl
BSA 100x	0,4 µl	BSA 100x	0,4 µl
DNA T4 polymerase (0,3U)	1,2 µl	DNA T4 polymerase (0,3U)	1,2 µl
H_2O	7,4 µl	H_2O	2,4 µl
Final volume	20 µl	Final volume	20 µl

Each one of the reaction mix was incubated at 22ºC for 25 minutes. After the incubation period, the reaction was stopped by the addition of 2 µL of dCTP (10mM) and then, kept on ice.

2.7.4 Ligation

The ligation of the products was performed using a 1:1 molar ratio insert to vector with 150ng of the digested vector (pET28a). The reaction was prepared as described in table 10 and incubated at 37ºC for 30 minutes.

Table 10 – Annealing reaction mix.

Annealing reaction	
Components	**Volume (µl)**
DeaD insert	1,5 µl
pET28a vector	3 µl
1x DNA Ligation buffer (NEB)	1 µl
H_2O	4,5 µl
Final volume	10 µl

2.7.5 Transformation

The transformation was followed as described in 2.3 section, using 5 µL of the previous reaction and 100 µl of DH5α *E. coli* competent cells. Plasmid extraction was performed from 10 of the obtained transformants using the ZR Plasmid Miniprep kit.

Subsequently, pET28a:DeaD was digested with *Sal*I restriction enzyme (Fermentas) following the supplier indications to confirm the ligation of DeaD to pET28a plasmid. The digestion reaction mix was prepared as described in table 11 and incubated at 37ºC for 60 minutes. The cleavage products were visualized in a 0.8% agarose gel (Fig. 13).

Table 11 – Digestion of pET28a:DeaD with *Sal*I restriction enzyme.

Digestion reaction	
Components	**Volume (µl)**
pET28a:DeaD (60 ng/µl)	4 µl
10x Fast digest buffer	1 µl
*Sal*I (10U/µl)	0,5 µl
H_2O	4,5 µl
Final volume	10 µl

2.8 Protein overexpression by IPTG induction

To allow the expression of proteins upon IPTG induction, constructions of pET15b:rnr (pABA-RNR) (6) , pET15b:rnb (pFCT6.1) (27) or pET28a:DeaD were transformed into BL21 (DE3) competent cells as described in 2.3 section.

A single colony of each strain was inoculated in LB medium with kanamycin (50µg/ml) (for pET28a:DeaD) or ampicilin (100µg/ml) (for pET15b:rnr and pET15b:rnb), and incubated overnight at 37ºC. The pre-inoculum was diluted in 250 ml of LB to a final OD_{600} of 0.05 containing kanamycin (50 µg/ml) or ampicilin (100 µg/ml) and incubated at 37ºC. After cells reached exponential growth phase (OD_{600} of approximately 0.5), the protein expression was induced by addition of IPTG (0,25 mM final concentration) to the culture. Cultures remained 3h shaking at 180 rpm. The cultures were then centrifuged at 5000 rpm for 10 minutes at 4°C and the pellet was stored at -80ºC.

2.9 HIS tag purification

HIS-tag purification was performed from the pellet obtained above (section 2.8). Each pellet was resuspended with 1.5 ml of IPP150-A (supplemented with 50 µl DTT

1M, 400 µl PMSF 0,2M and 500 µl imidazol 20mM). Cells were lysed with one of this two methods: **1)** suspensions were lysed by two passages in French press or, **2)** suspensions were added to glass beads (1/3 of the final volume) and cell lysis was made using FastPrep instrument in a speed of 6.0 M/s during 45s. In both cases, supernatant was collected and the pellet was discarded. A fraction from total proteins (20 µl) was separated for further SDS-PAGE gel analysis.

After lyses 0.5µl of benzonase (250 U/µl) was added to degrade the nucleic acids in the sample. Samples were incubated on ice for 10 minutes and centrifuged at 17000 rpm, 30 minutes at 4ºC. 20 µl of the soluble proteins fraction were separated for further SDS-PAGE gel analysis. The HIS tag recombinant proteins were purified by affinity chromatography columns. The supernatant was loaded in a column equilibrated with 1ml of HIS tag agarose beads (Ni-NTA) previously washed with 1ml of IPP150-A. The column remained at 4ºC with gentle agitation for 1h. After incubation, the column was washed twice with 5ml of IPP150-B. As a final step, elution was performed with 1ml of IPP150-C into an eppendorf.

2.10 Total Proteins extraction

To allow the protein extraction, BW25113 *E. coli* cells were grown in two different growth conditions: until exponential phase or in cold shock conditions. A single colony was picked from a freshly culture plate, inoculated in LB medium and incubated overnight at 37ºC. The pre-inoculum was diluted in 200 ml of LB to a final OD_{600} of 0,05 and then incubated at 37ºC at 180 rpm. After cells reached exponential growth phase (OD_{600} of approximately 0.5), one of the cultures was centrifuged at 5000 rpm for 10 minutes at 4ºC. The other culture was incubated for 3h at 15ºC (cold shock induction) and then centrifuged at 5000 rpm for 10 minutes at 4ºC. Each pellet was resuspended with 2 ml of IPP150-A, centrifuged at 4000 rpm for 5 minutes at 4ºC and frozen. The pellets were unfrozen and resuspended with 1 ml of IPP150-A (supplemented with 50 µl DTT 1M, 400 µl PMSF 0,2M and 500 µl imidazol 20mM). Cells were lysed using FastPrep instrument sample with a speed of 6.0 M/s during 45s

after addition of glass beads to the suspension (1/3 of the volume). Samples were collected, centrifuged at 17000 rpm for 30 minutes at 4ºC. After centrifugation, supernatant was filtered (0.45 µm) and protein separation was performed by gel filtration column.

2.11 Protein migration on a gel filtration column

In order to visualize the protein pattern in different growth conditions proteins were separated using a gel filtration column (Superdex 200) and the AKTA FPLC™ System (*GE Healthcare*). Gel filtration is a chromatographic method that allows separating protein according to their size.

The column was selected according to the fractionation range so that the expected molecular weight of our proteins falls approximately in the middle of the range for this column. The Superdex 200 column has a high resolution in the separation of proteins, peptides or other biomolecules according to the size, with a range from 10 kDa up to 600 kDa.

The equilibration of the column was performed following the supplier recommendations (*GE Healthcare*). Since the column has been stored in 20% ethanol, we started to wash first with 1 column volume of distilled water. Subsequently, we equilibrated the column with 1 column volume of the buffer IPP150 A (Annex I, table 14). We monitored the changes in the conductivity to confirm that the buffer filled all the column and that were no more water in the column.

To determine in which column volume a protein is expected to be detected we analyzed proteins with known sizes: Ferritin (440 kDa), Aldolase (158 kDa), RNase II (72 kDa) and Chymotripsin (25 kDa).The protein migration profile was monitored by UV spectra at 280nm.

Migration in the column was performed with the proteins purified by HIS tag method (see Materials and Methods 2.9) or from protein extracts obtained as described in section 2.10. The protein sample (2 ml) was applied into the column. The elution of the proteins was determined by monitoring the UV spectra at 280nm.

2.12 Western Blot

Western blot is a technique that allows to identify the target protein and to quantify relative amounts of that protein in different samples.

This technique was performed with samples obtained from two different experiments: **1)** sample obtained in section 2.9 were loaded in a 10% SDS-PAGE gel. After applying a voltage of 200V during 45 minutes the gel was washed in 1x Transfer buffer 20% ethanol and proteins were transferred into a nitrocellulose membrane using Trans-Blot SD semi-dry equipment (20v for 45 minutes) or, **2)** 20 µl of the samples obtained in section 2.11 were loaded into a nitrocellulose membrane using a Dot Blot System.

After transfer, the membranes were washed with water and stained with Ponceau solution to see if the transfer was well succeeded. Subsequently, membranes were washed with 50 ml of water and then with 50 ml of 1x TBS Tween. After the washes, protein blocking was performed by incubation of the membrane in 10 ml of 5% non-fat milk with gentle agitation for 1h at 4ºC. After blocking, membrane was incubated overnight with 10 ml of 5% non-fat milk with α-rnr (antibody against RNase R protein) (1:10000), with gentle agitation at 4ºC. Membrane was washed 3 times with 50ml of 1x TBS Tween for 5 minutes and incubated with 5% non-fat milk with anti-Rabbit (1:20000) for 1h with gentle agitation at 4ºC. After incubation, membrane was washed 3 times with 1x TBS Tween for 5 minutes and then chemiluminescent detection was performed using Western Lightning Plus-ECL reagents. Chemiluminescent signal was detected and quantified with ChemiDoc XRS software.

2.13 Silver staining protocol for SDS-PAGE gels

- Fix the gel in 30% ethanol, 10% acetic acid at least 30 minutes
- Rinse the gel twice in 20% ethanol, for 10 minutes for each wash

- Rinse twice in water for 10 minutes for each wash
- Sensitize the gels by soaking for 1 minute in 0.8 mM sodium thiosulfate (0.02% if pentahydrate salt is used)
- Rinse the gel twice for 1 minute for each wash in water
- Impregnate with 12mM (0.2% stock) silver nitrate from 20 to 120 minutes
- Remove the gel from the silver solution and dip it for 10 seconds in the water bath
- Transfer it in the developer solution (Annex I) (prepared in the day of use, and formaldehyde added at most 1hour before use). A brown or grey precipitate normally develops within a few seconds. This precipitate must be dissolved by shaking. The most intense bands take a few minutes to appear (from 5 to 45 minutes depending of the temperature)
- Stop the reaction by transferring the gel to the Tris stop solution (Annex I) (prepared in the day of use) from 30 to 120 minutes
- Store gels in the water.

3. Results

This section is divided in three main parts. The first part includes the purification of *E. coli* RNase R by the TAP tag method and the data obtained from the Mass Spectrometry analysis. The second part presents the studies of interaction between RNase R and DeaD helicase, and the third part reveals the assays performed in order to study RNase R behaviour after cold shock induction.

3.1 Purification of *E. coli* ribonuclease R

RNase R levels increase under certain stress conditions, particularly during cold shock. We hypothesized if other proteins could cooperate with this ribonuclease in different conditions causing its stabilisation and level increase. We also thought that the reason why RNase R levels increase in the cold shock can be partially elucidated by identifying which proteins are interacting with this exoribonuclease. We decided to investigate which proteins are interacting with RNase R in different growth conditions, and see if there are any relevant differences.

To achieve this goal, we purified RNase R using the tandem affinity purification method (TAP tag) (130). This technique is commonly used to purify protein complexes since it allows the recovery of highly purified complexes present in low amounts in the cell. There is also two different purification steps used in this method that reduce background contamination by abundant cellular proteins.

The TAP-tag is composed by two different parts which are separated by a cleavage site recognized by the *tobacco etch virus* (TEV) protease. The N-terminal part of the tag is the IgG-binding protein A from *Staphylococcus aureus* (ProtA) and the other is the calmodulin-binding peptide (CBP) (Fig 6). ProtA binding to rabbit IgG is

very efficient and highly specific (100), as well as the binding of CBP to calmodulin in the presence of calcium. With this technique, the protein of interest is fused with the TAP-tag. This will allow it to bind to the beads coated with IgG. After washing, protein elution was achieved by treatment with TEV protease. In the second purification step, the CBP part of the TAP tag binds reversibly to calmodulin beads. After washing, the protein is eluted using a chelating agent (like EDTA). After these two procedures, we can now examine the existence of binding partners by SDS-PAGE gel examination or mass spectrometry (Figure 7).

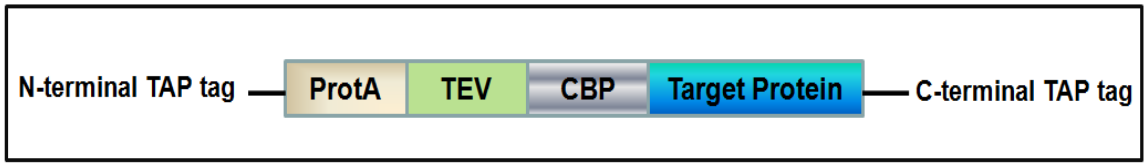

Figure 6 – Structure of the TAP tag consisting of protein A from *Staphylococcus aureus* (ProtA), a cleavage site for the *tobacco etch virus* protease (TEV), a calmodulin-binding peptide (CBP) and the target protein.

As already mentioned, our aim was to identify possible RNase R binding partners. For that purpose, we used a *E. coli* (BW25113) with RNase R protein fused with TAP tag, which was previously constructed in our laboratory. The TAP tag sequence was introduced into the bacterial genome using the lambda red recombination method. We also constructed a strain with the TAP tag sequence fused with one of the RNA polymerase subunits, RPOC. This fusion was examined in previous works (23) and since RNA polymerase is a well studied protein complex, we considered it to be an excellent control for our purification method and mass spectrometry technique.

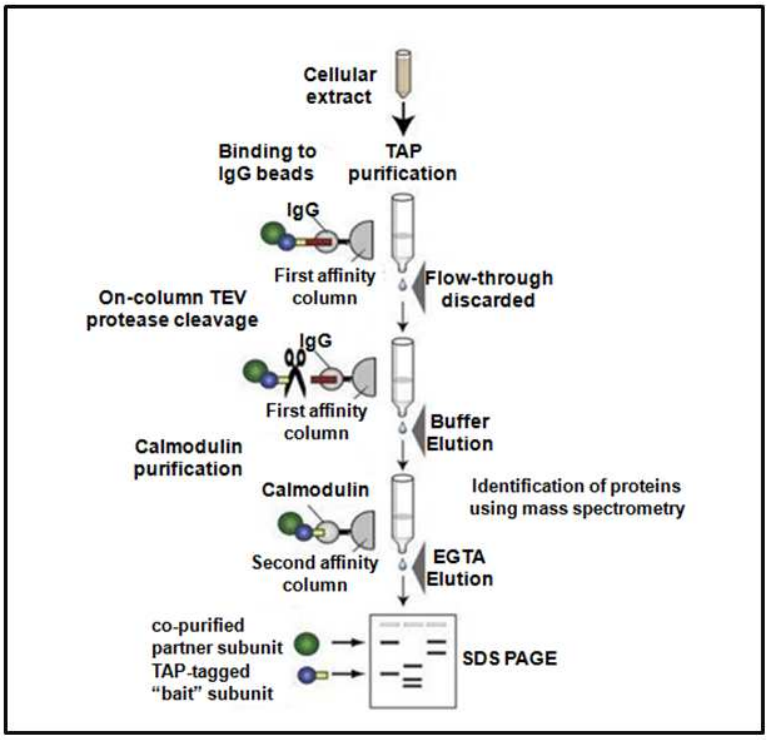

Figure 7 – Overview of the TAP tag purification method. Adapted from Rigaut et al 1999 (130).

The first aim was to purify RNase R by this method from cells that were grown in different conditions. By using SDS-PAGE gels, we wanted to compare the pattern of proteins that co-purify with our target. This would allow us to see if there are any relevant differences between samples from different conditions. Cells encoding the TAP tagged RNR were harvested in exponential, in stationary growth phase, and after cold shock. Purification was performed according to the protocol described in Materials and Methods, section 2.4. Protein elution fractions from both purification steps (IgG and calmodulin (CM)) were analysed in a SDS-PAGE gel. The gel was stained with silver following the protocol described in Materials and methods, section 2.13. The pattern of co-purifying proteins in all conditions was compared (Fig 8).

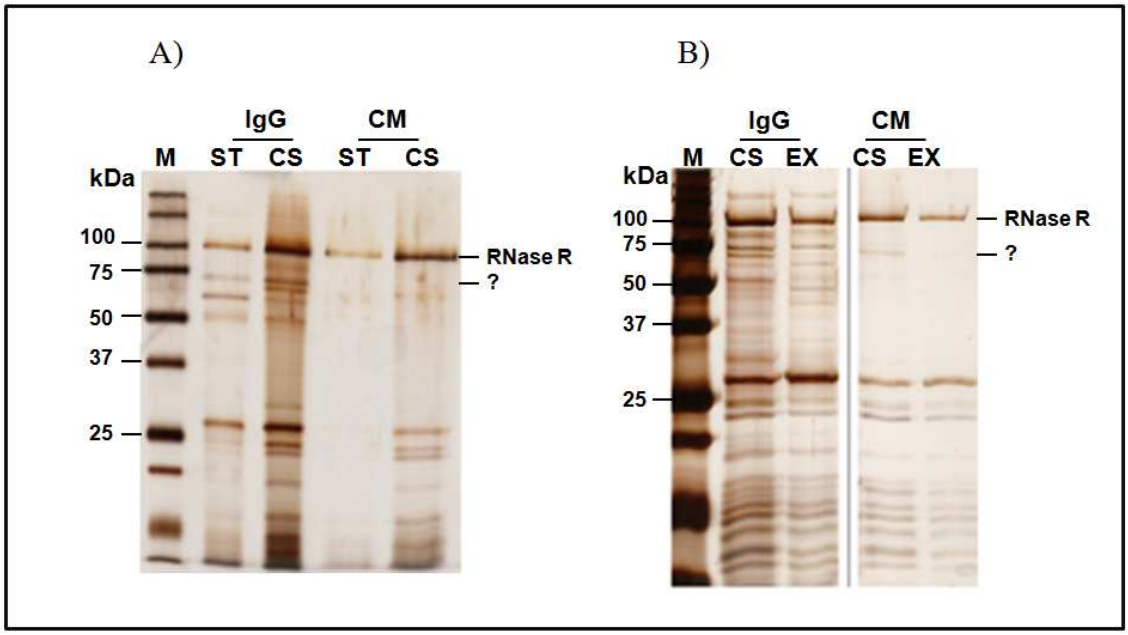

Figure 8 – TAP tag purification of RNase R. A) Comparison between elution fractions from cold shock (CS) and stationary cells (ST). B) - Comparison between elution fractions from cold shocked (CS) and exponentially growth (EX) cells. 12% SDS-PAGE gel silver stained with elution fractions, CM – elution from calmodulin resin, IgG – elution from immunoglobulin resin. RNase R and a protein detected during cold shock induction are indicated on the right side of the gel.

By analysing the SDS-PAGE gels, we can see slight differences in the protein patterns. The strongest difference is observed in the protein elution from cold shocked cells. If we look more carefully to these fractions, we can see a band (about 70 kDa) that is not detected in the samples from exponential or stationary growth cells (marked on figure 8). To discover the differences between them, we decided to analyse our elution fractions in more detail using mass spectrometry analysis. At this step, we decided to analyse only the cells from stationary and cold shock growth phase. We also included in our analysis the RPOC preparations purified with the same method and in the same conditions as a control.

Elutions from immunoglobulin and calmodulin resins obtained from TAP tag purification of RNase R and RPOC from cold shock or stationary growth phase were

precipitated with acetone as described in “Materials and Methods” (2.5). The obtained pellets were resuspended and sent for mass spectrometry analysis.

Mass spectrometry analysis was performed by an external laboratory using a qualitative method that requires several steps until protein identification (http://mslab-ibb.pl/en). Briefly, protein disulfide bonds are submitted to reduction, alkylation than tryptic digestion is performed to obtain a peptide mixture. Sample is separated by liquid chromatography, and peptides are introduced into the mass spectrometer to produce a complex spectrum from which the molecular weights of all the proteolytic fragments can be read. This spectrum with the molecular weight information is compared against a protein sequence database and, if the protein already exists, it can be identified (113).

As a result of mass spectrometry analysis we obtained lists of proteins identified in each sample with the respective scores (full lists are in Annex II, table 16). We verified that 80 proteins were detected in the RNase R calmodulin elution fraction from the cold shocked cells and 64 in cells from stationary phase. Table 12 shows the 15 proteins detected with highest scores in RNase R preparations (see all detected proteins in Annex II, table 16). For RPOC (control), 51 proteins were detected during cold shock induction and 50 during stationary phase. In RPOC preparations, the first proteins detected are the subunits of RNA polymerase complex, as expected (Table 12). In our analysis, we excluded ribosomal proteins, which were highly detected in all preparations. Taking into consideration that RNase R and RPOC are RNA binding proteins, specific interactions with ribosomes are hard to distinguish from contamination. However, it is worth to mention that our assumption does not exclude the possibility that some of the ribosome interactions may be specific.

To validate our purification method, we checked how many of the proteins detected in RPOC preparations were previously reported to interact with RNA polymerase. Our results showed that a total of 66 different proteins were detected in both conditions in calmodulin elution in RPOC preparations. From these 66 proteins, 25 were detected in a previous work using the same method (23). Other 26 were reported to physically or genetically interact with RNA polymerase or with polymerase-interacting factors. These results were based on data obtained using functional protein association networks (STRING) database (http://string-db.org/). Five of these 66 proteins were detected in all preparations, although with low scores. This suggests that

they may unspecific interact with the resin and can be considered as a background. Resuming, from the 66 proteins co-purified with our control, 84% were already reported to interact with RNA polymerase (RNAP). This calculation increases our confidence in the analysis of TAP tag purification and mass spectrometry results obtained for RNase R protein.

A)

RNase R			
Cold shock		Stationary	
Protein	Score	Protein	Score
rnr	19585	rnr	28151
deaD	3120	rplL	1779
rplL	2245	rpsG	1573
rpsG	1357	rpsE	1048
rplC	1192	rpsF	1011
rpsD	1101	rpsD	941
rplD	905	rplD	927
aceE	879	rplV	739
rplV	873	rplC	600
rplB	799	groL	585
rpsF	754	rpsQ	540
rplM	648	yfiF	538
rpsI	625	rpsR	493
rplI	598	rpmB	482
infC	570	hupA	476

B)

RPOC			
Cold shock		Stationary	
Protein	Score	Protein	Score
rpoB	50429	rpoB	46141
rpoC	45026	rpoC	30835
rpoA	24984	rpoA	18217
rpoD	5070	rpoD	7314
rapA	4099	rapA	3106
rpoZ	2761	rpoZ	1141
rpoS	1024	rpoS	598
nusA	795	hupA	481
nusG	279	rplC	419
hupA	265	rpsG	406
rplC	257	rpsO	380
usg	235	hupB	301
greB	234	rplV	267
infB	187	rpsD	262
yacL	156	rplJ	192

Table 12 – Mass spectrometry results from TAP tag purification of RNase R or RPOC from cold shock and stationary growth phase cells. List of the first 15 proteins identified and respective scores during stationary or cold shocked cells that co-purified with RNase R (A) or with RPOC (B) from calmodulin elution resin. In green are represented the proteins previously related to be interacting with RNA polymerase (RNAP), and in gray are represented the proteins found in all preparations.

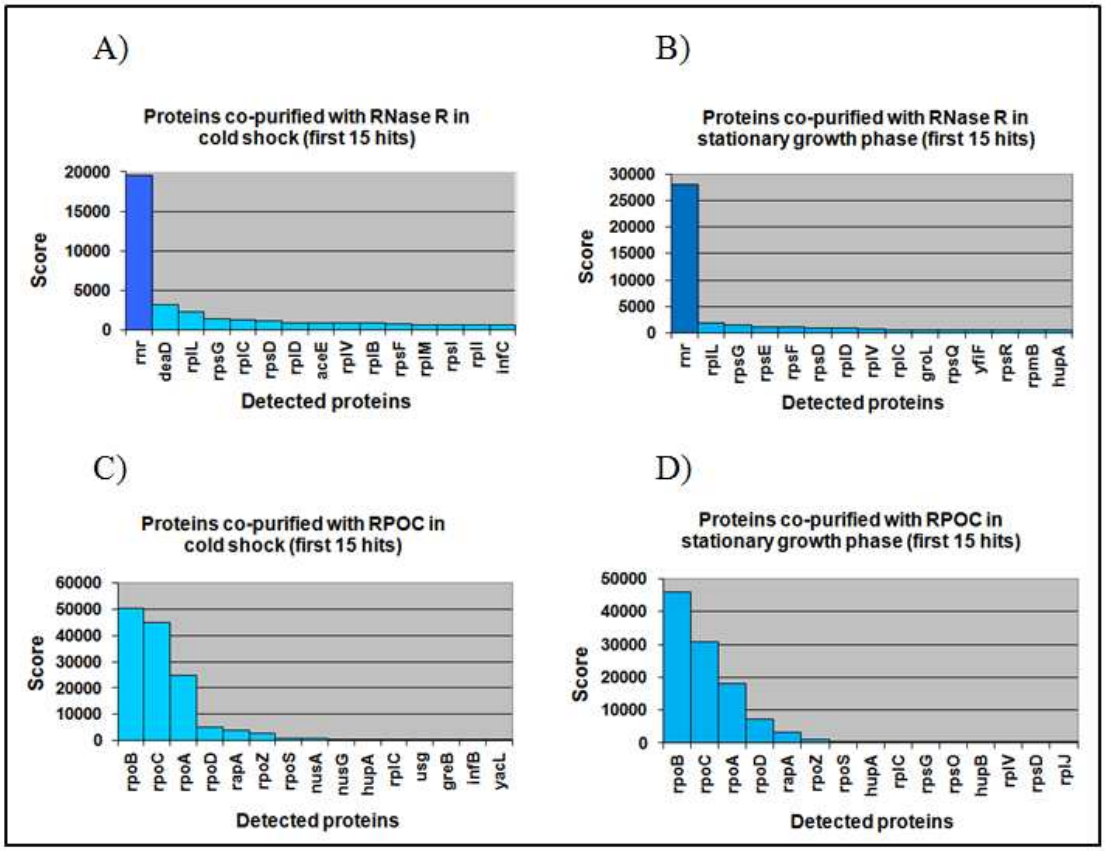

Figure 9 – Mass spectrometry results from TAP tag purification of RNase R or RPOC from cold shock or stationary growth phase cells. Graphs with scores of identified proteins co-purified with RNase R in cold shock (A) or stationary phase (B) and co-purified proteins with RPOC in cold shock (C) or stationary phase (D), from calmodulin elution resin.

By observing the results from mass spectrometry obtained for RNase R purification samples, we identified more proteins during cold shock (80) than in stationary growth phase (64). For RPOC, we had co-purified only one more protein during cold shock (51) than in stationary growth phase (50). In the purifications of RPOC, RPOB was detected with the highest score, followed by RPOC and the other subunits of RNA polymerase that were also detected with high scores. This result

confirms that RPOC forms a stable interaction with the other RNA polymerase subunits. Contrary, for RNase R the protein detected with highest score was RNase R and, the follow co-purified proteins were detected with much lower scores in both conditions. A similar pattern was also observed in the SDS-PAGE gels (Fig. 8). This difference is clearly visible on the graphs (Figure 9). This result suggests that, in contrast to RPOC, RNase R probably does not form stable complexes with defined stechiometry. This does not exclude the possibility that RNase R can transiently interact with other proteins or/and form unstable complexes that are not well preserved in our purification conditions.

A)

RNase R			
Cold shock		Stationary	
Protein	Score	Protein	Score
deaD	3120	yfiF	538
aceE	879	adhE	454
infC	570	ppk	166
adhE	442	deaD	88
lon	258	aceF	86
yfiF	171	aceE	70
lpdA	163	lon	68
rne	139	sra	52
rimM	117	seqA	37
pnp	116		
aceF	96		
rhlB	82		
degP	78		
rlmL	73		
mreB	69		

B)

Protein	Description
deaD	ATP-dependent RNA helicase, 50S ribosomal subunit biogenesis
aceE	Pyruvate dehydrogenase, decarboxylase component E1
infC	Protein chain initiation factor IF3; unusual AUU start codon
adhE	Alcohol dehydrogenase, largely anaerobic; aerobic antioxidant
lon	DNA-binding, ATP-dependent protease LA; lon mutants form long cells
yfiF	Predicted RNA methyltransferase, function unknown; rlmB paralog
lpdA	Lipoamide dehydrogenase, NADH-dependent
rne	RNase E; component of RNA degradosome; mRNA turnover; 5S and 16S RNA maturation
rimM	Factor required for 16S RNA processing; 30S maturation; binds S19
pnp	Exoribonuclease; PNPase component of RNA degradosome; cold shock protein
aceF	Pyruvate dehydrogenase, dihydrolipoamide acetyltransferase E2; acetate requirement
rhlB	ATP-dependent RNA helicase; unwinds dsRNA; component of RNA degradosome; also binds PNPase directly; DEAD box family
degP	Periplasmic, membrane-associated serine endoprotease; protease Do, required for high-temperature growth
rlmL	23S rRNA m(2)G2445 methyltransferase, SAM-dependent
mreB	MreB filaments participate in directional chromosome movement and segregation; mecillinam resistance
ppk	Polyphosphate kinase, reversible
sra	Peptide chain release factor 3, RF-3
seqA	Sequesters newly replicated hemimethylated oriC origins to prevent re-initiation; binds hemimethylated GATC sequences

Table 13 – Mass spectrometry results from TAP tag purification of RNase R. List of non ribosomal proteins detected from TAP tag purification of RNase R cells in the stationary phase or after the cold shock induction (A) and description of the proteins with higher scores (B). Scores are indicated for proteins identified in calmodulin resin elution fraction (excluding RNase R). Proteins found only in RNase R are identified in blue, and in purple are represented the proteins found in RNase R only in stationary phase.

To investigate the possible interactions formed by RNase R during cold shock adaptation, we compared the co-purified proteins in cold shock and stationary growth phase. As already mentioned, from the total of proteins detected for RNase R in both conditions we excluded the ribosomal proteins (Table 13A). In samples derived from cold shocked cells, we detected 16 non ribosomal proteins that co-purified with RNase R, while from cells in stationary growth phase we only detected 9 proteins. From these, 4 proteins were found in both conditions, 3 were specific for stationary phase and 14 for cold shock (Table 13). Two proteins, DeaD and AceE were also found in RPOC preparations from the cold shocked cells, although with lower scores (Table 13). Interestingly, many of the proteins found in the cold shock condition are known to be

involved in the RNA metabolism. We also detected most of the subunits of the bacterial degradosome, the main complex involved in RNA degradation in *E. coli*.

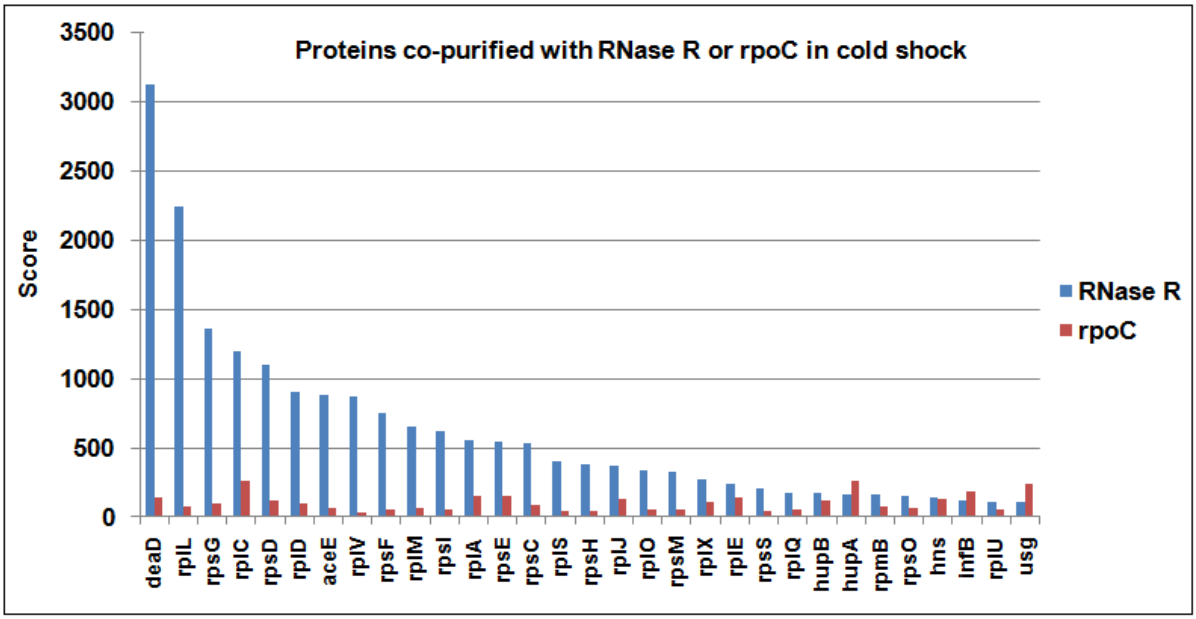

Figure 10 – Comparison of mass spectrometry results from TAP tag purification of RNase R or RPOC from cold shocked cells.

In the purification of TAP tagged RNase R from the cold shocked cells, DeaD protein was detected with the highest score, which made it the most probable candidate for RNase R interactor (Fig 9). DeaD seems to appear in our RNase R preparations specifically in the cold shock conditions. DeaD was also detected in the sample of RNase R purification from stationary grown cells, although with a much lower score, which may suggests the existence of cross contamination between samples. DeaD is an RNA helicase and one of the proteins that are induced in the cold shock adaptation process (137). This protein (70 kDa) has the estimated size of the band observed in the SDS-PAGE gels (Fig. 8). Moreover, it was already described in the literature that RNase R can complement the cold shock functions of DeaD, which suggests a functional interaction between this two proteins (14). DeaD was also detected in RPOC

preparations from cold shocked cells (Annex II, Table 16). This result suggests that this protein could be a "cold shock" specific contamination. We excluded this hypothesis based on the fact that the score for DeaD in RNase R preparation was much higher than the one in RPOC preparations. The comparison of the scores of the other proteins found in both preparations suggests a specific increase of DeaD co-purified with RNase R in cold shock response. It is worth to mention that there is specific enrichment, although to smaller extent, for some of the ribosomal proteins. Resuming, among the potential proteins interacting with RNase R during cold shock, DeaD helicase seemed to be a good candidate to further investigations.

3.1.1 Amplification and cloning of DeaD helicase

In order to determine if DeaD helicase interacts with RNase R we decided to purify both proteins and perform *in vitro* interaction studies. RNase R overexpression plasmid was already constructed and tested (6) so, the first step was to clone DeaD sequence into an expression vector. The coding sequence of the DeaD gene from *E. coli* was amplified by PCR from genomic DNA. As mentioned in Material and Methods section, during the PCR we introduced *Sal*I and *Not*I restriction sites into the product sequence. The PCR product was cloned into pUC18 by blunt-end ligation as described in "Materials and Methods" (2.6 – table 2 to 4). The positive clones were detected using the white/blue selection with *E. coli* DH5α cells and were subsequently digested with *Sal*I and *Not*I restriction enzymes, as described in "Materials and Methods" (2.6 – table 5), to check if the insert was introduced into the plasmid (Fig. 11B).

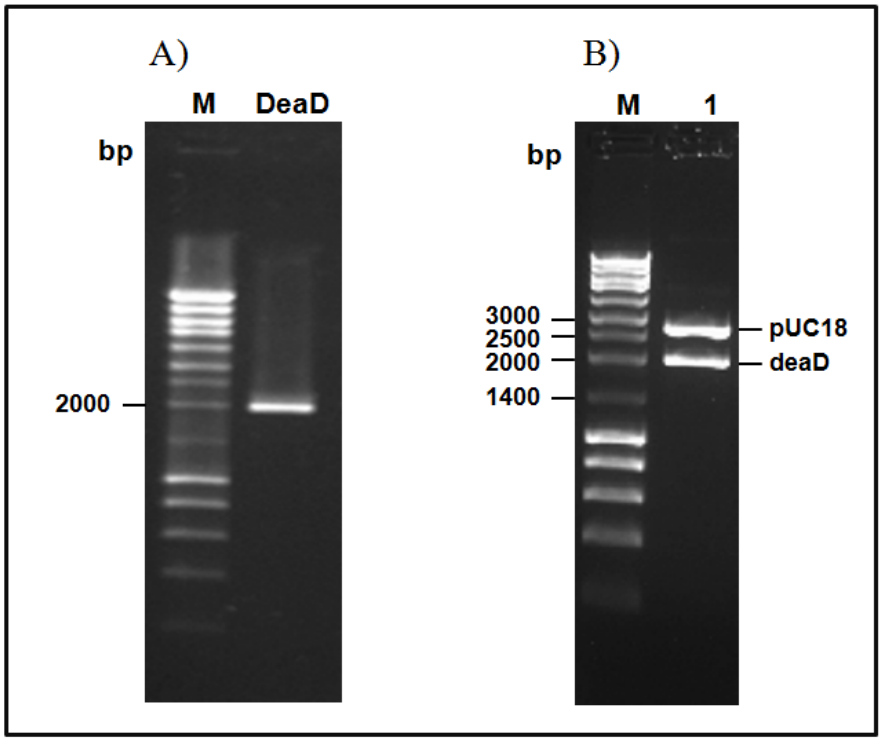

Figure 11 – A) Visualization of DeaD amplification (1890 bps) by PCR on a 0.8% agarose gel. **B)** pUC18-DeaD digestion with *Sal*I and *Not*I (**1 -** DeaD 1890 bps and pUC18 2686 bps), visualized on a 0,8% agarose gel. The DNA size marker (M) is the commercial NZYDNA ladder III (NZYtech)**.** In both gels, bands were visualized by ethidium-bromide staining.

The insertion of DeaD helicase gene into the expression vector was made by Sequence and Ligation Independent Cloning (SLIC), a method based on homologous recombination. The pET28a was the expression vector used to provide a high level of protein overproduction. This plasmid carries an N-terminal HIS tag, which allows the purification of the protein with a HIS-tag. It also contains a gene for kanamycin resistance, important for colony selection (Fig. 12).

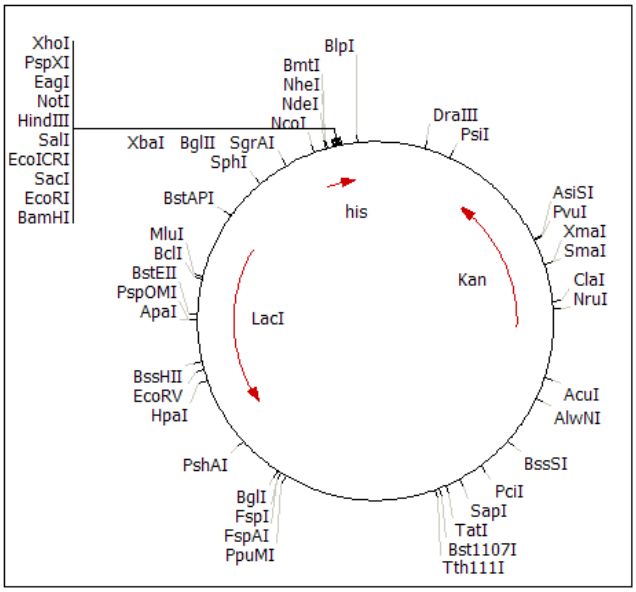

Figure 12 – Schematic diagram of pET28a plasmid. HIS, *Kan* and *Lac*I regions are represented by red arrows pointing the direction of transcription. Restriction sites are also indicated in the figure. This image was obtained by Clone Manager software program.

SLIC is a cloning method that takes advantage of the annealing between a PCR product with about 30bp of sequence that is homologue to the ends of the target vector (94) (Fig. 13). To perform this method we amplified DeaD with new primers (Annex III, table 17) as described in "Materials and Methods" (2.7 – table 6 and 7). Using DeaD cloned in pUC18 as a PCR template, we amplify its gene using primers with 30bp homology to the vector pET28a. The vector was then digested with *Sal*I/*Not*I pair of enzymes. Subsequently, single stranded ends in the vector and insert were generated using T4 DNA polymerase. After ligating the vector and insert, the mixture was used to transform *E. coli* DH5α cells. The positive clones were digested with *Sal*I (see Materials and Methods 2.7 – table 8) to check if the insert was introduced into the pET28a plasmid (Fig. 14). Subsequently, protein expression was induced by IPTG and protein levels were analysed in SDS-PAGE gel (Fig. 15).

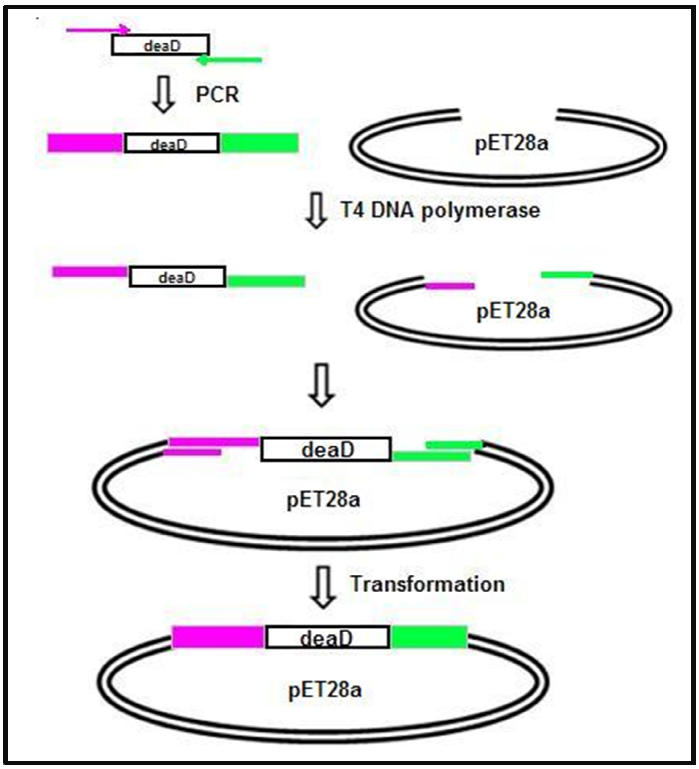

Figure 13 – Representation of DeaD gene insertion in pET28a vector by homologous recombination and single strand annealing. Scheme adapted from Li & Elledge, 2007 (94).

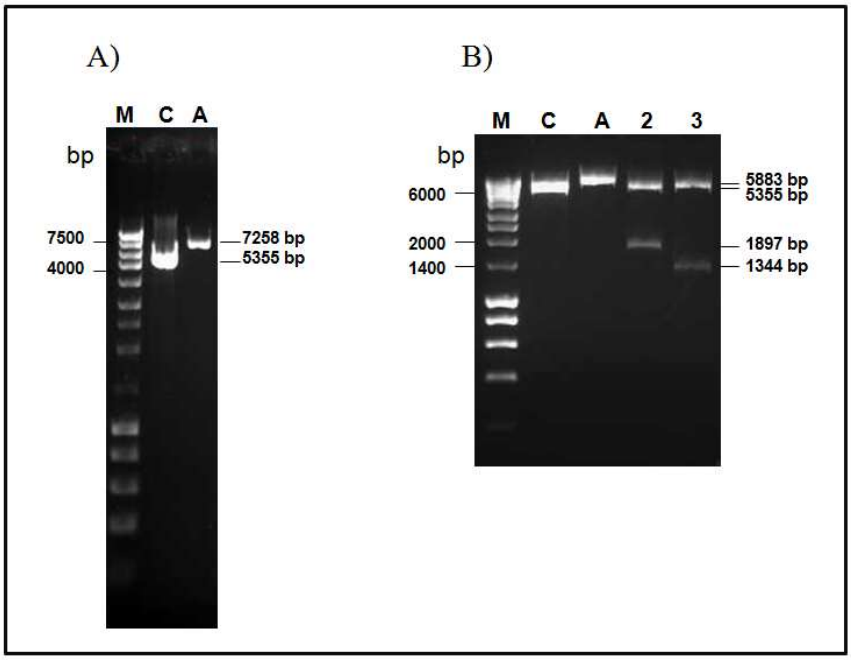

Figure 14 – A – B) Restriction analysis of the recombinants derived from SLIC reaction. Plasmid DNA from one independent kanamycin resistant colony digested with *Sal*I (A), with *Sal*I and *Not*I (2), or with *Ecor*I (3). As a control (C) pET28a was digested with *Sal*I and *Not*I. Digestion products were separated on a 0,8% agarose gel and visualized by staining with ethidium-bromide. The DNA size marker (M) is the commercial NZYDNA ladder III (NZYtech).

The goal of this first experiment was accomplished. We cloned DeaD helicase in the expression vector pET28a and we were able to overexpress this protein by IPTG induction.

3.2 Studies of interaction between RNase R and DeaD helicase

3.2.1 Purification of overexpressed DeaD helicase

In the subsequent experiments we wanted to overexpress and purify HIS tagged DeaD, RNase R and RNase II proteins. As first step, we decided to check the solubility of each of the enzymes. DeaD helicase was cloned on pET28a plasmid as already described in chapter 3.1.1. RNase R and RNase II were available in our laboratory. They were previously cloned on pET15b plasmid. *E. coli* BL21 (DE3) cells were transformed with the appropriate plasmids as described in “Materials and Methods” (2.3). Cells were grown at 37°C at 180 rpm until OD_{600} 0.5 and protein expression was induced with IPTG for three hours (see Materials and Methods 2.8). Purification was performed by HIS tag method and proteins bound to the Ni-NTA beads were analysed in SDS-PAGE gel. The three proteins showed to be soluble and this purification method revealed to be efficient (Fig. 15). For this reason this protocol was repeated in other assays.

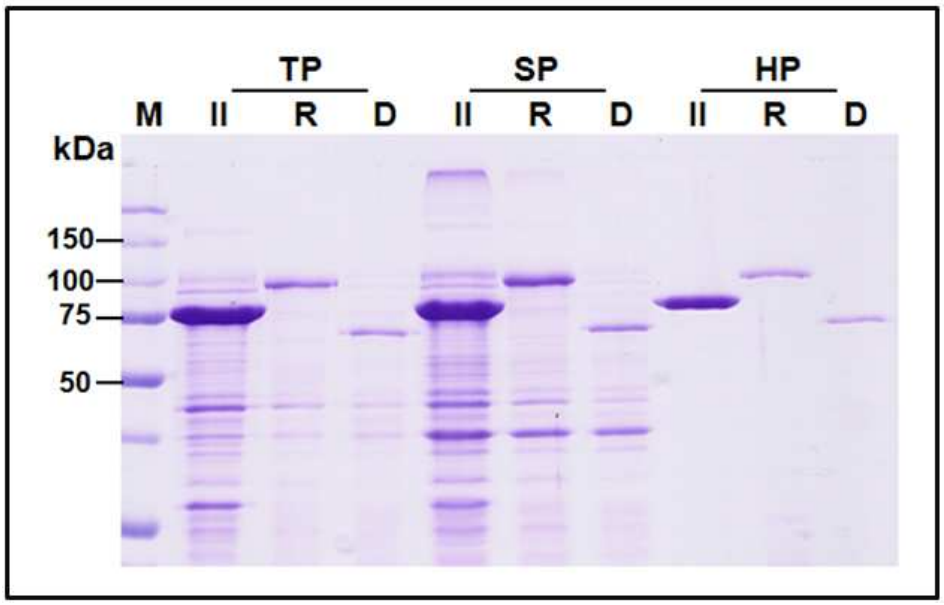

Figure 15 – HIS tag purification of RNase R (R), DeaD (D) or RNase II (II) from exponentially grown cells. The protein fractions were visualized in a 10% SDS-PAGE gel stained with Coomassie Blue solution, TP – Elution Total Proteins, SP – Elution Soluble Proteins, HP – Proteins bound to Ni-NTA beads resin, M – Precision plus Protein Standards (BioRad).

In order to evaluate if DeaD helicase was interacting with RNase R we checked if, while purifying DeaD by the HIS tag method, we could also detect endogenous RNase R enrichment in our preparations. As a control, HIS tagged RNase II was also overexpressed and purified in the same conditions, allowing the comparison of the RNase R levels in both preparations. *E. coli* BL21 (DE3) cells containing the plasmids with HIS tagged proteins were grown until OD_{600} 0.5 and protein expression was induced with IPTG for three hours (see Materials and Methods 2.8). Purification was performed by HIS tag method and after several washes steps, the Ni-NTA beads with the protein bound were analysed in SDS-PAGE and compared with total proteins fractions (Fig. 16). To determine the amount of RNase R co-purified with HIS tagged DeaD and, as a control, with HIS tagged RNase II, RNase R levels were detected by

Western blot using anti-RNase R antibodies (following the protocol as described in "Materials and Methods" (2.12)).

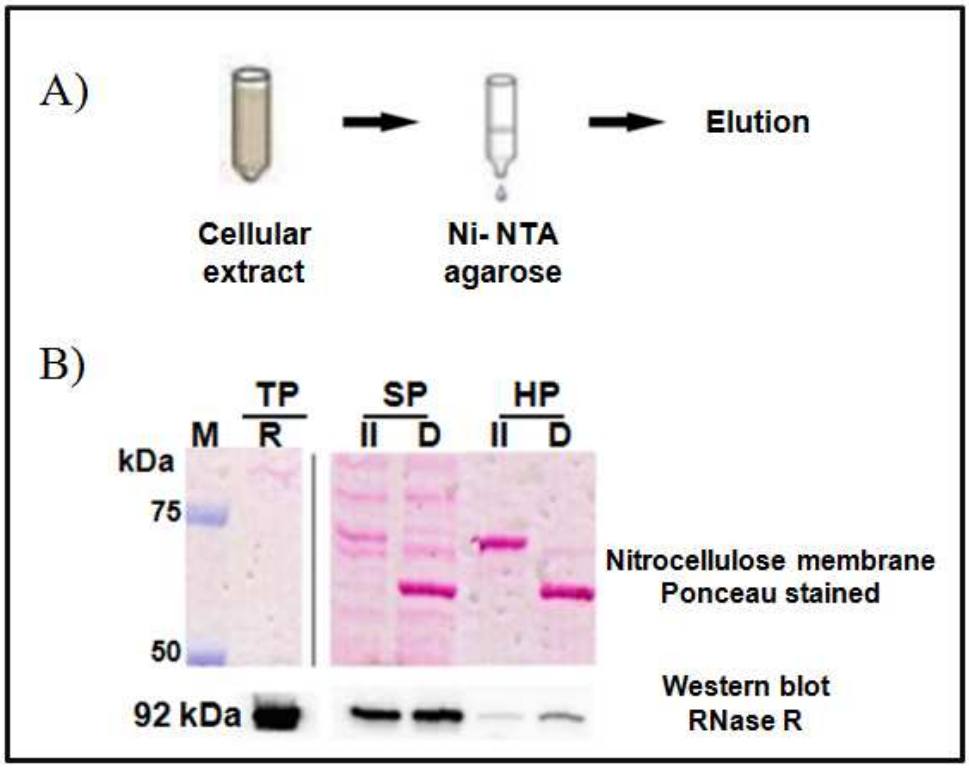

Figure 16 – Scheme of the investigation procedure used to study interaction between HIS tagged DeaD helicase and RNase R. A) Cells overexpressing HIS tag DeaD were purified using Ni-NTA agarose and B) HIS tag elutions were analysed in a 6% SDS-PAGE, transferred into a nitrocellulose membrane and Ponceau stained. The amount of RNase R was detected by Western blot (α-rnr). TP – Total protein fraction of purified RNase R (R), SP – soluble protein fractions from cells overexpressing DeaD (D) or RNase II (II); HP – purified proteins fractions, M – Precision plus Protein Standards (BioRad).

We saw that the RNase R levels detected during purification of DeaD and RNase II were the same. If we compare the determined values in the soluble fraction prior to

purification, no enrichment was observed. The results obtained did not prove any direct interaction between RNase R and DeaD helicase.

3.2.2 In vitro interaction between RNase R and DeaD proteins

Since the first experiment did not revealed any direct interaction between RNase R and DeaD, we wondered if it was because endogenous RNase R could be already involved in some other complexes in the cell in such a way that would not interact with overexpressed DeaD. The second strategy used was, after purification, proteins were mixed and then separated on the gel filtration column to observe protein migration. Gel filtration is a chromatographic method that allows separating proteins based on their molecular weight. The Superdex 200 column allows the separation of proteins from 10 kDa to 600 kDa in the way that smaller proteins diffuse through the column slower than the largest that move quickly. By migrating proteins with known size it is possible to determine accurately in which column volume a protein are expected to be detected by UV spectra. RNase R and DeaD proteins have sizes of 92 and 70 kDa, respectively. We will run a mixture of both proteins on the column. If they do not interact directly, with this chromatographic procedure they will migrate separately, if they interact, an additional peak will be observed around the volume corresponding to the sum of the sizes of DeaD and RNase R, or higher (depending on the complex stechiometry).

To perform this study, after protein purification (described above in section 3.2.1), the proteins were mixed in the end of the purification procedure. Then, they were separated on a gel filtration column (Superdex 200) and monitored by UV spectra. As a control, overexpressed RNase R alone was also separated by this procedure. The UV spectrum at 280nm obtained for the mixture of RNase R and DeaD was compared with the one obtained for RNase R alone. To analyse RNase R and DeaD behavior, fractions collected during gel filtration were analysed in SDS-PAGE gel stained with Coomassie blue solution (Fig. 17).

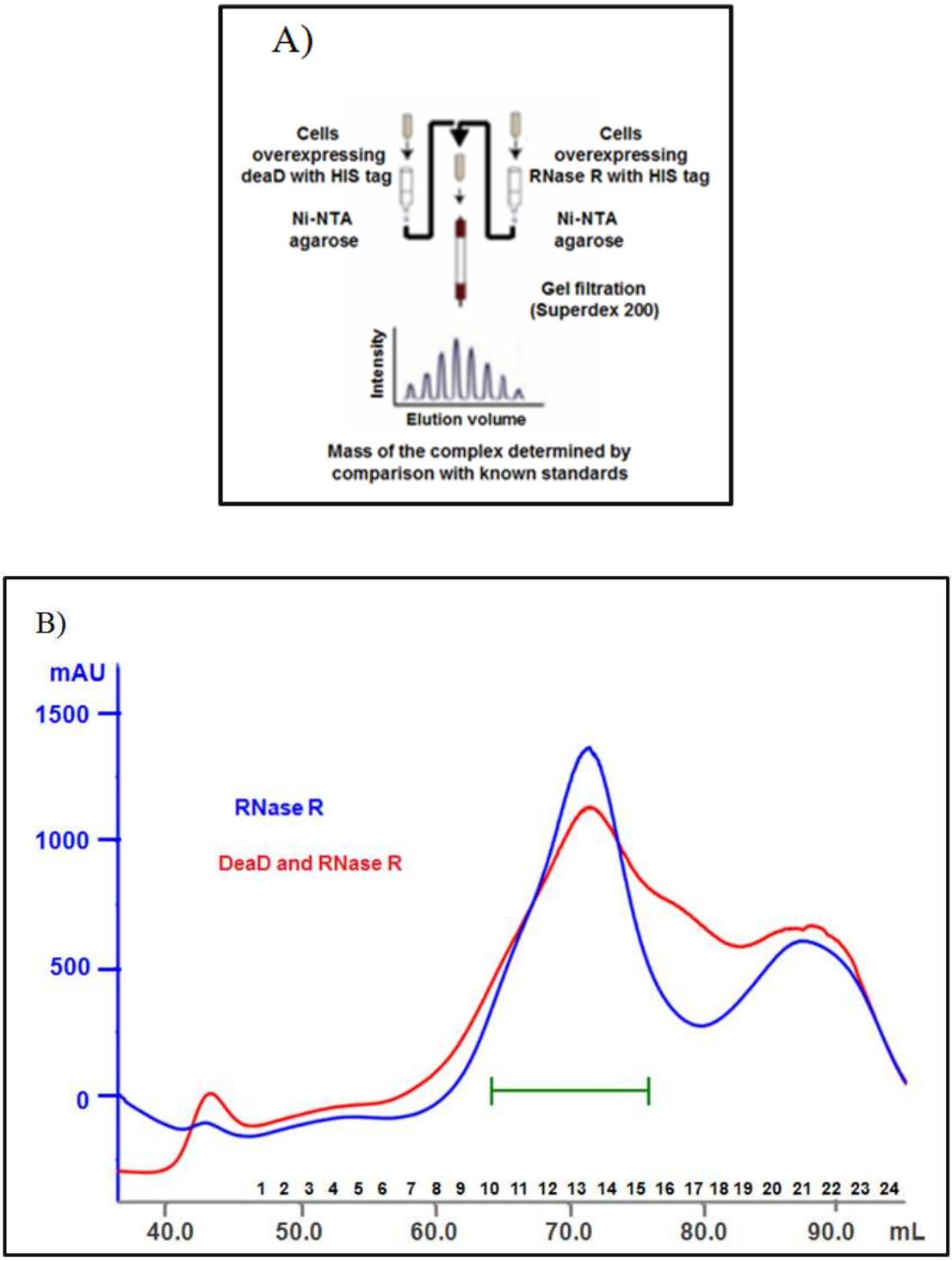

Figure 17 – A) Scheme of RNase R and DeaD purification by the HIS tag method. Purified proteins were mixed, separated on gel filtration column (Superdex 200) and monitorized by UV spectra (280nm). **B)** UV spectra (280nm) obtained after gel

filtration of the mixture of RNase R with DeaD (red) or RNase R alone (blue). The green line represents the expected peak corresponding to RNase R protein (volumes from 65 to 75ml).

Gel filtration method allows determining in which volume a protein with a certain size is going to be detected. The peak obtained for RNase R alone (around 70ml) is the expected one because the volume where it appears is according to the size of RNase R (92 kDa). By observing the migration of the two proteins together, the behaviour is almost the same when compared to RNase R alone. However, there is a small shift between 75 and 85 ml. According to DeaD size (70 kDa), this small shift probably corresponds to the detection of DeaD. If RNase R and DeaD would interact directly, we should detect a peak around 60 ml that is the volume were a protein of more less 170 kDa should be detected (sum of DeaD and RNase R sizes). Since no peak was detected around this volume, it suggests that these two proteins do not interact directly, or they do not interact strongly enough to migrate together.

3.3 RNase R behaviour after cold shock induction

We have performed different attempts to prove the interaction between DeaD and RNase R but direct interaction could not be confirmed. Since that in mass spectrometry analysis other proteins were detected and some could also be involved in cold shock response, we wondered if RNase R could be interacting with other proteins. Other option could be the interaction of RNase R with DeaD through other proteins, forming a stable complex.

In order to investigate this possibility, the third part of this investigation consisted in the separation of *E. coli* total proteins extracts from different growth conditions by gel filtration. After, RNase R migration was monitored using anti-RNase R antibodies. If there is any difference in protein levels and proteins interactions during cold shock or exponential growth phase, this should be detected by protein migration in the gel filtration column. Isolated proteins should give a single peak in the UV spectra and, if RNase R is incorporated into a protein complex, migration pattern of this protein

should be detected in the beginning of the graph. The RNase R detection (by Western blot) from elutions collected during gel filtration allows its comparison with the UV spectra patterns obtained from protein migration on gel filtration column (Fig. 18).

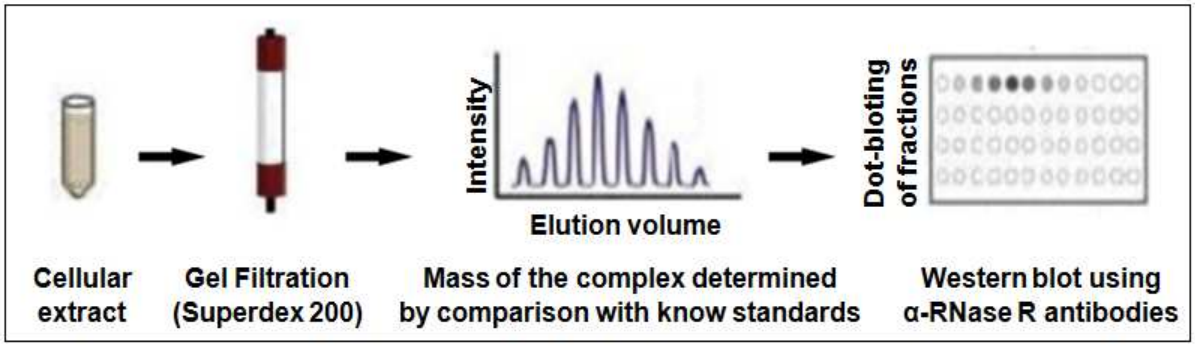

Figure 18 – Representation of the experimental steps for total protein separation and then, RNase R monitoring by Western blot technique. Total proteins were extracted from cells in different growth conditions and extracts were subjected to the gel filtration procedure. Fractions collected were loaded on nitrocellulose membrane using dot blot device and proteins were detected using anti-RNase R antibodies.

To perform this study, BW25113 *E. coli* cells were grown until exponential growth phase or in cold shock conditions. Total protein extracts were collected as described in "Materials and Methods" (2.10) and separated by gel filtration column (Superdex 200). Protein migration was monitorized by UV spectra and fractions collected during gel filtration procedure were loaded on a nitrocellulose membrane using a Dot blot device. Subsequently, the membrane was used to perform Western blot technique with anti RNase R antibodies following the procedure described in "Materials and Methods" (2.12). Results from this method were quantified using ChemiDoc XRS software and compared with the gel filtration protein patterns.

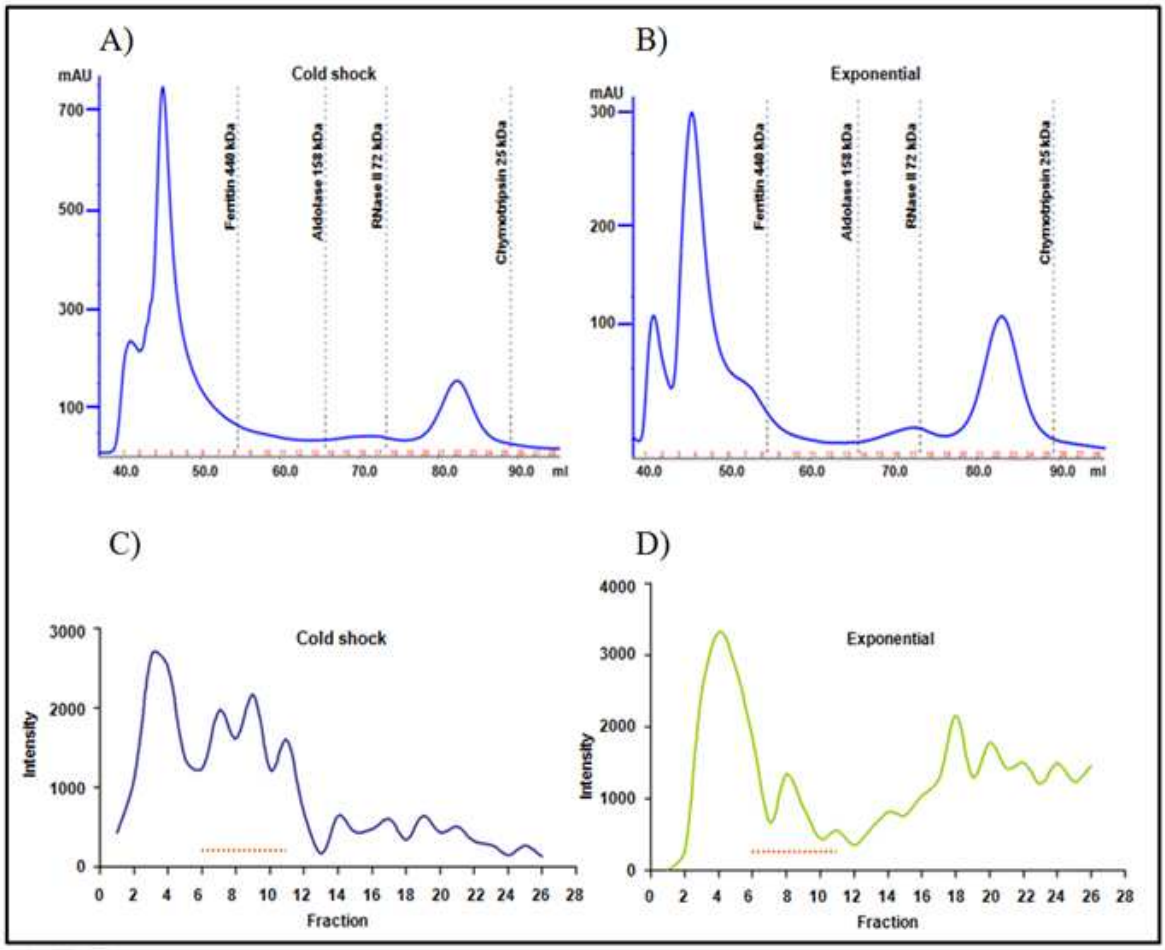

Figure 19 – A, B) Protein spectra (280nm) obtained during filtration of *E. coli* total protein extract on Superdex 200 column. Size of the markers used for column calibration is indicated by dashed lines and fraction numbers are indicated in the bottom part of the graph. UV spectra of protein extracts from *E. coli* after cold shock induction (A) or during exponential growth phase (B). **C, D)** Fractions collected during gel filtration procedure were loaded on nitrocellulose membrane and proteins were detected by Western blot, using anti-RNase R antibodies. Intensity of each fraction (y-axis) was quantified using ChemiDoc XRS software. Graphic representation of protein extracts from *E. coli* after cold shock induction (C) or during exponential growth phase (D).

By observing the results from the gel filtration technique, we can see small differences between the protein pattern after cold shock induction and exponential grown phase. However, the differences are more obvious in the graphs obtained from RNase R detection by Western blot technique. From fractions 6 to 11 (corresponding to 50 - 60 ml) resultant from cold shock induction, the detection of RNase R increased dramatically when compared with the protein pattern observed in exponential phase (Fig. 19C and D). According to the size of this protein (92 kDa), the volume where the peak of detection should be detected was 70 ml (corresponding to fractions 16 - 18). In exponential growth phase a peak was detected around 70 ml but, in cold shock conditions, RNase R seemed to be part of a high molecular mass protein complex (represented in figure 19C with red dots).

These results suggested that, after cold shock induction RNase R is probably recruited into a high molecular mass protein complex (over 450 kDa) in the way that is interacting with more than one protein. Since during purification of RNase R a higher number of ribosomal proteins were detected, we hypothesised that RNase R, DeaD and some ribosomal proteins can interact during cold shock response by forming a protein complex.

4. Discussion and Conclusions

Bacteria are extremely resistant and adaptable microorganisms. They can survive in a variety of stress conditions, one of them is the abrupt change in temperature. As a consequence of the temperature downshift there is a biochemical alteration in the cells and bacteria have to face significant problems. There is a decrease in the cell metabolism and protein synthesis almost stops (82). To prevent the cells from the deleterious effect of the low temperature, bacteria developed the cold shock response, a specific sequence of events that lead to adaptation to the new environmental conditions. Bacteria perform the mentioned changes during acclimation period that follows rapid temperature downshift. One of the characteristic of acclimation is the blocking of translation of the majority of the proteins excepting the group of the cold inducible proteins (CIPs). These proteins are constantly translated but, in most of cases, their level increases (68, 117, 137). CIPs is a group of proteins of different kinds and functions, but all of them are considered to have a role in the bacterial adaptation to low temperatures.

One of the alterations that appear with low temperature is the stabilization of RNA secondary structures. The stabilization of pre-existed structures and the introduction of new ones is considered to lead to disorders in the vital cellular processes like translation, transcription and RNA degradation, secondary structures can mask ribosome binding sites, impede RNA polymerase or ribosome movement, form artificial transcription terminators, and impede RNA degradation. Finally, the cell has to adapt its RNA metabolism to the new conditions.

The adaptation of RNA degradation machinery seems to involve two exoribonucleases, which levels elevate in response to temperature downshift. These are PNPase and RNase R. PNPase is not essential for the cell at 37°C but, at lower temperatures, became vital (13). At low temperatures it is probably involved in the general degradation of RNAs. PNPase is able to digest secondary structures, which

seems to be important in the cold conditions. It was also proved that, at the end of the acclimation phase, PNPase is specifically required for the degradation of unnecessary transcripts of cold shock induced proteins such, as *csp* RNAs. During the acclimation period of the cold shock response, the protein level of PNPase increases about 2-fold (104).

RNase R is also able to degrade double-stranded RNAs and, in normal growth conditions, is responsible for the degradation of different subtracts (28, 43, 44, 95). This protein modulates its level according to the environmental conditions (8, 26) and, after cold shock induction can increase about 10-fold. Despite this dramatic induction, RNase R is not essential for *E. coli* survival in the cold, although its absence originates a small colony phenotype. The exact function of this protein in the cold adaptation process is unclear. However, it was suggested that, according to its enzymatic activity this protein serves in general degradation of RNAs with secondary structures. The elevation of the protein level in response to low temperatures can be a consequence of the increase in the amount and stability of *rnr* message (28). However, recently it was proposed that a post-transcriptional mechanism could explain this phenomenon (96). In fact, it was shown that RNase R protein is very unstable in exponentially growing cells and strongly stabilized in stress conditions. This stabilization, achieved by the action of SmpB/tmRNA leads to an increase in the protein level (96).

The high increase of RNase R level upon temperature downshift suggests its important role in the adaptation to the new conditions. However, until date, the exact function is not known. In our research, we intended to answer to this question. Many times researchers can have the information about the protein function by identifying which proteins are interacting with the target one. With this strategy and using the information about the function of the identified proteins, one can conclude about the metabolic pathways in which our target protein takes part. It can also allow to see if it is involved in any stable protein complexs. We decided to use this strategy to identify and compare proteins that can co-purify with RNase R in different growth conditions. To achieve this goal, we decided to use a construct of an *E. coli* strain with a genome encoded RNase R fused with TAP tag sequence, followed by Tandem Affinity Purification (TAP) method.

4.1 Identification of proteins co-purifying with RNase R

Tandem affinity purification method (TAP tag) is a technique to investigate protein-protein interactions (130). This method was previously successfully used by other researchers, and revealed to be an excellent method to purify protein complexes.

In our experiments we first constructed an *E. coli* strain with a genome encoding RNase R fused with TAP tag sequence. Subsequently tagged protein was purified from the cells grown in three different conditions: exponential phase, stationary phase or exponentially grown cells introduced to low temperatures for three hours (after this time we could observe the highest induction of RNase R from all the times tested). We have compared the protein patterns in exponential, stationary growth phase and after cold shock induction and detected some differences (Fig. 8). The most visible one was the appearing of a band of about 70 kDa, which was observed in the protein elution from cold shocked cells. This protein band was not detected in the preparations from exponential or stationary phase grown cells.

The detection of these differences led us to analyse them in more detail. For a deeper analysis we decided to use mass spectrometry protein identification for the elution samples. For this experiment we only used the elutions from cold shocked and stationary grown cells. The identification of RNase R co-purifying proteins from exponentially grown cells was already performed by other research group using the same method, the TAP-tag. The results obtained suggested that there are no strong interactions formed by RNase R in this growth state (23). Since we suspected that mass spectrometry analysis could give us many false positive hits that will correspond to proteins that will unspecifically interact with resin or TAP tag, we used a proper negative control that will let us to exclude this. As a control we decided to use one of the RNA polymerase subunits, the RPOC protein. We prepared the strain with endogenous RPOC (RNA polymerase subunit C) protein fused with TAP tag and then RPOC interacting proteins were purified in the same conditions and with the same method as the one used for RNase R. We assumed that the proteins that unspecifically bind to the resin should be the same for RNase R and RPOC preparations. Moreover, RPOC forms a well defined protein complex, the RNA polymerase (RNAP). Also, the

interactions of RNAP with different bacterial proteins in different conditions are well known (23). Thus, by searching these well defined interactions in our preparations, we can show the quality of our procedures. As a last argument, RNAP, similarly to what happens with RNase R, is a RNA binding protein. If there are any unspecific interactions with other RNA binding proteins carried by RNA molecules, we also should be able to exclude this by comparing results obtained for our target and for the control. To decrease this last possibility, we were also using benzonase (RNase and DNase) in all preparations of our protein extracts.

Finally, the elution fractions obtained for RPOC from stationary and cold shocked cells and for RNase R from stationary and cold shocked cell were analysed using mass spectrometry method.

As a result of mass spectrometry analysis we obtained lists of proteins detected in each elution. The first step was the exclusion from these lists of the proteins present with similar scores in all preparations. We considered them as contamination.

We obtained 80 proteins in RNase R calmodulin elution fraction from the cold shock cells and 64 proteins from cells in stationary phase. For RPOC we obtained 51 proteins during cold shock and 50 proteins from stationary phase cells.

To verify the quality of our procedures, we compared the results obtained for RPOC elutions with the available literature and databases. In our study, we detected 66 different proteins interacting with RPOC, 84% of which were already reported to interact with RNA polymerase (STRING), (23). This result shows that our work is of good quality and that, among the proteins identified for RNase R, we also should have a high percentage of specific interactions.

In RNase R preparations we detected 80 proteins in cold shock and 64 in stationary growth phase. We also could observe differences in the score pattern for the results obtained for RNase R and RPOC. In the purifications of RPOC, RPOB was detected with the highest score followed by RPOC and the other subunits of RNA polymerase. This result confirms that RPOC forms a stable interaction with the other RNA polymerase subunits. For RNase R the results were different. After RNR that was detected with the highest score, the follow co-purified proteins were detected with much lower scores in both conditions. This result suggests that, in contrast to RPOC, RNase R probably do not form a stable complex with defined stechiometry.

A high number of proteins identified in all purifications were ribosomal components. The presence of ribosomes in our preparations could be due to the unspecific interactions carried by RNA molecules to our proteins in study. At this point, there was no way to exclude this hypothesis and we decided to exclude this hits from our interaction analysis. Without taking into account the ribosomal proteins, we detected 9 proteins co-purifying with RNase R in stationary phase and 19 proteins in the preparations from cold shocked cells. Three of this where found in both stationary and cold shock preparations. The fact that we did not detect these proteins in RPOC preparations and only in both RNase R preparations suggests that these are strong candidates for interactors of RNase R. These proteins are: alcohol dehydrogenase (AdhE) and pyruvate dehydrogenase (AceF), both involved in crucial cell respiration processes. The other protein was Lon protease. Interestingly, Lon protease was suggested a candidate for the enzyme that control the differences in stability of RNase R protein in different growth conditions (42).

We detected more non-ribosomal proteins in preparations of RNase R from cold shocked cells (19) when compared with the ones obtained from stationary phase cells (10). This fact can be explained by the presence of a higher protein concentration in the cell in these conditions (temperature downshift causes a 10-fold increase of RNase R level). We can observe that some of the proteins identified in preparations from cold shocked cells are enzymes involved in the RNA degradation process (4 out of 19). These proteins were not detected in preparations from stationary cells. This may suggests that, in the cold conditions, RNase R is much more involved in RNA degradation than in stationary or exponential phase. This may also be related to the stabilization of the secondary structures of the RNA molecules.

Among the potential interactions that we detected, one especially caught our attention. In the purification of TAP tagged RNase R from the cold shocked cells, DeaD protein was detected with the highest score, which makes it the most probable candidate for RNase R interactor (Table 12). DeaD seems to appear in our RNase R preparations specifically in the cold shock conditions. DeaD was also detected in samples of RNase R purification from stationary grown cells but with a much lower score, which can suggests cross contamination between samples during mass spectrometry analysis (Annex II, Table 16). DeaD is an RNA helicase and one of the proteins that are induced

in the cold shock adaptation process (137). This protein (70 kDa) has the estimated size of the band observed in the SDS-PAGE gels, specifically in RNase R purifyed from cold shocked cells (Fig. 8). Moreover, it was already described in the literature that RNase R can complement the cold shock functions of DeaD, which suggests at least a functional interaction between this two proteins (14). DeaD was also detected in RPOC preparations from cold shocked cells (Annex II, Table 16). This could suggest that this protein is a "cold shock" specific contamination. We partially excluded this hypothesis based on the fact that the score for DeaD in RNase R preparation was much higher than the one in RPOC preparations.

Finally we considered DeaD as the best candidate to check for interaction with RNase R using a different method, which would confirm our mass spectrometry results.

4.2 Studies of interaction between RNase R and DeaD helicase

In the subsequent experiments we were trying to prove the existence of direct interaction between RNase R and DeaD helicase. In the first attempt, we wanted to investigate if during the purification of overexpressed HIS tagged DeaD we could also co-purify endogenous RNase R.

To perform this experiment, we cloned *E. coli* DeaD helicase sequence into the bacterial expression vector pET28a. Then, DeaD was overexpressed and purified and the elution fractions were monitored for RNase R enrichment by Western blot using anti-RNase R antibodies. As a control, we performed the purification of the HIS tagged RNase II (an RNase R homologue that is not induced in cold shock conditions) instead of the DeaD, and we also monitored RNase R levels in the elution fractions.

The results showed no enrichment of RNase R in DeaD purification fractions compared with RNase II purifications (Fig. 16). We concluded that, by using this method we cannot detect any direct interactions between RNase R and DeaD helicase. We also performed this experiment at low temperature, since we hypothesize that the interaction could be temperature dependent. The result again was clearly negative. We decided to use another strategy to ensure that our conclusions are true. We were still

considering the possibility that we were not seeing an interaction between DeaD and RNase R because endogenous RNase R in the cell was already involved in more stable complexes and could not be able to interact with the overexpressed DeaD protein.

To overcome this possibility we decided to overexpress and purify both proteins and to check if we can detect any interaction in vitro. Proteins were purified separately, mixed and then analysed by gel filtration to observe if there would be any changes in protein migration. This chromatographic method allows separating proteins according to their size in the native conditions. Since native conditions are used with this method, the interactions between proteins should be preserved and the protein complexes should migrate differently when compared to the single proteins.

Once again, the results obtained with this experiment did not prove any specific interaction between the investigated proteins. The UV pattern of RNase R migration on gel filtration column was not changed with the addition of equimolar amounts of DeaD protein.

Thus, by using two independent strategies we proved that there is no direct interaction between RNase R and DeaD helicase. These results are in the opposition to the ones obtained using the TAP tag technique followed by mass spectrometry analysis, which was pointing to the existence of an interaction between these two proteins. Our research than led us into the assumption that the interaction between DeaD and RNase R observed by the TAP tag technique was artificial.

From the other hand, there was still one more explanation that could conciliate the two opposite results. The interaction between DeaD and RNase R detected by TAP tag technique might not have been direct. We can imagine that RNase R in the cold shock may interact with other proteins that can subsequently interact with DeaD. In this situation, RNase R would co-purify with DeaD *via* other proteins. This does not mean that both proteins can directly interact. To check this hypothesis we went to see if RNase R could be incorporated into any complex in cold conditions.

4.3 RNase R behavior after cold shock induction

Since we could purify RNase R together with other cellular proteins using TAP tag strategy we assumed that RNase R, at least in the cold shock conditions, could be incorporated into a protein complex. To prove this, we decided to isolate total bacterial soluble proteins in similar conditions to the ones used in the TAP tag purification experiments. Subsequently, we separated the total soluble proteins in the native conditions, using gel filtration chromatography, and we monitored the fractions using anti RNase R antibodies.

By using the gel filtration technique, we are able to separate proteins according to their size. Since we used native conditions, we are also able to separate protein complexes. The calibration of the column allows us to know which elution fraction corresponds to which protein size. Subsequently, we analysed the amount of RNase R signal in each fraction and we could see which fractions have the highest amounts of RNase R and check the approximate protein size characteristic for them. In our experiments we compared protein extracts isolated from exponentially grown and cold shocked cells.

RNase R is a 90 kDa protein so, if it would be a soluble cytosolic protein not involved in any interactions, we would expect it to migrate in the gel filtration column accordingly to its size. In our results this situation was observed in the case of protein extracts isolated from exponentially grown cells. Interestingly, in the case of extracts isolated from cold shocked cells, RNase R signal enrichment was observed in different fractions corresponding to protein sizes of 400-500kDa. This result suggests that RNase R can be incorporated into a protein complex of this size. Moreover, in both cases we could also observe high signal enrichment in the beginning of the fractionation, in the void volume. At this volume migrates all the particles that are too big to be separated by the column. This includes aggregated proteins, but also big particles like bacterial ribosomes (over 2MDa).

The obtained result suggests that, at low temperatures RNase R can be incorporated into a high molecular mass protein complex. From this data we cannot say if DeaD helicase is the part of the complex that we observe on gel filtration column. More analysis will be needed to clarify this hypothesis.

The high RNase R signal in column void volume suggests either that RNase R in our extracts is partially aggregated (which is a probable hypothesis, since this is not very stable protein). On the other hand, it can also suggest the interaction with some high molecular mass particles, like ribosomes. In our mass spectrometry results we could identify several ribosomal proteins. Moreover, DeaD was previously reported to be ribosome bound. Facing this fact, it may be interesting in a near future to check eventual interactions between ribosomes and RNase R.

4.4 Final Conclusions

RNase R increases its level in the cold shock about ten-fold. In spite of this dramatic increase, the role of this exonuclease in the cell adaptation to low temperatures is not clear. In our work, we are trying to find out the function of this protein in cold adaptation, starting with the identification of proteins interacting with RNase R. As a result of our work, we identified proteins that co-purify with RNase R in different grow conditions: stationary phase and low temperature. Our results brought many candidates that can potentially interact with RNase R. The next step is to investigate the most probable interactions, and to find its biological meaning.

The first interaction we wanted to investigate was with DeaD helicase. Our research showed that the interaction between RNase R and DeaD is not direct. Then, we showed that RNase R, in low temperature conditions, is probably incorporated into a high molecular mass protein complex. More research is needed to check if DeaD is also part of this complex.

5. References

1. **Agafonov, D. E., V. A. Kolb, I. V. Nazimov, and A. S. Spirin.** 1999. A protein residing at the subunit interface of the bacterial ribosome. Proc Natl Acad Sci U S A **96:**12345-9.

2. **Agafonov, D. E., V. A. Kolb, and A. S. Spirin.** 2001. Ribosome-associated protein that inhibits translation at the aminoacyl-tRNA binding stage. EMBO Rep **2:**399-402.

3. **Aguilar, P. S., J. E. Cronan, Jr., and D. de Mendoza.** 1998. A *Bacillus subtilis* gene induced by cold shock encodes a membrane phospholipid desaturase. J Bacteriol **180:**2194-200.

4. **Aguilar, P. S., A. M. Hernandez-Arriaga, L. E. Cybulski, A. C. Erazo, and D. de Mendoza.** 2001. Molecular basis of thermosensing: a two-component signal transduction thermometer in *Bacillus subtilis*. Embo J **20:**1681-91.

5. **Albanesi, D., M. C. Mansilla, and D. de Mendoza.** 2004. The membrane fluidity sensor DesK of *Bacillus subtilis* controls the signal decay of its cognate response regulator. J Bacteriol **186:**2655-63.

6. **Amblar, M., A. Barbas, P. Gomez-Puertas, and C. M. Arraiano.** 2007. The role of the S1 domain in exoribonucleolytic activity: substrate specificity and multimerization. RNA **13:**317-27.

7. **Anderson, K. L., and P. M. Dunman.** 2009. Messenger RNA Turnover Processes in *Escherichia coli*, *Bacillus subtilis*, and Emerging Studies in *Staphylococcus aureus*. Int J Microbiol **2009:**525491.

8. **Andrade, J. M., F. Cairrao, and C. M. Arraiano.** 2006. RNase R affects gene expression in stationary phase: regulation of *ompA*. Mol Microbiol **60:**219-28.

9. **Andrade, J. M., V. Pobre, I. J. Silva, S. Domingues, and C. M. Arraiano.** 2009. The role of 3'-5' exoribonucleases in RNA degradation. Prog Mol Biol Transl Sci **85:**187-229.

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

11. **Atlung, T., and F. G. Hansen.** 1999. Low-temperature-induced DnaA protein synthesis does not change initiation mass in *Escherichia coli* K-12. J Bacteriol **181:**5557-62.

12. **Atlung, T., and H. Ingmer.** 1997. H-NS: a modulator of environmentally regulated gene expression. Mol Microbiol **24:**7-17.

13. **Awano, N., M. Inouye, and S. Phadtare.** 2008. RNase activity of polynucleotide phosphorylase is critical at low temperature in *Escherichia coli* and is complemented by RNase II. J Bacteriol **190:**5924-33.

14. **Awano, N., V. Rajagopal, M. Arbing, S. Patel, J. Hunt, M. Inouye, and S. Phadtare.** *Escherichia coli* RNase R has dual activities, helicase and RNase. J Bacteriol **192:**1344-52.

15. **Bae, W., B. Xia, M. Inouye, and K. Severinov.** 2000. *Escherichia coli* CspA-family RNA chaperones are transcription antiterminators. Proc Natl Acad Sci U S A **97:**7784-9.

16. **Barbas, A., R. G. Matos, M. Amblar, E. Lopez-Vinas, P. Gomez-Puertas, and C. M. Arraiano.** 2008. New insights into the mechanism of RNA degradation by ribonuclease II: identification of the residue responsible for setting the RNase II end product. J Biol Chem **283:**13070-6.

17. **Beran, R. K., and R. W. Simons.** 2001. Cold-temperature induction of *Escherichia coli* polynucleotide phosphorylase occurs by reversal of its autoregulation. Mol Microbiol **39:**112-25.

18. **Blaha, G., D. N. Wilson, G. Stoller, G. Fischer, R. Willumeit, and K. H. Nierhaus.** 2003. Localization of the trigger factor binding site on the ribosomal 50S subunit. J Mol Biol **326:**887-97.

19. **Brandi, A., P. Pietroni, C. O. Gualerzi, and C. L. Pon.** 1996. Post-transcriptional regulation of CspA expression in *Escherichia coli*. Mol Microbiol **19:**231-40.

20. **Brandi, A., R. Spurio, C. O. Gualerzi, and C. L. Pon.** 1999. Massive presence of the *Escherichia coli* 'major cold-shock protein' CspA under non-stress conditions. Embo J **18:**1653-9.

21. **Briani, F., M. Del Favero, R. Capizzuto, C. Consonni, S. Zangrossi, C. Greco, L. De Gioia, P. Tortora, and G. Deho.** 2007. Genetic analysis of polynucleotide phosphorylase structure and functions. Biochimie **89:**145-57.

22. **Briegel, K. J., A. Baker, and C. Jain.** 2006. Identification and analysis of *Escherichia coli* ribonuclease E dominant-negative mutants. Genetics **172:**7-15.

23. **Butland, G., J. M. Peregrin-Alvarez, J. Li, W. Yang, X. Yang, V. Canadien, A. Starostine, D. Richards, B. Beattie, N. Krogan, M. Davey, J. Parkinson, J. Greenblatt, and A. Emili.** 2005. Interaction network containing conserved and essential protein complexes in *Escherichia coli*. Nature **433:**531-7.

24. **Bylund, G. O., L. C. Wipemo, L. A. Lundberg, and P. M. Wikstrom.** 1998. RimM and RbfA are essential for efficient processing of 16S rRNA in *Escherichia coli*. J Bacteriol **180:**73-82.

25. **Cairrao, F., C. Arraiano, and S. Newbury.** 2005. Drosophila gene tazman, an orthologue of the yeast exosome component Rrp44p/Dis3, is differentially expressed during development. Dev Dyn **232:**733-7.

26. **Cairrao, F., and C. M. Arraiano.** 2006. The role of endoribonucleases in the regulation of RNase R. Biochem Biophys Res Commun **343:**731-7.

27. **Cairrao, F., A. Chora, R. Zilhao, A. J. Carpousis, and C. M. Arraiano.** 2001. RNase II levels change according to the growth conditions: characterization of *gmr*, a new *Escherichia coli* gene involved in the modulation of RNase II. Mol Microbiol **39:**1550-61.

28. **Cairrao, F., A. Cruz, H. Mori, and C. M. Arraiano.** 2003. Cold shock induction of RNase R and its role in the maturation of the quality control mediator SsrA/tmRNA. Mol Microbiol **50:**1349-60.

29. **Caldas, T., S. Laalami, and G. Richarme.** 2000. Chaperone properties of bacterial elongation factor EF-G and initiation factor IF2. J Biol Chem **275:**855-60.

30. **Cao-Hoang, L., F. Dumont, P. A. Marechal, and P. Gervais.** Inactivation of *Escherichia coli* and *Lactobacillus plantarum* in relation to membrane permeabilization due to rapid chilling followed by cold storage. Arch Microbiol **192:**299-305.

31. **Cao-Hoang, L., P. A. Marechal, M. Le-Thanh, and P. Gervais.** 2008. Synergistic action of rapid chilling and nisin on the inactivation of *Escherichia coli*. Appl Microbiol Biotechnol **79:**105-9.

32. **Carpousis, A. J.** 2002. The *Escherichia coli* RNA degradosome: structure, function and relationship in other ribonucleolytic multienzyme complexes. Biochem Soc Trans **30:**150-5.

33. **Carpousis, A. J.** 2007. The RNA degradosome of *Escherichia coli*: an mRNA-degrading machine assembled on RNase E. Annu Rev Microbiol **61:**71-87.

34. **Cartier, G., F. Lorieux, F. Allemand, M. Dreyfus, and T. Bizebard.** Cold adaptation in DEAD-box proteins. Biochemistry **49:**2636-46.

35. **Carty, S. M., K. R. Sreekumar, and C. R. Raetz.** 1999. Effect of cold shock on lipid A biosynthesis in *Escherichia coli*. Induction At 12 degrees C of an acyltransferase specific for palmitoleoyl-acyl carrier protein. J Biol Chem **274:**9677-85.

36. **Chaikam, V., and D. T. Karlson.** Comparison of structure, function and regulation of plant cold shock domain proteins to bacterial and animal cold shock domain proteins. BMB Rep **43:**1-8.

37. **Chapot-Chartier, M. P., C. Schouler, A. S. Lepeuple, J. C. Gripon, and M. C. Chopin.** 1997. Characterization of cspB, a cold-shock-inducible gene from Lactococcus lactis, and evidence for a family of genes homologous to the Escherichia coli cspA major cold shock gene. J Bacteriol **179:**5589-93.

38. **Charollais, J., M. Dreyfus, and I. Iost.** 2004. CsdA, a cold-shock RNA helicase from *Escherichia coli*, is involved in the biogenesis of 50S ribosomal subunit. Nucleic Acids Res **32:**2751-9.

39. **Charollais, J., D. Pflieger, J. Vinh, M. Dreyfus, and I. Iost.** 2003. The DEAD-box RNA helicase SrmB is involved in the assembly of 50S ribosomal subunits in *Escherichia coli*. Mol Microbiol **48:**1253-65.

40. **Charpentier, X., S. P. Faucher, S. Kalachikov, and H. A. Shuman.** 2008. Loss of RNase R induces competence development in *Legionella pneumophila*. J Bacteriol **190:**8126-36.

41. **Chen, C., and M. P. Deutscher.** 2005. Elevation of RNase R in response to multiple stress conditions. J Biol Chem **280:**34393-6.

42. **Chen, C., and M. P. Deutscher.** RNase R is a highly unstable protein regulated by growth phase and stress. Rna **16:**667-72.

43. **Cheng, Z. F., and M. P. Deutscher.** 2005. An important role for RNase R in mRNA decay. Mol Cell **17:**313-8.

44. **Cheng, Z. F., and M. P. Deutscher.** 2003. Quality control of ribosomal RNA mediated by polynucleotide phosphorylase and RNase R. Proc Natl Acad Sci U S A **100:**6388-93.

45. **Cheng, Z. F., Y. Zuo, Z. Li, K. E. Rudd, and M. P. Deutscher.** 1998. The *vacB* gene required for virulence in *Shigella flexneri* and *Escherichia coli* encodes the exoribonuclease RNase R. J Biol Chem **273:**14077-80.

46. **Cleveland, J., T. J. Montville, I. F. Nes, and M. L. Chikindas.** 2001. Bacteriocins: safe, natural antimicrobials for food preservation. Int J Food Microbiol **71:**1-20.

47. **Coburn, G. A., X. Miao, D. J. Briant, and G. A. Mackie.** 1999. Reconstitution of a minimal RNA degradosome demonstrates functional coordination between a 3' exonuclease and a DEAD-box RNA helicase. Genes Dev **13:**2594-603.

48. **Cordin, O., J. Banroques, N. K. Tanner, and P. Linder.** 2006. The DEAD-box protein family of RNA helicases. Gene **367:**17-37.

49. **Cummings, H. S., and J. W. Hershey.** 1994. Translation initiation factor IF1 is essential for cell viability in *Escherichia coli*. J Bacteriol **176:**198-205.

50. **Dammel, C. S., and H. F. Noller.** 1995. Suppression of a cold-sensitive mutation in 16S rRNA by overexpression of a novel ribosome-binding factor, RbfA. Genes Dev **9:**626-37.

51. **Dersch, P., S. Kneip, and E. Bremer.** 1994. The nucleoid-associated DNA-binding protein H-NS is required for the efficient adaptation of *Escherichia coli* K-12 to a cold environment. Mol Gen Genet **245:**255-9.

52. **Donovan, W. P., and S. R. Kushner.** 1986. Polynucleotide phosphorylase and ribonuclease II are required for cell viability and mRNA turnover in *Escherichia coli* K-12. Proc Natl Acad Sci U S A **83:**120-4.

53. **Drlica, K.** 1992. Control of bacterial DNA supercoiling. Mol Microbiol **6:**425-33.

54. **Erova, T. E., V. G. Kosykh, A. A. Fadl, J. Sha, A. J. Horneman, and A. K. Chopra.** 2008. Cold shock exoribonuclease R (VacB) is involved in *Aeromonas hydrophila* pathogenesis. J Bacteriol **190:**3467-74.

55. **Fairman, M. E., P. A. Maroney, W. Wang, H. A. Bowers, P. Gollnick, T. W. Nilsen, and E. Jankowsky.** 2004. Protein displacement by DExH/D "RNA helicases" without duplex unwinding. Science **304:**730-4.

56. **Feng, Y., H. Huang, J. Liao, and S. N. Cohen.** 2001. *Escherichia coli* poly(A)-binding proteins that interact with components of degradosomes or impede RNA decay mediated by polynucleotide phosphorylase and RNase E. J Biol Chem **276:**31651-6.

57. **Frazao, C., C. E. McVey, M. Amblar, A. Barbas, C. Vonrhein, C. M. Arraiano, and M. A. Carrondo.** 2006. Unravelling the dynamics of RNA degradation by ribonuclease II and its RNA-bound complex. Nature **443:**110-4.

58. **Friedman, D. I., E. R. Olson, C. Georgopoulos, K. Tilly, I. Herskowitz, and F. Banuett.** 1984. Interactions of bacteriophage and host macromolecules in the growth of bacteriophage lambda. Microbiol Rev **48:**299-325.

59. **Gao, H., Z. K. Yang, L. Wu, D. K. Thompson, and J. Zhou.** 2006. Global transcriptome analysis of the cold shock response of *Shewanella oneidensis* MR-

1 and mutational analysis of its classical cold shock proteins. J Bacteriol **188:**4560-9.

60. **Garcia-Mena, J., A. Das, A. Sanchez-Trujillo, C. Portier, and C. Montanez.** 1999. A novel mutation in the KH domain of polynucleotide phosphorylase affects autoregulation and mRNA decay in *Escherichia coli*. Mol Microbiol **33:**235-48.

61. **Giangrossi, M., A. M. Giuliodori, C. O. Gualerzi, and C. L. Pon.** 2002. Selective expression of the beta-subunit of nucleoid-associated protein HU during cold shock in *Escherichia coli*. Mol Microbiol **44:**205-16.

62. **Goldenberg, D., I. Azar, and A. B. Oppenheim.** 1996. Differential mRNA stability of the cspA gene in the cold-shock response of *Escherichia coli*. Mol Microbiol **19:**241-8.

63. **Goldstein, J., N. S. Pollitt, and M. Inouye.** 1990. Major cold shock protein of *Escherichia coli*. Proc Natl Acad Sci U S A **87:**283-7.

64. **Grau, R., and D. de Mendoza.** 1993. Regulation of the synthesis of unsaturated fatty acids by growth temperature in *Bacillus subtilis*. Mol Microbiol **8:**535-42.

65. **Graumann, P., and M. A. Marahiel.** 1997. Effects of heterologous expression of CspB, the major cold shock protein of *Bacillus subtillis*, on protein synthesis in *Escherichia coli*. Mol Gen Genet **253:**745-52.

66. **Graumann, P., and M. A. Marahiel.** 1996. Some like it cold: response of microorganisms to cold shock. Arch Microbiol **166:**293-300.

67. **Graumann, P., K. Schroder, R. Schmid, and M. A. Marahiel.** 1996. Cold shock stress-induced proteins in *Bacillus subtilis*. J Bacteriol **178:**4611-9.

68. **Graumann, P. L., and M. A. Marahiel.** 1998. A superfamily of proteins that contain the cold-shock domain. Trends Biochem Sci **23:**286-90.

69. **Gualerzi, C. O., A. M. Giuliodori, and C. L. Pon.** 2003. Transcriptional and post-transcriptional control of cold-shock genes. J Mol Biol **331:**527-39.

70. **Gualerzi, C. O., and C. L. Pon.** 1990. Initiation of mRNA translation in prokaryotes. Biochemistry **29:**5881-9.

71. **Hanna, M. M., and K. Liu.** 1998. Nascent RNA in transcription complexes interacts with CspE, a small protein in *E. coli* implicated in chromatin condensation. J Mol Biol **282:**227-39.

72. **Higgins, C. F., C. J. Dorman, D. A. Stirling, L. Waddell, I. R. Booth, G. May, and E. Bremer.** 1988. A physiological role for DNA supercoiling in the

osmotic regulation of gene expression in S. typhimurium and E. coli. Cell **52:**569-84.

73. **Horn, G., R. Hofweber, W. Kremer, and H. R. Kalbitzer.** 2007. Structure and function of bacterial cold shock proteins. Cell Mol Life Sci **64:**1457-70.

74. **Jarrige, A. C., N. Mathy, and C. Portier.** 2001. PNPase autocontrols its expression by degrading a double-stranded structure in the pnp mRNA leader. Embo J **20:**6845-55.

75. **Jiang, W., L. Fang, and M. Inouye.** 1996. The role of the 5'-end untranslated region of the mRNA for CspA, the major cold-shock protein of *Escherichia coli*, in cold-shock adaptation. J Bacteriol **178:**4919-25.

76. **Jiang, W., Y. Hou, and M. Inouye.** 1997. CspA, the major cold-shock protein of *Escherichia coli*, is an RNA chaperone. J Biol Chem **272:**196-202.

77. **Jones, P. G., M. Cashel, G. Glaser, and F. C. Neidhardt.** 1992. Function of a relaxed-like state following temperature downshifts in *Escherichia coli*. J Bacteriol **174:**3903-14.

78. **Jones, P. G., and M. Inouye.** 1994. The cold-shock response--a hot topic. Mol Microbiol **11:**811-8.

79. **Jones, P. G., and M. Inouye.** 1996. RbfA, a 30S ribosomal binding factor, is a cold-shock protein whose absence triggers the cold-shock response. Mol Microbiol **21:**1207-18.

80. **Jones, P. G., R. Krah, S. R. Tafuri, and A. P. Wolffe.** 1992. DNA gyrase, CS7.4, and the cold shock response in *Escherichia coli*. J Bacteriol **174:**5798-802.

81. **Jones, P. G., M. Mitta, Y. Kim, W. Jiang, and M. Inouye.** 1996. Cold shock induces a major ribosomal-associated protein that unwinds double-stranded RNA in *Escherichia coli*. Proc Natl Acad Sci U S A **93:**76-80.

82. **Jones, P. G., R. A. VanBogelen, and F. C. Neidhardt.** 1987. Induction of proteins in response to low temperature in *Escherichia coli*. J Bacteriol **169:**2092-5.

83. **Kandror, O., A. DeLeon, and A. L. Goldberg.** 2002. Trehalose synthesis is induced upon exposure of *Escherichia coli* to cold and is essential for viability at low temperatures. Proc Natl Acad Sci U S A **99:**9727-32.

84. **Kandror, O., and A. L. Goldberg.** 1997. Trigger factor is induced upon cold shock and enhances viability of *Escherichia coli* at low temperatures. Proc Natl Acad Sci U S A **94:**4978-81.

85. **Kasai, T., R. S. Gupta, and D. Schlessinger.** 1977. Exoribonucleases in wild type *Escherichia coli* and RNase II-deficient mutants. J Biol Chem **252:**8950-6.

86. **Khemici, V., and A. J. Carpousis.** 2004. The RNA degradosome and poly(A) polymerase of *Escherichia coli* are required in vivo for the degradation of small mRNA decay intermediates containing REP-stabilizers. Mol Microbiol **51:**777-90.

87. **Khemici, V., I. Toesca, L. Poljak, N. F. Vanzo, and A. J. Carpousis.** 2004. The RNase E of *Escherichia coli* has at least two binding sites for DEAD-box RNA helicases: functional replacement of RhlB by RhlE. Mol Microbiol **54:**1422-30.

88. **Kido, M., K. Yamanaka, T. Mitani, H. Niki, T. Ogura, and S. Hiraga.** 1996. RNase E polypeptides lacking a carboxyl-terminal half suppress a mukB mutation in *Escherichia coli*. J Bacteriol **178:**3917-25.

89. **Kushner, S. R.** 2002. mRNA decay in *Escherichia coli* comes of age. J Bacteriol **184:**4658-65; discussion 4657.

90. **La Teana, A., A. Brandi, M. Falconi, R. Spurio, C. L. Pon, and C. O. Gualerzi.** 1991. Identification of a cold shock transcriptional enhancer of the *Escherichia coli* gene encoding nucleoid protein H-NS. Proc Natl Acad Sci U S A **88:**10907-11.

91. **Lalonde, M. S., Y. Zuo, J. Zhang, X. Gong, S. Wu, A. Malhotra, and Z. Li.** 2007. Exoribonuclease R in *Mycoplasma genitalium* can carry out both RNA processing and degradative functions and is sensitive to RNA ribose methylation. Rna **13:**1957-68.

92. **Lee, S. J., A. Xie, W. Jiang, J. P. Etchegaray, P. G. Jones, and M. Inouye.** 1994. Family of the major cold-shock protein, CspA (CS7.4), of *Escherichia coli*, whose members show a high sequence similarity with the eukaryotic Y-box binding proteins. Mol Microbiol **11:**833-9.

93. **Lelivelt, M. J., and T. H. Kawula.** 1995. Hsc66, an Hsp70 homolog in *Escherichia coli*, is induced by cold shock but not by heat shock. J Bacteriol **177:**4900-7.

94. **Li, M. Z., and S. J. Elledge.** 2007. Harnessing homologous recombination in vitro to generate recombinant DNA via SLIC. Nat Methods **4:**251-6.

95. **Li, Z., S. Reimers, S. Pandit, and M. P. Deutscher.** 2002. RNA quality control: degradation of defective transfer RNA. Embo J **21:**1132-8.

96. **Liang, W., and M. P. Deutscher.** A novel mechanism for ribonuclease regulation: transfer-messenger RNA (tmRNA) and its associated protein SmpB regulate the stability of RNase R. J Biol Chem **285:**29054-8.

97. **Liang, W., A. Malhotra, and M. P. Deutscher.** Acetylation Regulates the Stability of a Bacterial Protein: Growth Stage-Dependent Modification of RNase R. Mol Cell **44:**160-6.

98. **Lim, J., T. Kuroki, K. Ozaki, H. Kohsaki, T. Yamori, T. Tsuruo, S. Nakamori, S. Imaoka, M. Endo, and Y. Nakamura.** 1997. Isolation of murine and human homologues of the fission-yeast dis3+ gene encoding a mitotic-control protein and its overexpression in cancer cells with progressive phenotype. Cancer Res **57:**921-5.

99. **Liou, G. G., W. N. Jane, S. N. Cohen, N. S. Lin, and S. Lin-Chao.** 2001. RNA degradosomes exist in vivo in *Escherichia coli* as multicomponent complexes associated with the cytoplasmic membrane via the N-terminal region of ribonuclease E. Proc Natl Acad Sci U S A **98:**63-8.

100. **Lohman, T. M., K. Chao, J. M. Green, S. Sage, and G. T. Runyon.** 1989. Large-scale purification and characterization of the *Escherichia coli rep* gene product. J Biol Chem **264:**10139-47.

101. **Lopez, M. M., and G. I. Makhatadze.** 2000. Major cold shock proteins, CspA from *Escherichia coli* and CspB from *Bacillus subtilis*, interact differently with single-stranded DNA templates. Biochim Biophys Acta **1479:**196-202.

102. **Lu, J., H. Aoki, and M. C. Ganoza.** 1999. Molecular characterization of a prokaryotic translation factor homologous to the eukaryotic initiation factor eIF4A. Int J Biochem Cell Biol **31:**215-29.

103. **Maier, R., B. Eckert, C. Scholz, H. Lilie, and F. X. Schmid.** 2003. Interaction of trigger factor with the ribosome. J Mol Biol **326:**585-92.

104. **Mathy, N., A. C. Jarrige, M. Robert-Le Meur, and C. Portier.** 2001. Increased expression of *Escherichia coli* polynucleotide phosphorylase at low temperatures is linked to a decrease in the efficiency of autocontrol. J Bacteriol **183:**3848-54.

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

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

107. **Mitta, M., L. Fang, and M. Inouye.** 1997. Deletion analysis of *cspA* of *Escherichia coli*: requirement of the AT-rich UP element for *cspA* transcription and the downstream box in the coding region for its cold shock induction. Mol Microbiol **26:**321-35.

108. **Mizushima, T., K. Kataoka, Y. Ogata, R. Inoue, and K. Sekimizu.** 1997. Increase in negative supercoiling of plasmid DNA in *Escherichia coli* exposed to cold shock. Mol Microbiol **23:**381-6.

109. **Moll, I., S. Grill, A. Grundling, and U. Blasi.** 2002. Effects of ribosomal proteins S1, S2 and the DeaD/CsdA DEAD-box helicase on translation of leaderless and canonical mRNAs in *Escherichia coli*. Mol Microbiol **44:**1387-96.

110. **Moll, I., M. Huber, S. Grill, P. Sairafi, F. Mueller, R. Brimacombe, P. Londei, and U. Blasi.** 2001. Evidence against an Interaction between the mRNA downstream box and 16S rRNA in translation initiation. J Bacteriol **183:**3499-505.

111. **Morita, T., H. Kawamoto, T. Mizota, T. Inada, and H. Aiba.** 2004. Enolase in the RNA degradosome plays a crucial role in the rapid decay of glucose transporter mRNA in the response to phosphosugar stress in *Escherichia coli*. Mol Microbiol **54:**1063-75.

112. **Nakashima, K., K. Kanamaru, T. Mizuno, and K. Horikoshi.** 1996. A novel member of the cspA family of genes that is induced by cold shock in *Escherichia coli*. J Bacteriol **178:**2994-7.

113. **Nesvizhskii, A. I., and R. Aebersold.** 2004. Analysis, statistical validation and dissemination of large-scale proteomics datasets generated by tandem MS. Drug Discov Today **9:**173-81.

114. **Neuhaus, K., S. Rapposch, K. P. Francis, and S. Scherer.** 2000. Restart of exponential growth of cold-shocked *Yersinia enterocolitica* occurs after down-regulation of cspA1/A2 mRNA. J Bacteriol **182:**3285-8.

115. **Perl, D., C. Welker, T. Schindler, K. Schroder, M. A. Marahiel, R. Jaenicke, and F. X. Schmid.** 1998. Conservation of rapid two-state folding in mesophilic, thermophilic and hyperthermophilic cold shock proteins. Nat Struct Biol **5:**229-35.

116. **Phadtare, S.** 2004. Recent developments in bacterial cold-shock response. Curr Issues Mol Biol **6:**125-36.

117. **Phadtare, S., J. Alsina, and M. Inouye.** 1999. Cold-shock response and cold-shock proteins. Curr Opin Microbiol **2:**175-80.

118. **Phadtare, S., and M. Inouye.** 2001. Role of CspC and CspE in regulation of expression of RpoS and UspA, the stress response proteins in *Escherichia coli*. J Bacteriol **183:**1205-14.

119. **Phadtare, S., and M. Inouye.** 1999. Sequence-selective interactions with RNA by CspB, CspC and CspE, members of the CspA family of *Escherichia coli*. Mol Microbiol **33:**1004-14.

120. **Phadtare, S., and K. Severinov.** 2005. Nucleic acid melting by *Escherichia coli* CspE. Nucleic Acids Res **33:**5583-90.

121. **Portier, C., L. Dondon, M. Grunberg-Manago, and P. Regnier.** 1987. The first step in the functional inactivation of the *Escherichia coli* polynucleotide phosphorylase messenger is a ribonuclease III processing at the 5' end. Embo J **6:**2165-70.

122. **Portier, C., and P. Regnier.** 1984. Expression of the rpsO and pnp genes: structural analysis of a DNA fragment carrying their control regions. Nucleic Acids Res **12:**6091-102.

123. **Prud'homme-Genereux, A., R. K. Beran, I. Iost, C. S. Ramey, G. A. Mackie, and R. W. Simons.** 2004. Physical and functional interactions among RNase E, polynucleotide phosphorylase and the cold-shock protein, CsdA: evidence for a 'cold shock degradosome'. Mol Microbiol **54:**1409-21.

124. **Py, B., C. F. Higgins, H. M. Krisch, and A. J. Carpousis.** 1996. A DEAD-box RNA helicase in the *Escherichia coli* RNA degradosome. Nature **381:**169-72.

125. **Raetz, C. R.** 1993. Bacterial endotoxins: extraordinary lipids that activate eucaryotic signal transduction. J Bacteriol **175:**5745-53.

126. **Raetz, C. R.** 1990. Biochemistry of endotoxins. Annu Rev Biochem **59:**129-70.

127. **Raynal, L. C., and A. J. Carpousis.** 1999. Poly(A) polymerase I of *Escherichia coli*: characterization of the catalytic domain, an RNA binding site and regions for the interaction with proteins involved in mRNA degradation. Mol Microbiol **32:**765-75.

128. **Reiner, A. M.** 1969. Characterization of polynucleotide phosphorylase mutants of *Escherichia coli*. J Bacteriol **97:**1437-43.

129. **Resch, A., B. Vecerek, K. Palavra, and U. Blasi.** Requirement of the CsdA DEAD-box helicase for low temperature riboregulation of *rpoS* mRNA. RNA Biol **7:**796-802.

130. **Rigaut, G., A. Shevchenko, B. Rutz, M. Wilm, M. Mann, and B. Seraphin.** 1999. A generic protein purification method for protein complex characterization and proteome exploration. Nat Biotechnol **17:**1030-2.

131. **Schmid, S. R., and P. Linder.** 1992. D-E-A-D protein family of putative RNA helicases. Mol Microbiol **6:**283-91.

132. **Schroder, K., P. Graumann, A. Schnuchel, T. A. Holak, and M. A. Marahiel.** 1995. Mutational analysis of the putative nucleic acid-binding surface of the cold-shock domain, CspB, revealed an essential role of aromatic and basic residues in binding of single-stranded DNA containing the Y-box motif. Mol Microbiol **16:**699-708.

133. **Shi, Z., W. Z. Yang, S. Lin-Chao, K. F. Chak, and H. S. Yuan.** 2008. Crystal structure of *Escherichia coli* PNPase: central channel residues are involved in processive RNA degradation. Rna **14:**2361-71.

134. **Six, D. A., S. M. Carty, Z. Guan, and C. R. Raetz.** 2008. Purification and mutagenesis of LpxL, the lauroyltransferase of *Escherichia coli* lipid A biosynthesis. Biochemistry **47:**8623-37.

135. **Soderberg, M. A., and N. P. Cianciotto.** Mediators of lipid A modification, RNA degradation, and central intermediary metabolism facilitate the growth of *Legionella pneumophila* at low temperatures. Curr Microbiol **60:**59-65.

136. **Tanner, N. K., and P. Linder.** 2001. DExD/H box RNA helicases: from generic motors to specific dissociation functions. Mol Cell **8:**251-62.

137. **Thieringer, H. A., P. G. Jones, and M. Inouye.** 1998. Cold shock and adaptation. Bioessays **20:**49-57.

138. **Tobe, T., C. Sasakawa, N. Okada, Y. Honma, and M. Yoshikawa.** 1992. *vacB*, a novel chromosomal gene required for expression of virulence genes on the large plasmid of *Shigella flexneri*. J Bacteriol **174:**6359-67.

139. **Toone, W. M., K. E. Rudd, and J. D. Friesen.** 1991. *deaD*, a new *Escherichia coli* gene encoding a presumed ATP-dependent RNA helicase, can suppress a mutation in *rpsB*, the gene encoding ribosomal protein S2. J Bacteriol **173:**3291-302.

140. **Tsao, M. Y., T. L. Lin, P. F. Hsieh, and J. T. Wang.** 2009. The 3'-to-5' exoribonuclease (encoded by HP1248) of *Helicobacter pylori* regulates motility and apoptosis-inducing genes. J Bacteriol **191:**2691-702.

141. **VanBogelen, R. A., and F. C. Neidhardt.** 1990. Ribosomes as sensors of heat and cold shock in *Escherichia coli*. Proc Natl Acad Sci U S A **87:**5589-93.

142. **Vasina, J. A., and F. Baneyx.** 1997. Expression of aggregation-prone recombinant proteins at low temperatures: a comparative study of the *Escherichia coli cspA* and *tac* promoter systems. Protein Expr Purif **9:**211-8.

143. **Vorachek-Warren, M. K., S. M. Carty, S. Lin, R. J. Cotter, and C. R. Raetz.** 2002. An *Escherichia coli* mutant lacking the cold shock-induced palmitoleoyltransferase of lipid A biosynthesis: absence of unsaturated acyl

chains and antibiotic hypersensitivity at 12 degrees C. J Biol Chem **277:**14186-93.

144. **Walker, G. C.** 1984. Mutagenesis and inducible responses to deoxyribonucleic acid damage in *Escherichia coli*. Microbiol Rev **48:**60-93.

145. **Wang, J. Y., and M. Syvanen.** 1992. DNA twist as a transcriptional sensor for environmental changes. Mol Microbiol **6:**1861-6.

146. **Wang, N., K. Yamanaka, and M. Inouye.** 1999. CspI, the ninth member of the CspA family of *Escherichia coli*, is induced upon cold shock. J Bacteriol **181:**1603-9.

147. **Weber, M. H., C. L. Beckering, and M. A. Marahiel.** 2001. Complementation of cold shock proteins by translation initiation factor IF1 in vivo. J Bacteriol **183:**7381-6.

148. **Willimsky, G., H. Bang, G. Fischer, and M. A. Marahiel.** 1992. Characterization of *cspB*, a *Bacillus subtilis* inducible cold shock gene affecting cell viability at low temperatures. J Bacteriol **174:**6326-35.

149. **Xia, B., H. Ke, and M. Inouye.** 2001. Acquirement of cold sensitivity by quadruple deletion of the cspA family and its suppression by PNPase S1 domain in *Escherichia coli*. Mol Microbiol **40:**179-88.

150. **Xia, B., H. Ke, W. Jiang, and M. Inouye.** 2002. The Cold Box stem-loop proximal to the 5'-end of the *Escherichia coli csp*A gene stabilizes its mRNA at low temperature. J Biol Chem **277:**6005-11.

151. **Xia, B., H. Ke, U. Shinde, and M. Inouye.** 2003. The role of RbfA in 16S rRNA processing and cell growth at low temperature in *Escherichia coli*. J Mol Biol **332:**575-84.

152. **Yamada, M., H. Nagamitsu, H. Izu, K. Nakamura, and A. A. Talukder.** 2002. Characterization of the ves gene, which is expressed at a low temperature in *Escherichia coli*. J Mol Microbiol Biotechnol **4:**163-9.

153. **Yamanaka, K., L. Fang, and M. Inouye.** 1998. The CspA family in *Escherichia coli*: multiple gene duplication for stress adaptation. Mol Microbiol **27:**247-55.

154. **Yamanaka, K., and M. Inouye.** 2001. Selective mRNA degradation by polynucleotide phosphorylase in cold shock adaptation in *Escherichia coli*. J Bacteriol **183:**2808-16.

155. **Yamanaka, K., T. Mitani, T. Ogura, H. Niki, and S. Hiraga.** 1994. Cloning, sequencing, and characterization of multicopy suppressors of a mukB mutation in *Escherichia coli*. Mol Microbiol **13:**301-12.

156. **Zangrossi, S., F. Briani, D. Ghisotti, M. E. Regonesi, P. Tortora, and G. Deho.** 2000. Transcriptional and post-transcriptional control of polynucleotide phosphorylase during cold acclimation in *Escherichia coli*. Mol Microbiol **36:**1470-80.

157. **Zeeb, M., and J. Balbach.** 2003. Single-stranded DNA binding of the cold-shock protein CspB from *Bacillus subtilis*: NMR mapping and mutational characterization. Protein Sci **12:**112-23.

158. **Zuo, Y., and M. P. Deutscher.** 2001. Exoribonuclease superfamilies: structural analysis and phylogenetic distribution. Nucleic Acids Res **29:**1017-26.

6. Annex

Annex I – Materials

1. Culture Media, solutions and gels

Table 14 – Culture media, solutions and gels used in the experimental procedures.

LB Medium	**LA Medium**
10g Tryptone 5g Yeast Extract 10g NaCl ddH2O to 1000 ml Adjust pH to 7.0 and autoclave	10g Tryptone 5g Yeasts Extract 10g NaCl 15g Agar ddH2O to 1000 ml Adjust pH to 7.0 and autoclave
Blue Coomassie Dye	**Destaining solution**
0.05% Blue Coomassie 10% Acetic acid 50% Methanol	7.5% Acetic acid 20% Ethanol
TBS 10X	**1X TBS + Tween**
12.10g Tris base 40g NaCl H_2O MQ to 500 ml adjust pH to 7.6 with HCl	100 ml TBS 10X 0.9 ml Tween H_2O MQ to 1000 ml
SDS-PAGE Running buffer 10X	**Transfer Buffer 10X**
5g SDS 15.13g Tris base 72.05g Glycin H_2O MQ to 500 ml	1.6g SDS 32g Tris base 16g Glycin H_2O MQ to 400 ml
IPP150 A	**IPP150 A + Triton X-100**
10 mM Tris-HCl pH 8.0 150 mM NaCl	10 mM Tris-HCl pH 8.0 150 mM NaCl 0.1% Triton X-100
IPP150 B	**IPP150 C**
10 mM Tris-HCl pH 8.0 150 mM NaCl 20 mM Imidazol	10 mM Tris-HCl pH 8.0 150 mM NaCl 250 mM Imidazol

CaCl2 I	**CaCl2 II**
2.94g $CaCl_2$ ddH_2O to 200 ml	1.47g $CaCl_2$ 20 ml Glycerol ddH_2O to 100 ml
Kanamycin (50mg/mL)	**Ampicilin (100mg/mL)**
0.5g kanamycin in 10ml H_2O Filter sterilization, aliquot and store at -20ºC	25 mg/ml in deionized water Filter sterilization, aliquot and store at -20ºC
Cloranfenicol (50mg/mL)	**Ponceau Staining**
0.5g Cloranfenicol in 10ml H_2O Filter sterilization, aliquot and store at -20ºC	1% (v/v) Acetic acid 0.1% (v/v) Ponceau S
Lysis buffer	**TEV Cleavage buffer (TEVClevBuf)**
2 mM PMSF 1 mM DTT 50 mM Tris-HCl pH 8.0 250 mM NaCl	10 mM Tris-HCl pH 8.0 150 mM NaCl 0.5 mM EDTA 1 mM DTT
CBB	**CELUT**
10 mM β-mercaptoethanol 10 mM Tris-HCl pH 8.0 150 mM NaCl 0.1% Triton X-100 2 mM $CaCl_2$	10 mM β-mercaptoethanol 10 mM Tris-HCl pH 8.0 150 mM NaCl 1 mM $CaCl_2$
Sodium thiosulfate (10%)	**Potassium carbonate (3%)**
5g sodium thiosulfate H_2O MQ to 50 ml	1.5g Potassium carbonate H_2O MQ to 50 ml
Developer solution	**STOP solution**
1.5g Potassium carbonate 12.5 µl formaline 6.25 µl Sodium thiosulfate 10% H_2O MQ to 50 ml	2g Tris 1 ml Acetic acid H_2O MQ to 50 ml

SDS-PAGE Gel	
A - Running Gel 40% acrylamide mix 1.5 M Tris (pH 8.8) 10% SDS 10% APS 0.04% TEMED	**B - Stacking Gel** 40% acrylamide mix 1.0 M Tris (pH 6.8) 10% SDS 10% APS 0.1% TEMED
6X DNA Loading buffer Orange G	**TBE 10X Buffer**
10 mM Tris-HCl pH 7.6 0.15% Orange G 60% Glycerol 60 mM EDTA	890 mM Tris base 890 mM Boric Acid 20 mM EDTA
2X Loading buffer proteins	**0.8% Agarose Gel**
100 mM Tris-HCl pH 6.8 4% SDS 0.2% Bromophenol Blue 20% Glycerol add β-mercaptoethanol to a 5% final volume	0.8 g Agarose 100 ml TBE 1X
LA + X-Gal	**5% Non-Fat Milk**
400 ml LA medium 800 µl X-Gal (2%) 400 µl Ampicilin (100mg/ml) 80 µl IPTG 1M	0.5 g non-fat milk 10 ml 1x TBS Tween

2. Strains

Table 15 – Strains used in the experimental procedures.

Strain	Relevant Genotype
E.coli BL21 DE(3) (Novagen)	F^- *omp*T *hsd* S_B ($r_B^- m_B^-$) *gal dcm* (DE3)
E.coli DH5α (Novagen)	F^- ϕ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17($rk^- mk^+$) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1
E. coli BW25113 (KEIO Collection)	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), λ^-, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*

3. Plasmids

Plasmids used in the experimental procedures:

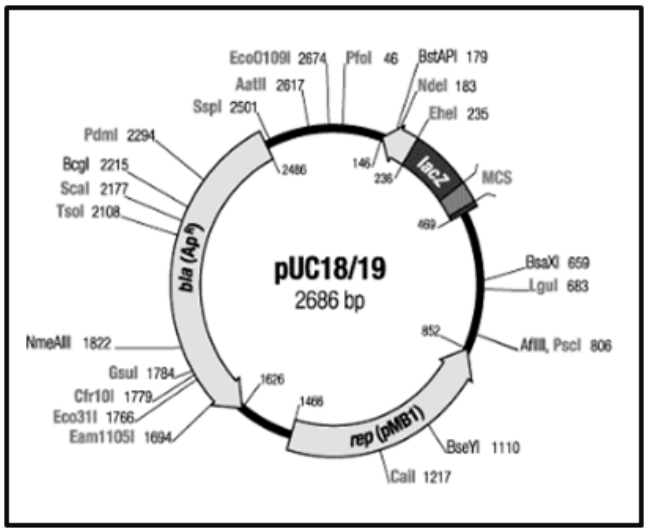

Figure 20 – Representation of pUC18 plasmid, a commercial plasmid used as a cloning vector. This image was obtained from “Fermentas”.

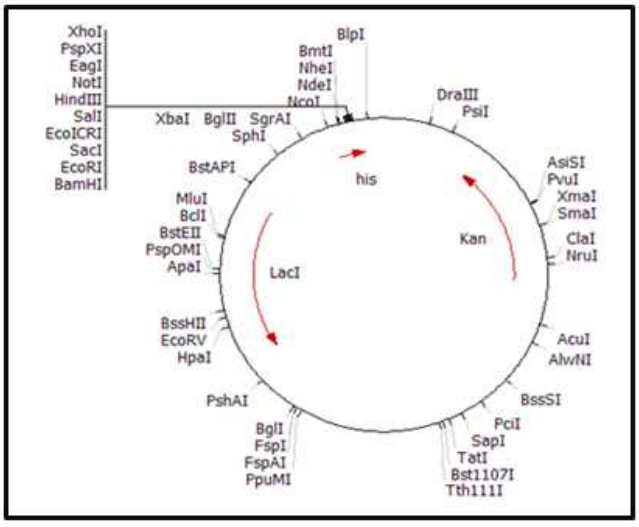

Figure 21 – Representation of pET28a plasmid, used as an expression vector. This image was obtained by Clone Manager software program.

Annex II – Mass Spectrometry results

1. Results from mass spectrometry analysis of RNase R or RPOC TAP tag purification

Table 16 – List of proteins detected to co-purify with RNase R or RPOC purification by TAP tag method during cold shock induction or in stationary growth cells. In green are represented the proteins previously related to interact with RNA polymerase (RNAP) and in gray are represented the proteins found in all preparations.

RNase R			
Cold shock		Stationary	
Protein	Score	Protein	Score
rnr	19585	rnr	28151
deaD	3120	rplL	1779
rplL	2245	rpsG	1573
rpsG	1357	rpsE	1048
rplC	1192	rpsF	1011
rpsD	1101	rpsD	941
rplD	905	rplD	927
aceE	879	rplV	739
rplV	873	rplC	600
rplB	799	groL	585
rpsF	754	rpsQ	540
rplM	648	yfiF	538
rpsI	625	rpsR	493
rplI	598	rpmB	482
infC	570	hupA	476
rplA	554	rplM	475
rpsE	545	adhE	454
rpsC	534	rplB	414
adhE	442	rplQ	400
rplS	400	rpsP	389
rplT	395	rapA	388
rpsJ	384	rpsH	374

RPOC			
Cold shock		Stationary	
Protein	Score	Protein	Score
rpoB	50429	rpoB	46141
rpoC	45026	rpoC	30835
rpoA	24984	rpoA	18217
rpoD	5070	rpoD	7314
rapA	4099	rapA	3106
rpoZ	2761	rpoZ	1141
rpoS	1024	rpoS	598
nusA	795	hupA	481
nusG	279	rplC	419
hupA	265	rpsG	406
rplC	257	rpsO	380
usg	235	hupB	301
greB	234	rplV	267
infB	187	rpsD	262
yacL	156	rplJ	192
rplA	151	rpsF	185
rpsE	151	yacL	165
deaD	140	rpsE	164
rplE	136	rplQ	128
hns	135	cpxR	120
rplJ	134	rplS	114
cpxR	126	nusG	112

rpsH	381	infC	373
rplJ	367	rplP	345
rplO	332	rplS	344
rpsM	331	rpsI	344
rpsR	311	rplX	330
rpsP	304	rpsJ	325
rplX	270	rpsK	243
lon	258	trmH	232
rplE	244	rpsC	230
rpsN	238	rpsT	223
rpsK	233	rplA	220
rplP	218	rpsL	210
rpsS	211	rpsU	196
dnaK	206	rplN	196
rplN	182	hupB	185
ptsI	181	ppk	166
rplQ	179	rplK	157
rhlE	172	rplJ	152
yfiF	171	ihfA	145
rpsB	169	rplI	130
hupB	169	rpsO	128
hupA	168	rplU	127
lpdA	163	rplT	126
rpmB	161	rplR	118
rpsL	160	rpsM	116
rpsO	147	usg	102
hns	143	rpsS	94
rne	139	rplO	89
rplR	138	deaD	88
rpsT	131	aceF	86
infB	122	rpsN	76
rplY	118	aceE	70
rimM	117	lon	68
pnp	116	ihfB	65
rplU	112	dnaK	53
usg	106	sra	52
proQ	102	rpmC	50
aceF	96	rpmE	46
rpsQ	88	hns	38
rplW	85	seqA	37
rpmE	82	proQ	35
rhlB	82	rpml	31

rpsD	123	rplD	98
hupB	121	rplL	97
stpA	119	ihfA	90
rplX	106	rpmB	88
rplD	98	rplX	78
rpsG	95	rpsI	77
rpsC	88	trmH	70
rpoH	87	ihfB	69
fis	85	greB	69
rpmB	81	rpsP	68
rplL	81	nusA	65
rpsO	69	rpsL	65
aceE	68	rpsC	64
rplM	65	rplM	59
rplQ	55	rpsU	58
dnaJ	53	proQ	56
rplU	53	rpsK	56
rplO	53	rplU	56
rpsF	53	rplT	50
rpsI	52	dnaK	50
rpsM	50	recB	46
rplS	47	rplK	46
rplK	47	groL	46
rpsH	45	tdcB	46
rpsS	39	infC	45
ygfB	38	rpsS	40
rplV	37	cedA	38
tdcB	32	rpsJ	33
mnmE	32		

rpsU	80	
degP	78	
rlmL	73	
mreB	69	
rpsA	66	
rpmG	64	
rpml	50	
groL	50	
purR	47	
rluC	46	
ihfB	46	
rpmF	38	
pstA	37	
rpmA	36	
ihfA	33	
yjgR	33	

Annex III – Primers and Sequences

1. Oligonucleotides used in this work

Table 17 – Primers used for DeaD amplification. Forward primers (Lprimer) include the *Sal*I restriction site on their sequence and the reverse primers (Rprimer) contained the *Not*I restriction site (*Sal*I and *Not*I restriction sites are underlined).

	Primers	Sequence 5' to 3'
DeaD	Lprimer	AAGTCGACATGGCTGAATTCGAAACCAC
	RPrimer	TTGCGGCCGCTTACGCATCACCACCGAAACG

Table 18 – Primers used for DeaD amplification to perform the assembly of DeaD and pET28a fragments by homologous recombination using SLIC method. Forward primers (Lprimer) include the *Sal*I restriction site and on their sequence and the reverse primers (Rprimer) contained the *Not*I restriction site (*Sal*I and *Not*I restriction sites are underlined).

	Primers	Sequence 5' to 3'
DeaD	Lprimer	GGGTCGGGATCCGAATTCGAGCTCCGTCGACATGGCTGAATTCGAAACCAC
	RPrimer	TGGTGGTGGTGGTGGTGCTCGAGTGCGGCCGCTTACGCATCACCACCGAAACG

Annex IV – DeaD helicase sequence

1. DeaD helicase sequence

ATGGCTGAATTCGAAACCACTTTTGCAGATCTGGGCCTGAAGGCTCCTATCC
TTGAAGCCCTTAACGATCTGGGTTACGAAAAACCATCTCCAATTCAGGCAG
AGTGTATTCCACATCTGCTGAATGGCCGCGACGTTCTGGGTATGGCCCAGAC
GGGGAGCGGAAAAACTGCAGCATTCTCTTTACCTCTGTTGCAGAATCTTGAT
CCTGAGCTGAAAGCACCACAGATTCTGGTGCTGGCACCGACCCGCGAACTG
GCGGTACAGGTTGCTGAAGCAATGACGGATTTCTCTAAACACATGCGCGGC
GTAAATGTGGTTGCTCTGTACGGCGGCCAGCGTTATGACGTGCAATTACGCG
CCCTGCGTCAGGGGCCGCAGATCGTTGTCGGTACTCCGGGCCGTCTGCTGGA
CCACCTGAAACGTGGCACTCTGGACCTCTCTAAACTGAGCGGTCTGGTTCTG
GATGAAGCTGACGAAATGCTGCGCATGGGCTTCATCGAAGACGTTGAAACC
ATTATGGCGCAGATCCCGGAAGTCATCAGACCGCTCTGTTCTCTGCAACCAT
GCCGGAAGCGATTCGTCGCATTACCCGCCGCTTTATGAAAGAGCCGCAGGA
AGTGCGCATTCAGTCCAGCGTGACTACCCGTCCTGACATCAGCCAGAGCTA
CTGACTGTCTGGGGTATGCGCAAAAACGAAGCACTGGTACGTTTCCTGGAA
GCGGAAGATTTTGATGCGGCGATTATCTTCGTTCGTACCAAAAACGCGACTC
TGGAAGTGGCTGAAGCTCTTGAGCGTAACGGCTACAACAGCGCCGCGCTGA
ACGGTGACATGAACCAGGCGCTGCGTGAACAGACACTGGAACGCCTGAAA
GATGGTCGTCTGGACATCCTGATTGCGACCGACGTTGCAGCCCGTGGCCTGG
ACGTTGAGCGTATCAGCCTGGTAGTTAACTACGATATCCCGATGGATTCTGA
GTCTTACGTTCACCGTATCGGTCGTACCGGTCGTGCGGGTCGTGCTGGCCGC
GCGCTGCTGTTCGTTGAGAACCGCGAGCGTCGTCTGCTGCGCAACATTGAAC
GTACTATGAAGCTGACTATTCCGGAAGTAGAACTGCCGAACGCAGAACTGC
TAGGCAAACGCCGTCTGGAAAAATTCGCCGCTAAAGTACAGCAGCAGCTGG
AAAGCAGCGATCTGGATCAATACCGCGCACTGCTGAGCAAAATTCAGCCGA
CTGCTGAAGGTGAAGAGCTGGATCTCGAAACTCTGGCTGCGGCACTGCTGA
AAATGGCACAGGGTGAACGTACTCTGATCGTACCGCCAGATGCGCCGATGC
GTCCGAAACGTGAATTCCGTGACCGTGATGACCGTGGTCCGCGCGATCGTA
ACGACCGTGGCCCGCGTGGTGACCGTGAAGATCGTCCGCGTCGTGAACGTC
GTGATGTTGGCGATATGCAGCTGTACCGCATTGAAGTGGGCCGCGATGATG
GTGTTGAAGTTCGTCATATCGTTGGTGCGATTGCTAACGAAGGCGACATCAG
CAGCCGTTACATTGGTAACATCAAGCTGTTTGCTTCTCACTCCACCATCGAA
CTGCCGAAAGGTATGCCGGGTGAAGTGCTGCAACACTTTACGCGCACTCGC
ATTCTCAACAAGCCGATGAACATGCAGTTACTGGGCGATGCACAGCCGCAT
ACTGGCGGTGAGCGTCGTGGCGGTGGTCGTGGTTTCGGTGGCGAACGTCGT
GAAGGCGGTCGTAACTTCAGCGGTGAACGCCGTGAAGGTGGCCGTGGTGAT
GGTCGTCGTTTTAGCGGCGAACGTCGTGAAGGCCGCGCTCCGCGTCGTGAT
GATTCTACCGGTCGTCGTCGTTTCGGTGGTGATGCGTAA