UNIVERSIDADE NOVA DE LISBOA

DISSERTATION PRESENTED TO OBTAIN THE MASTER DEGREE IN MEDICAL MICROBIOLOGY

NEW INSIGHTS ON RIBOSOME BIOGENESIS:

THE CRITICAL ROLE OF HFQ AND RNASE R ON RRNA QUALITY CONTROL

RICARDO FILIPE DA CRUZ DUARTE DOS SANTOS

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"Talent is cheaper than table salt.

What separates the talented individual from the successful one is a lot of hard work."

– Stephen King

Acknowledgments

Gostaria de começar por agradecer à comissão científica e organizadora da quinta edição do Mestrado em Microbiologia Médica pela oportunidade proporcionada e pela excelência de ensino.

Agradeço à minha orientadora Prof. Dra. Cecilia M. Arraiano pela oportunidade, orientação e afeição. Obrigado por tudo!

Agradeço ao meu supervisor Dr. José M. Andrade pela orientação, aconselhamento, paciência, dedicação, ensino excepcional e amizade. Obrigado por tudo o que me ensinas-te, jamais o esquecerei!

Agradeço à minha colega e mentora Joana Pissarra por tudo o que me ensinou e por todo o apoio prestado.

Agradeço a todos os meus companheiros de laboratório por me terem recebido tão bem, e por toda a força que me deram. Um agradecimento especial à Patrícia Apura, Andreia Aires, Margarida Saramago e Inês Silva. Obrigado pelos risos, sorrisos e lágrimas de alegria!

Agradeço a todos os meus amigos que me ajudaram durante esta etapa. A lista inclui, apesar de não exclusivamente: Anup Kakoo, Marta Oliveira e Elisabete Ferreira pela lealdade e companheirismo; Diana Kumar, pelo apoio; Pedro Rodrigues, pelos conselhos; Daniel Duarte, pela parceria. Obrigado a todos por tudo e muito mais!

Agradeço à minha amiga Susana Barahona pela amizade, companhia e carinho. Este caminho não teria sido o mesmo sem ti! Obrigado pela motivação e pela diversão!

Agradeço à minha namorada e companheira de vida Helena Pereira pelo apoio incondicional e paciência. Obrigado pelo teu amor!

Agradeço à minha querida família, Dário dos Santos, Maria José dos Santos e Carina Alexandra dos Santos, por acreditarem em mim. Obrigado pai pelo carácter; Obrigada mãe pelo carinho; Obrigada irmã pelo exemplo. Tornaram isto possível.

Abstract

RNase R and Hfq are two important proteins that are implicated in post-transcriptional gene expression that also integrate the bacterial RNA degradation mechanisms. RNase R is known to preferably bind to polyadenylated 3'-end tails of RNAs besides being also capable of degrading structured RNA molecules. Hfq also preferably binds to polyadenylated stretches near structured RNA regions. The apparent sharing of substrates has prompted us to further investigate the role of both these proteins in RNA degradation.

In this work we have found evidence of a possible functional cooperation between RNase R and Hfq. Cells lacking these proteins were found to accumulate products of ribosomal RNA degradation. Our data also indicates accumulation of rRNA precursors of the 16S, 23S and 5S ribosomal RNAs, yielding immature RNA molecules. The ribosomal biogenesis is a complex and precise process by which the ribosomes are generated and assembled. Our data evidences drastic defects in ribosomal biogenesis when both Hfq and RNase R are absent. In the Δ*hfq* Δ*rnr* double mutant, ribosomal profiles are shown to be defective as the amount of 70S particles in this mutant is lowered. The synergetic role between this proteins is further supported by our data concerning protein interaction assays. Here we have shown that RNase R and Hfq interact directly with each other, which nicely expands the interaction network of both these proteins. Taken all together, we provide evidence of a possible new RNA degradation pathway that imply the cooperative action of both the exoribonuclease RNase R and the RNA chaperone Hfq and that RNase R was found to be the major exoribonuclease for the removal of accumulated rRNA fragments in the absence of Hfq.

Resumo

As proteínas RNase R e Hfq são duas importantes proteínas implicadas na regulação pós-transcricional da expressão génica e integram ainda mecanismos bacterianos de degradação de RNA. A proteína RNase R liga-se preferencialmente a caudas poliadeniladas na extremidade 3' de moléculas de RNA, estando ainda envolvida na degradação de RNAs estruturados. A proteína Hfq também interage preferencialmente com extremidades poliadeniladas perto de regiões estruturadas do RNA. Esta aparente partilha de substratos levou-nos a investigar o papel destas duas proteínas na degradação de RNA.

Neste estudo apresentamos indícios de uma possível cooperação funcional entre as proteínas RNase R e Hfq. Na ausência de ambas as referidas proteínas foi observada a acumulação de fragmentos provenientes da degradação de RNA ribossomal. A acumulação de precursores dos rRNAs 16S, 23S e 5S foi ainda observada nestas condições, levando ao aumento de moléculas de RNA imaturas. A biogénese ribossomal é um processo complexo e preciso pelo qual os ribossomas são sintetizados e montados. Os nossos dados evidenciam defeitos drásticos na biogénese ribossomal aquando da ausência das proteínas Hfq e RNase R. No duplo mutante Δ*hfq* Δ*rnr* os perfis ribossomais encontram-se alterados verificando-se uma redução na quantidade de partículas 70S. O papel sinergético entre ambas as proteínas é ainda suportado pelos nossos dados relacionados com a interacção proteica. Mostramos aqui que as proteínas RNase R e Hfq interagem directamente expandindo assim a rede de interações de ambas. Em suma, fornecemos evidências que apontam para uma possível nova via de degradação de RNA que implica a cooperação da exoribonuclease RNase R com a chaperon de RNA Hfq, e onde proteína RNase R é a principal exoribonuclease responsável pela remoção de tais fragmentos quando a proteína Hfq está ausente.

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Thesis outline

The project of this Master Thesis was developed in the widely known bacterial model *Escherichia coli*. The main goal was to characterize an *E. coli* knockout mutants in *hfq* and *rnr* genes. The gene *hfq* encodes Hfq, a RNA chaperone, and the gene *rnr* encodes for the exoribonuclease RNase R. Both proteins have been implicated in post-transcriptional control of gene expression. The work performed focused mainly on the study how the absence of these two proteins in the single and double mutant strains could have an impact on ribosomal RNA and ribosome biogenesis. The first chapter is a bibliographic review introducing the theme of this Thesis. In the second chapter the experimental methodologies used during this work are explained in detail. The results are presented on the third chapter and finally discussion and main conclusions on the fourth.

1

Introduction

1 Introduction

1.1 Ribosome: The Decoder

The ribosome is a protein complex responsible for the orchestration of an essential cellular process – protein synthesis. It is an essential cellular organelle necessarily present in all domains of life. If protein synthesis is the expression of the genetic code, then the ribosome brings to action the design of life. For this reason, many times the ribosome is pointed as the foundation of life itself.

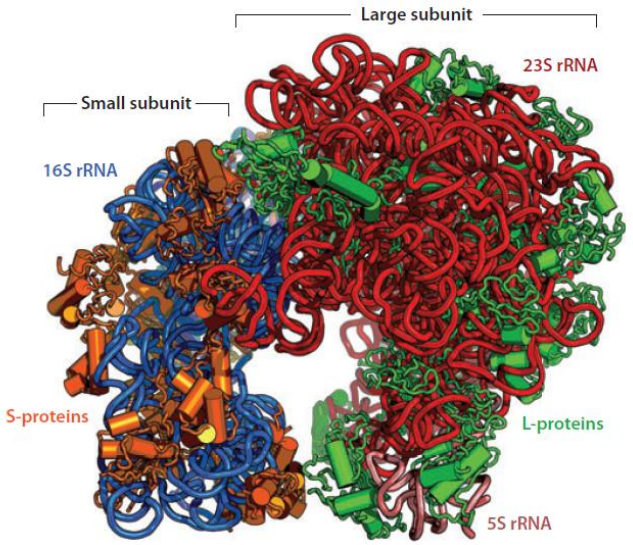

Figure 1 - ***E. coli*'s ribosome.** A computational rendering of the 70S particle from *Escherichia coli*. Large and small subunits are presented. In blue, the 16S rRNA; in red the 23S rRNA, in light red the 5S rRNA are represented. Moreover, small and large ribosomal proteins are in orange and green respectively (adapted from [88]).

The bacterial ribosome consists of two different subunits, a larger subunit, referred to as the 50S subunit, and a smaller one, the 30S subunit. Together they form the functional 70S ribosomal complex. Its eukaryotic homologues are the 60S large subunit, the 40S small subunit and the fully functional 80S ribosome. Their names refer to their sedimentation coefficient during ultracentrifugation, reflecting their differences in terms of mass and structure. Each subunit is a ribonucleoprotein (RNP) by itself that forms an interaction network between ribosomal proteins (r-proteins) and ribosomal RNA (rRNA). As this Master Thesis was developed in the bacterial model *Escherichia coli*, this introduction will focus on the the prokaryotic ribosome

The 30S bacterial small subunit comprises 21 r-proteins (S1-S21) and a 1542 nucleotide (nt) long 16S rRNA, whereas the 50S large subunit consists of 33 r-proteins (L1-L36) and two rRNA molecules, the 23S rRNA of 2904 nucleotides and the 5S rRNA with 120 nucleotides (Figure 1) [88]. The two subunits have two distinct functions: the 30S subunit is responsible for the association with the messenger RNA (mRNA) and decoding; and the 50S subunit's role is to provide the structural basis for the formation of the

polypeptide chain [27]. Although each subunit exists independently, the functional ribosomal particle is needed for efficient protein synthesis, and so both subunits associate with each other to carry out translation.

1.1.1 Translation

The translation of the nucleic acid messages consists of a systematic well-coordinated process that can be categorized in three main stages: Initiation, elongation and termination (Figure 2). The initiation stage takes off with the interaction between the mRNA and the 30S subunit, specifically with the Shine-Dalgarno (SD) sequence of the first with the anti-SD sequence located at the 3'-end of the 16S rRNA [90]. Initiation is promoted by initiation factors (IF 1-3) and is thought to be the rate limiting step of the translational process. Following the coupling of the 30S and 50S subunits is the GTP dependent elongation cycle leading to the addition of amino-acids to the nascent polypeptide chain at the peptidyl transferase center (PTC). Two proteins are essential for a correct elongation step, namely, the elongation factor Tu (EF-Tu) and the elongation factor G (EF-G). Finally, when a stop codon reaches the ribosome it is recognized by one of the two class I release factors in *E. coli* (RF1 and RF2), depending on the codon sequence. These release factors promote hydrolysis of the polypeptide chain, freeing it from the ribosomal exit channel. After that, RF 3 dissociates RF1 or RF2 from the ribosome, leaving the particle bound to the mRNA and the last deacylated tRNA. The final step of termination is one of the most significance because it frees both subunits, allowing a successful recycling of the ribosome. Ribosome recycling factor (RRF) and EF-G cooperate in this matter to dissociate the 30S and 50S subunit [53], and IF3 retrieves the deacylated tRNA from the 50S subunit. Therefore, the ribosome can now restart a new translation cycle with the same mRNA or proceed with the translation of other messengers [50,86].

The ribosomal particle is a 2.5 megadalton ribonucleoprotein whose perception has changed in the past few years [86]. Structural studies that took place on the past decade have shed a new light on this multicomponent complex and revealed a rRNA backbone garnished by r-proteins [11,73,87,107,110]. In fact, nearly two-thirds of the ribosome is RNA [87], and it is now considered a ribozyme, since its catalytic sites reside in the rRNA molecule [73]. Another interesting detail is that the association of the 30S with

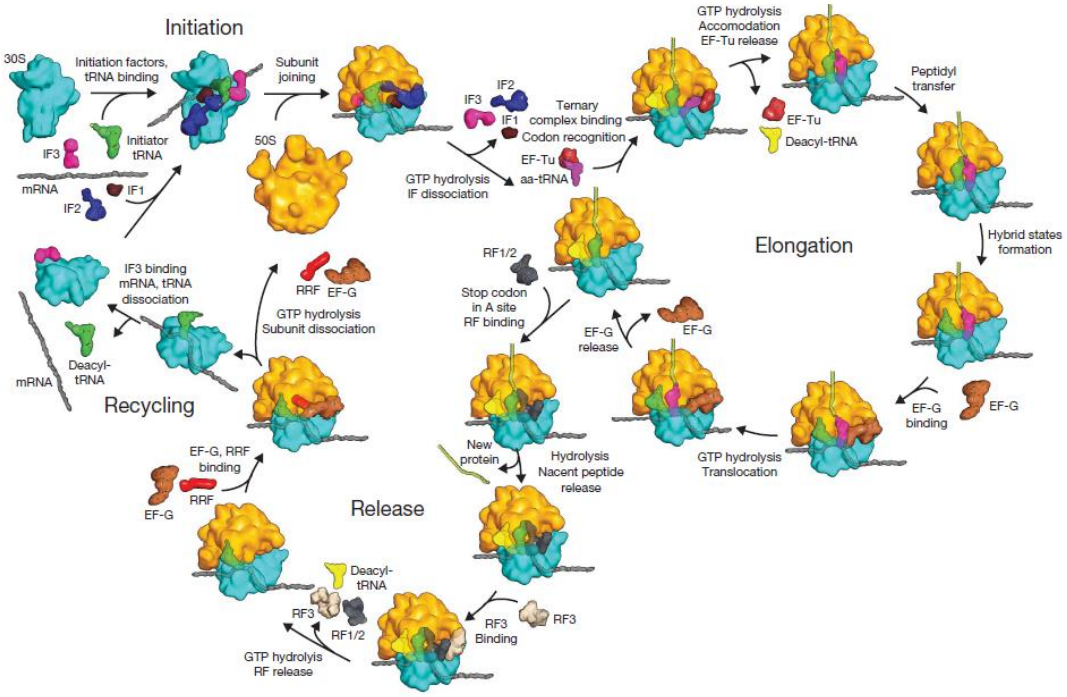

Figure 2 - **Bacterial translation stages.** An overlook on the translational process of the bacterial cell. The three main stages are illustrated: Initiation, Elongation, Termination (or Release) and Recycling. Mainstream proteins necessary for this process are also represented. (adapted from [86])

the 50S subunit upon translation initiation is made by a RNA enriched portion of both structures, forming a series of dynamic RNA bridges that can reorganize themselves as part of the translational process, giving the ribosome the plasticity needed for its movements during each elongation cycle [87]. This rRNA bridge can also serve as an interaction site with non-ribosomal elements [28], which, in some way, can modulate the ribosome's action. These and many other studies have not only brought us a precise look on the ribosomal structure but also new evidence to the overall process of translation [88,109]. Although these crystallographic approaches can help us to better understand how the ribosome fulfills its crucial goal, they provide only a still image of the particle at the time of crystallization, bypassing the dynamic event of how its individual components coordinate to assemble a fully functional ribosome.

1.2 Ribosome Biogenesis

The assembly of the bacterial ribosome is a thorough and well-coordinated biological process that, besides being metabolically demanding, it is also of the most importance to the cell [108]. Therefore, it is imperative that it occurs as smoothly and efficiently as possible.

Ribosome biogenesis comprises a series of steps leading to the correct formation of both subunits. These include transcription, processing and nucleotide modification of the rRNA; translation and modification of the r-proteins; rRNA folding; r-proteins folding; sequential biding of r-proteins and the action of ribosomal assembly factors [88]. Many of these steps are believed to occur simultaneously within the cell, for instances, the translation and modification of rRNA and r-proteins synthesis and binding [27,50]. The biogenesis program starts with transcription of the rRNAs 16S, 23S and 5S as a single transcript in what is pointed as the possible rate limiting step of the process [76]. This transcript also contains tRNA precursors [50]. As soon as the transcript arises from the RNA polymerase it begins to fold into specific secondary structures that can be recognized by r-proteins. Concomitantly nucleotides from rRNA molecules become chemically modified and extremity processing is performed by ribonucleases (RNases) [105].

1.2.1 rRNA chemical modification

Of the three rRNAs only the 5S is not chemically altered, whilst the 16S rRNA has 11 modifications (10 methylations and 1 pseudouridine) and the 23S contains 24 (14 methylations, 9 pseudouridines, 1 methylated pseudouridine) [50]. The majority of the modifications are grouped in important regions of the rRNA, the decoding region in the 16S and the PTC region in the 23S, which, along with the fact that there is a cross-species conservation of these modifications, suggest its importance. Some of these modifications are associated with antibiotic resistance/sensibility [25], others are known to be essential for correct assembly (*in vitro*) of the ribosome [37] and all are thought to contribute to a fine-tuning of the RNA folding and, ultimately, of the translational process [25,50].

1.2.2 rRNA maturation

rRNA maturation is crucial for correct RNA folding and function. Ribonucleases are the enzymes responsible for RNA processing and degradation. These proteins break down RNA molecules into its basic building blocks (ribose nucleotides). They can degrade RNA by the extremities of the RNA molecule, in which case they are classified as exoribonucleases, or cleave RNA internally thereby named endoribonucleases. The chemical reaction of the degradation can be either hydrolytic or phosphorolytic, depending on the RNase involved. After successful transcription of the primary RNA precursor, the first

step of maturation is an endoribonucleolytic cleavage mediated by RNase III. The double stranded portions of the 16S and 23S precursors are cleaved, resulting in the release of the 16S rRNA precursor (17S rRNA), the 23S rRNA precursor (with only approximately 3-7 nt at the 5'-end, and 7-9 nt at the 3') and, after tRNA processing by RNase P at the 3'-end of the primary transcript [46], the 5S rRNA precursor (9S rRNA) [50].

Final maturation of the 16S rRNA is not dependent on the initial RNase III cleavage, as the succeeding cuts will still occur in the absence of this enzyme [50]. RNase E and RNase G act at the 5'-terminus of the 17S rRNA at two different sites [58], and finally a combination of four different RNases (RNase II, RNase R, RNase PH and PNPase) process the 3'-terminus, completing 16S rRNA maturation [96]. On the other hand, 23S rRNA maturation is strictly dependent on RNase III cleavages. It is believed that RNase G participates in the 5'-end maturation [93], whereas RNase T, with help from RNase PH [38], is required for 3'-end processing [57]. Processing of the 9S rRNA (precursor of the 5S) is mediated by RNase E at both extremities [83], and specifically by RNase T at the 3'-end [56]. The RNase responsible for the 5' maturation of the 5S rRNA is still unknown [50].

It is evident from the interplay of RNases with a role on rRNA processing that these enzymes are of great relevance for the incorporation of correct rRNA molecules into both ribosomal subunits, and ultimately for the optimal functionality of the ribosome. Incorporation of immature rRNAs creates an heterogenic population of ribosomal subunits that weakens prokaryotic plasticity and adaptation to rapidly changing environments, observable, for instances, by a slow-growth phenotype in strains where such rRNA molecules accumulate, which may indicate some deficiencies in the overall translational process [26,29,49,55,57,88].

1.2.3 Modification of r-proteins

Many ribosomal proteins are posttranslationally modified, some contain even more than one modification. The implications of such modifications are still not clear, but their occurrence suggest relevance to the cell. It is thought that these modifications may tamper with the efficiency of r-proteins binding to rRNA or with the biding of non-ribosomal elements [50].

1.2.4 Assembly

The next step in ribosome biogenesis is the ribosomal assembly, where a series of binding events between the r-proteins and the rRNA takes place, along with ribosomal assembly factors that help to conclude the correct folding of both subunits. Our knowledge of the timeframe and physical pathways of assembly has been progressing over the years, and so we now begin to realize its complexity and importance [88]. Given its crucial role in cell physiology, the assembly process is unsurprisingly object of tight regulation [108], and, because of the high metabolic price paid for the end product, it has to flow unobstructed for the sake of optimal cell viability [27,97].

Early assembly studies were made with the 30S ribosomal subunit, since it has a few number of r-proteins and a simpler rRNA. Of both subunits, the assembly of the small subunit is the most characterized one, but relevant questions still remain unanswered, since there is little information about the temporal and sequential picture of the binding and folding events [108]. Prominent work dating back from the late 60's accomplished by Traub and Nomura [99] demonstrated that the 30S subunit could be assemble *in vitro* using only 16S rRNA and ribosomal proteins. This led to the conclusion that all the information needed to assemble the subunit resides in the rRNA and r-proteins, establishing the self-assembly principle [88]. Having successfully reconstituted an active ribosomal subunit *in vitro*, Mizushima and Nomura [68] determined that r-protein binding to the 16S rRNA is both cooperative and hierarchical, as early binding of the first r-proteins reorganizes rRNA, revealing binding sites for the late r-proteins [50,88]. The conclusions from this reconstitution studies were summarized in the Nomura Map that illustrates the hierarchical dependencies of the r-proteins during assembly [68]. Interestingly, after additional characterization of the assembly process in general and careful analysis of the

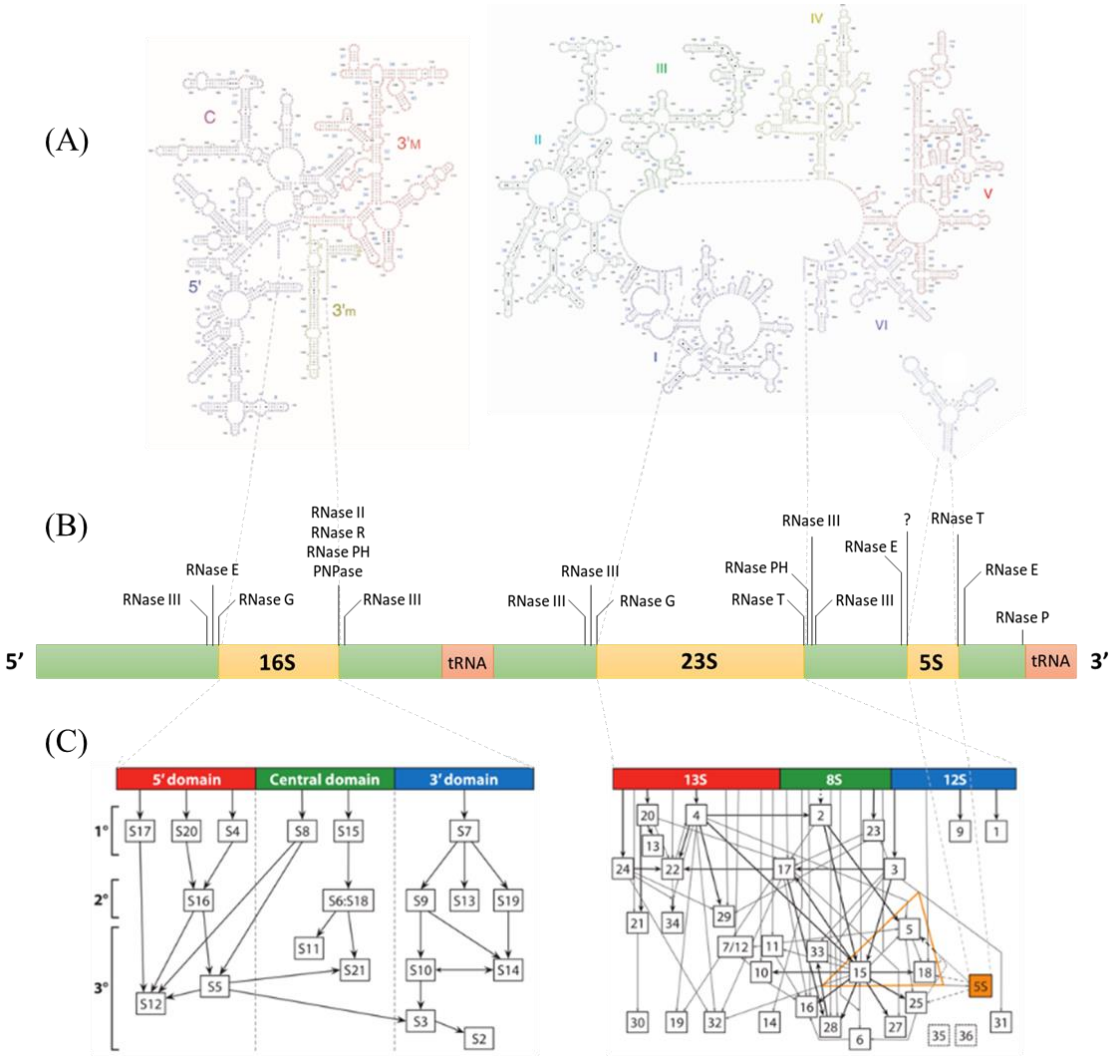

Figure 3 – **(A) Secondary structures and respective RNA domains of the three ribosomal RNAs.** (adapted from [110])**. (B) Schematization of the ribonucleases with characterized activity on rRNA maturation.** The primary single transcript is represented (green) with relative cleavage sites in acting order from the extremities to the center of the mature rRNA (yellow). **(C) Assembly maps of the 30S and 50S ribosomal subunits.** The 5S rRNA is represented in orange (adapted from [88]).

ribosomal proteins, ribosomal assembly intermediates and extra-ribosomal assembly factors, this map has remained nearly untouched for more than 40 years [50].

As consequence, small subunit ribosomal proteins were organized into three different groups: Primary r-proteins bind to rRNA as soon as it is transcribed, following a co-transcriptional direction (5' to 3') [108]; Secondary r-proteins first require the above interaction for proper recognition of their binding sites in the rRNA; Finally, tertiary r-proteins need at least one primary and one secondary r-protein to be associated with the rRNA for their proper binding to occur [50]. Following Nomura's lead, Nierhaus *et al* preformed similar experiments, but with the 50S ribosomal subunit [40]. Due to the elevated number of components in the 50S subunit it was expected for its assembly map to

be more complex, and this subunit its significantly less studied then the small subunit [88].

In vitro reconstitution experiments distinguished two reconstitution intermediates of the 30S subunit [99]. The first intermediate (RI) is formed at low temperatures (0-15ºC) after addition of 16S rRNA plus primary and secondary r-proteins. At this point the reconstitution becomes stalled in a particle of that sediments at 21S. To surpass this halt, energy is required. Heating (40ºC) of the 21S particle, promotes its rearrangement, forming a new intermediate (RI*) that sediments at 26S. This intermediate can now form an active 30S subunit after the addition of the remaining r-proteins [88]. Concerning the 50S subunit, Nierhaus *et al* identified three reconstitution intermediates [72]. The first one, the $RI_{50}(1)$, is formed at low temperatures and in the presence of Mg^{2+}, and it sediments at 33S. From here, temperature must be increased to 40ºC in order to allow conformational changes, leading to the formation of another intermediate, $RI_{50}*(1)$ that now sediments at 41S. After addition of the remaining r-proteins the final intermediate, $RI_{50}(2)$ is formed, and it sediments at 48S. From here, another temperature increase (50ºC) is needed, along with addition of more Mg^{2+} for the correct reconstitution of the 50S subunit [50]. Interestingly, similar precursors are found *in vivo*, with similar sedimentation coefficients, which supports the conclusion from the *in vitro* assembly reconstitutions mentioned above [60].

1.2.5 Assembly factors

The assembly process *in vivo* is much more complex than just joining rRNA and r-proteins. The ribosomal RNA is a very structured molecule, and, the larger the molecule, the easier it is to find a sequence with reasonable complementary. Because of this, misfold events occur quite frequently and the resulting structures may even be significantly stable, trapping the rRNA in secondary RNA structure intermediates [50,108]. One of the pointed roles of r-proteins is exactly to help prevent misfolding events; they bind the rRNA in specific locations, facilitating its proper folding [88,97,108]. It is a tremendous effort for the cell to assemble such large multicomponent complexes as the ribosome, and it is remarkable that such a complicated procedure is so successful. Part of this success is due to ribosomal maturation factors that come to play during ribosomal biogenesis. *In vitro* the kinetically trapped RNAs are thought to be the rate-limiting step on

ribosome reconstitution, hence the need for heating to promote structural rearrangements [50]. This factors may even lower the activation energy required *in vitro*, bypassing the heating step of reconstitution experiments. They are believed to facilitate *in vivo* assembly by acting on immature complexes, promoting its rearrangements by rescuing kinetically trapped molecules [86,97].

We can categorize this maturation factors into four groups: RNA chaperones, RNA helicases, ribosome-dependent GTPases and lastly maturation factors of unknown function [50].

RNA chaperones, like the bacterial Hfq, are proteins that help RNA to fold into its correct secondary structure [79]. Their putative role in ribosome biogenesis is exactly to help rRNA fold into its correct and fully functional form, keeping it out and rescuing it from kinetic traps. It is easy to understand how these proteins can drive ribosomal maturation, but there is little information about possible extra-ribosomal proteins with such function [50].

RNA helicases are extremely essential in many cellular processes like RNA processing, degradation, translation and also ribosome biogenesis. The DEAD-box protein family, a highly conserved family of which many of the RNA helicases belong to, are proteins that are capable of unwinding structured RNA molecules in an energy dependent manner, utilizing ATP to separate RNA duplexes [79]. Four of these helicases have recently been associated with ribosomal biogenesis, proving their role in the assembly process [20,21,45,89].

Another class of proteins that can help in RNA folding are the ribosome-dependent GTPases. The best studied example is the Era GTPase which can bind to the 16S rRNA and is also important for 16S rRNA processing [66]. Besides Era, another three GTPases whose deletion lead to ribosomal defects are the Der, $CgtA_E$, and the RsgA (previously YjeQ) proteins [41,43,47].

The final group of factors that are known to interfere with ribosome biogenesis is referred to as ribosome maturation factors, because their functional is yet to be uncovered, although some of these factors are known to have other physiological activities, like DnaK and GroEL protein chaperones [88]. The list of maturation factors is still growing, and many are brought to light by either proteomic or genetic approaches, but further characterization of these players is still needed [50]. RimM and RbfA proteins are known to

be essential to the processing of the 16S rRNA and both bind to the 30S subunit. They seem to work in conjunction with Era and RsgA, as they are shown to bind closely together in the decoding center of the 30S subunit [27,55].

A way to understand if any protein has a role in ribosome biogenesis is to evaluate the overall state of the ribosomal subunits and the ribosome particle, along with the processing of the rRNAs is being performed. Alterations of the ribosomal profile suggest that something is altering the stability of the process, and creating a heterogenic population of subunits, which results in a decrease of fully matured ribosomes (Figure 4) [50,55]. Yet mutants in assembly factors are viable and they still are capable of forming 70S particles, suggesting the existence of multiple parallel pathways for ribosome biogenesis [55,108]. This observation makes perfect sense in the light of evolutionary biology, as we are talking of a crucial biological process. Furthermore, as RNases are necessary to rRNA maturation, the lack of these essential enzymes can also alter ribosome profiling, leading to the integration of immature rRNAs into the ribosome [96].

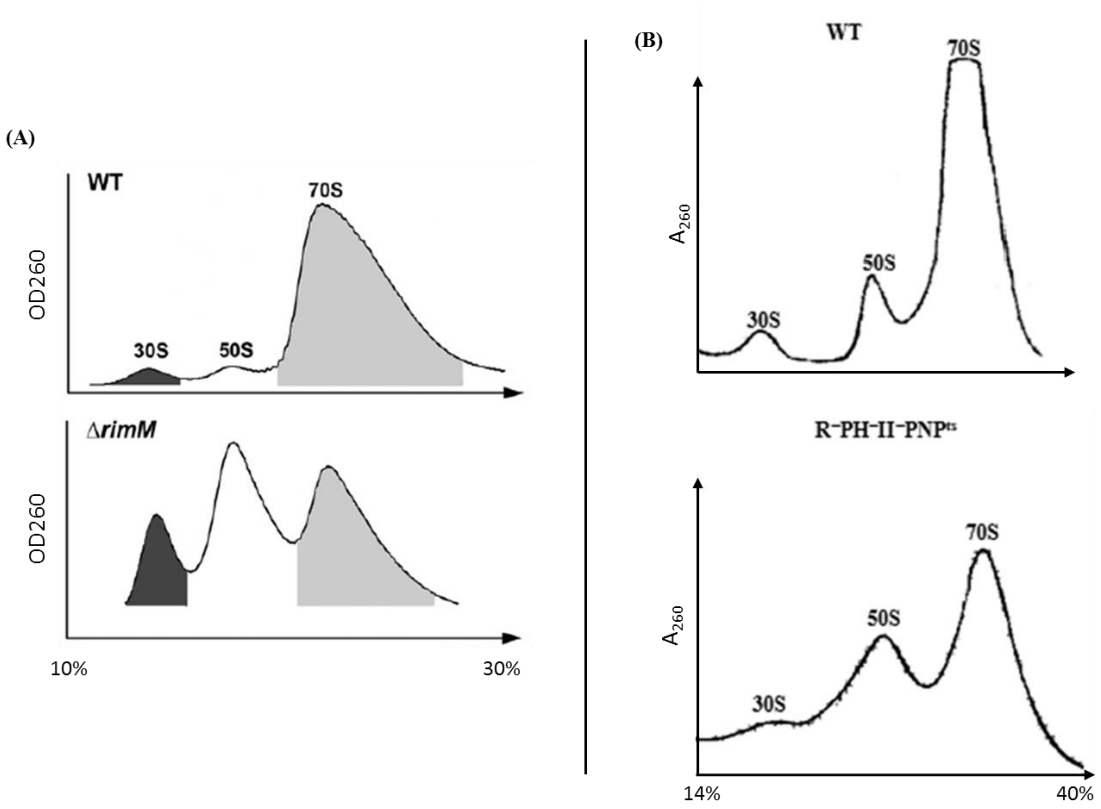

Figure 4 – **(A) Ribosomal profile comparison of a wild-type strain and the RimM ribosomal assembly factor.** The differences are notable. The 70S population of the mutant decreased, whereas the 50S and 30S subunit population increased. This is a typical example of a defective profile. (adapted from [55]). **(B) Ribosomal profile comparison between a wild-type strain and a lacking multiple RNases.** A second example is presented, this time the defective profile arises from the lack of multiple exoribonucleases within the cell. (adapted from [96])

The list of factors with a role in ribosome biogenesis is growing, drawing our attention to the complexity of this cellular process of such importance. Because of the vital nature of ribosome biogenesis, many of these factors are being pointed out as possible candidates for future drug targets [26,109]. Although there has been an exceptional effort into unraveling the secrets of protein translation and ribosome maturation, we are still far from holding an exact blueprint of this biological process.

1.3 RNA decay

Transcription is a central biological process that precedes translation. Not only it is indispensable for maintaining cellular function but also for regulating gene expression. The link between genes and proteins is the RNA, therefore, transcription is the moderator of the exchange between coded information and functionality, housing a great number of crucial regulation pathways. Specifically, mRNA is the molecular vehicle of this exchange, and the cellular concentration of a mRNA transcript available for translation is given by a ratio between its synthesis and degradation [7]. Thereby RNA decay is also a key factor for regulating gene expression. Controlling the half-life of a transcript by regulating its degradation rate is an effective and energy-saving way for the cell to rapidly adapt to environmental cues [84]. This type of regulation at the messenger level is especially important in small genome organisms, where transcription and translation are two coupled processes [92].

In order to regulate gene expression at a post-transcriptional level, cells resort to a class of enzymes, the ribonucleases. As mentioned above (1.2.2), RNases are responsible for rRNA processing and for tRNA maturation [63], being indispensable for the correct formation of ribosomal particles [7,50]. They are also required for RNA degradation, integrating a complex network of regulatory pathways that rely on RNA decay; and for the quality control of all RNA molecules, providing a mean for ribonucleotide recycling.

RNases are present in all domains of life and are necessary, among other things, for the post-transcriptional control of gene expression. They are the major component acting on RNA decay mechanisms. Many ribonucleases have an essential character, whereas others present overlapping functions [7,80]. In *E. coli* there are more than 20 different RNases that can either act alone or form protein complexes to potentiate their degradative function [7].

We can subdivide RNases into two major groups, according to the way they degrade their substrate. Endoribonucleases cleave RNA molecules internally, whereas exoribonucleases degrade RNA from the end, either from the 3'-end or from the 5'-end [63]. Moreover, exoribonucleases can act upon the RNA in two ways, hydrolytically and phosphorolytically. For being able to degrade RNA molecules, RNases are the core components of the prokaryotic degradation mechanism.

Nevertheless, there are other factors important to the fate of a RNA molecule, and so it is important to keep in mind that the stability of a specific RNA differs from the others, that the sequence determinants that regulate its stability can be found within the molecule at any region, and that the length of a transcript has no direct connection to its turnover [7]. Thus, factors like the sequence of the target can act as a protector or enhancer for a given RNase. Ribosomes can also protect the transcript from degradation by hiding them during translation [7]. The polyadenylation state of a RNA can contribute to its fast degradation, as some RNases are known to prefer poly(A) substrates [1]. *Trans*-acting elements that can rearrange a RNA molecules are known to affect their turnover rate by exposing/hiding specific sites for RNase recognition, for instances, RNA helicases can affect a transcript in such way, as well as other RNA binding proteins as Hfq (a RNA chaperone) which binds to small RNAs (sRNAs) and can influence the decay of certain transcripts [7].

1.3.1 RNA turnover in E. coli

As stated, RNases play a crucial role in the overall mechanism of RNA turnover. Only few of them are involved in RNA degradation, the ones that do not, however, hold a major part in other physiological processes [92]. Usually, RNA degradation in *E. coli* (Figure 5) starts with an endonucleolytic cleavage of the targeted RNA molecule. This initial cleavage can be catalyzed by two RNases: RNase III and RNase E. RNase E is thought to be the main endoribonuclease involved in RNA decay in *E. coli* [7]. RNase III is an endoribonuclease which binds to double-stranded RNA (dsRNA) molecules for cleavage and whose main role is exogenous mRNA degradation and rRNA processing [10]. RNase E is a single-strand specific endonuclease that seems to have a preference for A/U rich sites near stem loops, a characteristic also present in its paralogue, RNase G, which also has a role in degradation. Both RNase E and RNase G exhibit a higher activity

towards RNAs that are monophosphorylated at the 5'-end, but RNase E can cleave substrates regardless of their phosphorylation, due to exposure of single-stranded RNA (ssRNA) loci that allows its binding [18]. RppH can catalyze the conversion of 5'-triphosphate to 5'-monophosphate at the 5'-end, thus providing optimal substrate for RNase E endonucleolytic cleavage [30].

RNase E can be loaded into a protein complex – the degradosome – that include (under normal growth conditions) a RNA helicase, RhlB, a glycolytic enzyme, enolase, and an exoribonuclease, PNPase. This is the main complex for RNA degradation, coordinating both endo- and exonucleolytic activities. The combination of both endo- and exoribonucleolytic activities, alongside with an RNA helicase, provide enhanced efficiency to the overall RNA mechanism, as the RNA targets are degraded faster [54]. After the first cleavage by RNase E the originated segment from the 3'-end can suffer further RNase E activity, since it holds a 5'-monophosphate. On the other hand, the 5' segment can now be rapidly degraded by 3'-5' exonucleases, that were unable to access their substrate due to 3'-stem-loop protection [5].

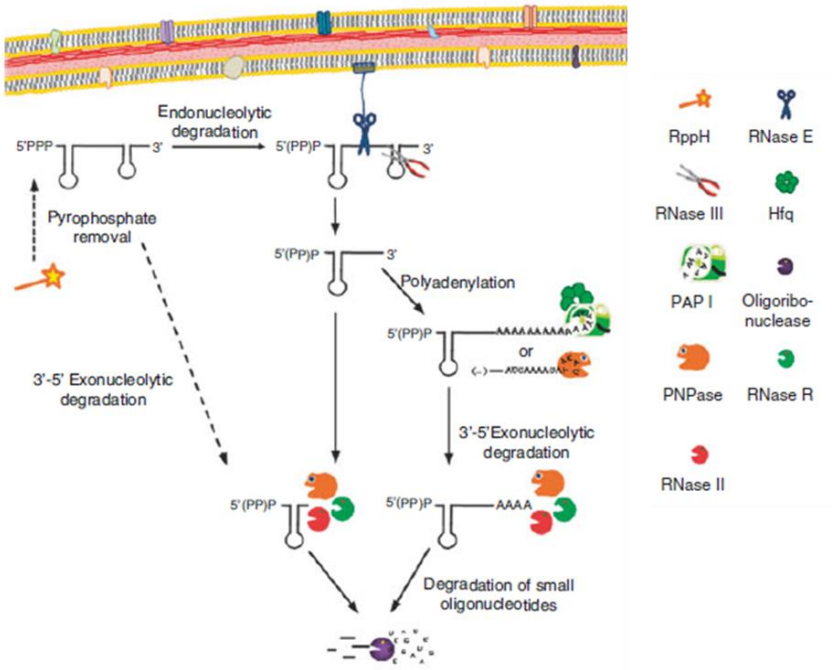

Figure 5 – **Overview of *E. coli*´s decay mechanisms.** For the majority of the transcripts endoribonucleolytic cleavage starts off the degradation process yield intermediate substrates suitable for both PAP I/Hfq activity or directly for exoribonucleolytic mediated degradation. (adapted from [92])

There are three major exoribonucleases in *E. coli*: RNase II, RNase R and PNPase. The first two are hydrolytic enzymes, releasing monophosphorylated nucleosides as an end product, the last one displays a phosphorolytic activity, resulting in the release of nucleoside diphosphates [63,92]. All three exoribonucleases are processive, but only RNase R is capable of degrading structured RNA molecules by itself [102]. PNPase and RNase II prefer polyadenylated susbtrates [7,62]. None of the three enzymes appears to be essential for cell viability, however the absence of both RNase II and PNPase or RNase R and PNPase is lethal, suggesting some overlapping functions [24]. Moreover, the polyadenylate polymerase I (PAP I) can further potentiate exoribonuclease activity by providing poly(A) tails to the RNA targets, providing optimal substrate for these RNases [92]. As the products from these processive exoribonucleases are 2 to 5 nucleotide long, another RNase is needed to fully recycle them. The oligoribonuclease acts as a scavenger of these end products from the other 3'-5' exoribonucleases, whose accumulation can lead to cell death. This step finishes the RNA metabolism pathway in *E. coli*, a critical biological process, not only for recycling purposes, but also for quality control of aberrant transcripts that can be prejudicial to the cell [92].

Studies in other species revealed the conservation of this biological process throughout the bacterial world, as many of the constituents of the *E. coli* RNA decay machinery have been found to be conserved in distantly related bacteria. Also, multicomponent enzymatic complex, similar to the degradosome, are found in other species, although not always with the same components [48].

1.4 Ribonuclease R

RNase R is a 3'-5' exoribonuclease encoded by the *rnr* gene (previously *vacB*), the second gene of an operon that includes a transcriptional regulator, *nsrR*, an rRNA methyltransferase, *rlmB*, and a protein of unknown function, *yjfI* [17]. Transcription of this operon ruled by a putative σ^{70} promoter, just upstream of the *nsrR* gene [17]. The *rnr*

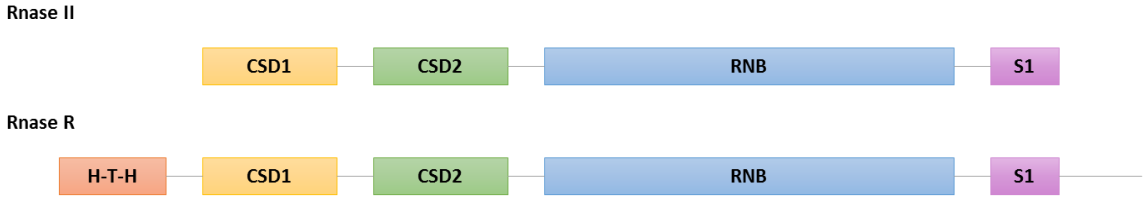

Figure 6 – **Representation of the domain arrangement of RNase II and RNase R.** From N to C-terminus. A putative nucleic acid binding site H-T-H (present in RNase R), the CSD1 and CSD2 RNA binding domains, the RNB catalytic domain and the S1 RNA binding domain.

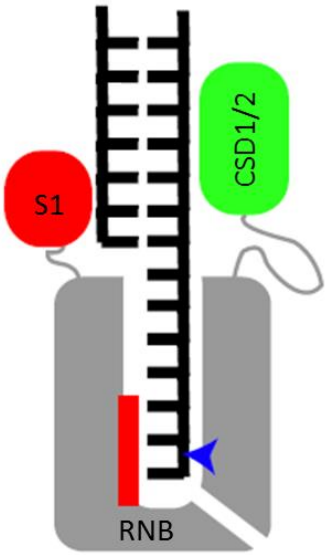

Figure 7 – **Model for RNA substrate binding.** Additional amino-acids from the N and C terminal are omitted. (adapted from[102])

transcript can be post-transcriptionally regulated by RNase E [16]. RNase R is a processive enzyme, meaning it degrades its substrate completely, and is also sequence-independent, requiring only a minimal overhang of 7 to 10 unpaired nucleotides at the 3'-end [102] (Figure 7). A unique feature of the RNase R is that it can degrade highly structured RNAs, the only exoribonuclease capable of doing it without the aid of a RNA helicase [5]. It is also pointed as a key enzyme in the degradation of polyadenylated transcripts. The role of RNase R in RNA quality control was shown to be crucial as it degrades defective tRNAs and rRNAs, whose accumulation can lead to defects in ribosomal maturation and assembly [24,102]. Moreover, RNase R also plays a role in protein quality control, as it is necessary for the correct processing of SsrA/tmRNA, affecting the *trans*-translation mechanism therefore leading the malfunctioning of the proteolysis tagging system [17]. The role of RNase R in RNA degradation has also emerged, despite the fact that RNase II is responsible for roughly 90% of the 3'-5' exonucleolytic activity [69]. During stationary phase growth there are specific mRNAs, like the *ompA* transcript, that can only be completely degraded when RNase R is present [2]. RNase R is also involved in the degradation of polyadenylated transcripts [3], and it has recently described that it can interact with the endoribonuclease YbeY, working together in the degradation of defective 70S ribosomes [44]. The levels of RNase R when the cell undergoes stress conditions like stationary growth or cold-shock, reveals a very important role for this protein in stress adaptation [2,17]. The increased levels of RNase R during stress induction is due to *rnr* transcript stabilization, alongside with the protein stabilization. The stabilization of the protein is regulated by post-translational acetylation [59]

1.4.1 Structural considerations

RNase R belongs to the RNB family of enzymes whose members are present in all domains of life. Members of this family include the bacterial RNase II and the eukaryotic Rrp44/Dis3 protein, all of them display a characteristic and well-conserved RNB domain with a unique αβ-fold [34]. The family name derived from the *rnb* gene encoding RNase B (also known as RNase II). In fact, RNase II is a representative of this family, since its structure is already determined [34], opposing to the case of RNase R whose crystal structure is yet to be determined [8]. Nevertheless, protein purification and characterization procedures determined a molecular mass of approximately 92kDa, in agreement with the expected 813 amino-acid composition of the protein [23]. Sequence analysis and modeling predicts that RNase R exhibits the typical domain organization of the RNB family (Figure 6): two RNA binding domains, CSD1 and CSD2 (for Cold Shock Domain 1 and 2), at the N-terminal region; the catalytic conserved RNB domain; and yet another RNA binding domain, the S1, at the C-terminal region [12]. Additionally it has also a helix-turn-helix domain (H-T-H, suggested to bind nucleic acids) near its N-terminus, and a lysine rich region at its C-terminus (Figure 6) [8]. The lysine rich region together with the RNA binding domains (mainly the CSD2 domain) are thought to act together to facilitate degradation of dsRNA, providing an helicase-like activity to RNase R [65]. Paradoxically, the RNB domain is capable to degrade double-stranded substrates by its own, even if the target has no 3'-overhang. This last observation suggests that the RNA binding domains of RNase R selectively bind to cellular RNAs tagged with a tail for degradation [64]. The yet to be resolved crystal structure of this protein will certainly bring to our understanding how this enigmatic exoribonuclease acts.

1.4.2 Implications in pathogenesis

Although most of what is known about RNase R was discovered in the *E. coli* model, RNase R homologues have been identified in a wide ranges of species, and it has also been implicated in the virulence mechanisms in a growing number of pathogens [7]. These include: *Shigella flexneri*, were it is necessary for the expression of invasion factors [98]; *Legionella pneumophila*, were it is necessary for viability at low temperatures and for inducing competence [22]; *Mycoplasma genitalium*, were RNase R is the only exoribonuclease known to date [42]; *Aeromonas hydrophila*, were its absence leads to reduced

motility and viability at low temperatures [32]; *Streptococcus pneumoniae*, were it is the unique homologue of the RNB family [31]; *Salmonella*, were differences in RNA affinity and activity suggests a its role in virulence [31]; *Pseudomonas syringae*, were it is involved in the maturation of rRNAs and tmRNA turnover [78]. Taken together, this observations indicate that bacterial strains lacking RNase R exhibit an attenuated virulence phenotype than their wild-type strains. Although there is still much work to be done in this field in order to understand what is causing the decreased virulence, RNase R may be seen as a potential future target for therapeutic drugs.

1.5 Hfq

Hfq was firstly identified in *E. coli* as a host factor necessary for the RNA bacteriophage Qβ, hence its name, host factor I (also known as host factor for phage Qβ) [33]. In the 90's it was recognized its fundamental role in cell physiology, as cells with no Hfq displayed a reduced fitness and capability to respond to stress [100]. It was later described as a regulator of RNA turnover [101,104], and today it is known to be a RNA chaperone involved in the stabilization/degradation of various RNAs. It is the core component of a post-transcriptional regulation network, mediating the base-pairing of regulatory sRNAs with their *trans* encoded mRNA targets [92,103]. Not far upstream of the *rnr* gene (roughly 6000 base pairs) there is the *hfq* gene. Null *hfq* mutants display a pleiotropic phenotype, which suggest its involvement in various pathways of *E. coli* metabolism [92]. sRNA mediated regulation has been studied over the last years due to its growing importance in the bacterial post-transcriptional regulation. *Trans* encoded sRNAs exhibit an imperfect base pairing to their mRNA target, thereby requiring the aid of a third-party molecule to ensure the match between both RNAs. Hfq favors binding of the sRNAs to

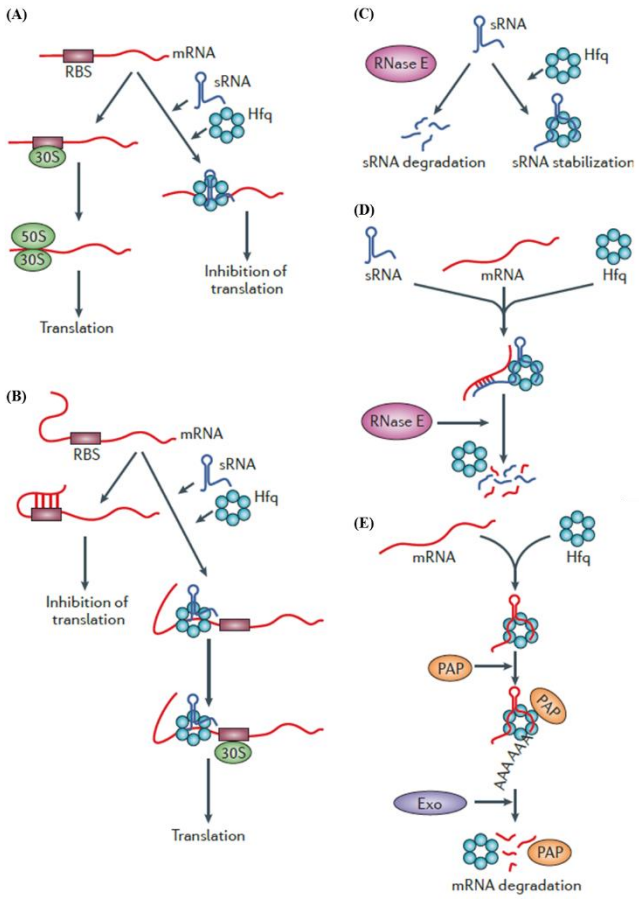

Figure 8 – **Representation of the diferente modes of Hfq activity in the sRNA mechanism.** (A), (C), (D) and (E) models lead to targeted degradation. (B) model leads to positive ribo-regulation and translation of the transcript (adapted from [103])

their targets [54]. There are general mechanisms of Hfq-mediated regulation of RNA levels or transcription (Figure 8). Firstly, Hfq, guided by a sRNA, can specifically block the SD sequence (also known as ribosome binding site – RBS) of the mRNA target, preventing translational initiation. Also, Hfq can boost translation initiation by disrupting possible secondary structures of the targeted mRNA that are blocking the SD sequence; Furthermore, it can help stabilize sRNAs, protecting them from ribonuclease degradation, or inversely, it can facilitate the base-pairing and help the formation of a sRNA/mRNA duplex that will serve as substrate for RNases; Finally, it is still implicated in RNA turnover as it can render the 3' extremity of the transcript accessible for polyadenylation (poly(A)) and favor subsequent 3'-5' degradation [103].

1.5.1 Structural considerations

Hfq is approximately 11kDa and belongs to a RNA-binding protein family which has representatives in all three domains of life [106], the Hfq-Sm-LSm family. A characteristic of this family is a doughnut-like shape accomplished by multimeric quaternary structure. Specifically, Hfq has a homohexameric ring architecture that displays a high affinity for short single-stranded sequences rich in adenines and uridins in close proximity of stem-loop structures [92]. The protein's structural core is well conserved, comprising an α-$\beta_{1\text{-}5}$ fold, where the family characteristic motifs, Sm1 ($\beta_{1\text{-}3}$) and Sm2 ($\beta_{4\text{-}5}$), form

specific secondary structures that allow RNA binding (Figure 9C) [6]. The C-terminal portion of the protein, however, is extremely disordered and poorly understood, both in terms of structure and function [103]. The ring like architecture of the homohexamer exposes two faces of the molecule (proximal and distal) and a lateral surface (rim) that bind RNA molecules with different specificities (Figure 9A) [54]. The proximal face of the protein preferentially binds uridine-rich sequences and it is thought to be the binding face of sRNAs due to Rho-independent terminators, which have characteristic poly-uridine tails. The distal face, on the other hand, binds more accurately to poly-adenine tails, found in some mRNA molecules [67]. Furthermore, it binds to mRNAs containing a ARN binding motif (where R is a purine, and N is any base) [61]. Recently there was a third interaction site described, the rim of the ring-like quaternary structure. This surface displays a positively charged region that has been implicated in sRNA binding and facilitating mRNA association and releasing of Hfq [85].

Besides its interaction capabilities with RNA molecules, Hfq was also shown to directly interact with the polyadenylate polymerase I, thereby promoting polyadenylation of mRNAs, targeting them for degradation [67]. Furthermore, interactions with RNase E and PNPase have been described, with the evident benefit of these interactions being the formation of more efficient ribonucleoprotein complexes able to degrade a specific mRNA target [70,71]. The number of putative protein partners of Hfq is still growing, suggesting that this ubiquitous protein plays a central role in many physiological pathways of the bacterial cell.

1.5.2 Implication in pathogenesis

The growing importance of this protein in the bacterial kingdom and the interplay of related pathways, makes it an interesting target for virulence studies of several pathogens. In several pathogenic models the lack of the Hfq protein was found to alter the virulence capability of the organism. Virulence phenotypes in *hfq* mutants have shown a more dramatic impact in Gram-negative bacteria [19]. Studies have tested different stress conditions in various pathogens, much expected results: *Brucella abortus* [81], *Neisseria meningitidis* [75], *Pseudomonas aeruginosa* [94], *Salmonella enterica* [52], and *Yersinia pestis* [35] have shown to increase sensibility in more than one stress condition and an overall reduction of virulence [19]. Acting as a global regulator of gene expression, Hfq

has drawn our attention to its impact on physiology and fitness. Its cross-species conservation enables a comparative view for the necessity for such regulator, proving that it is crucial for successful host-pathogen interactions.

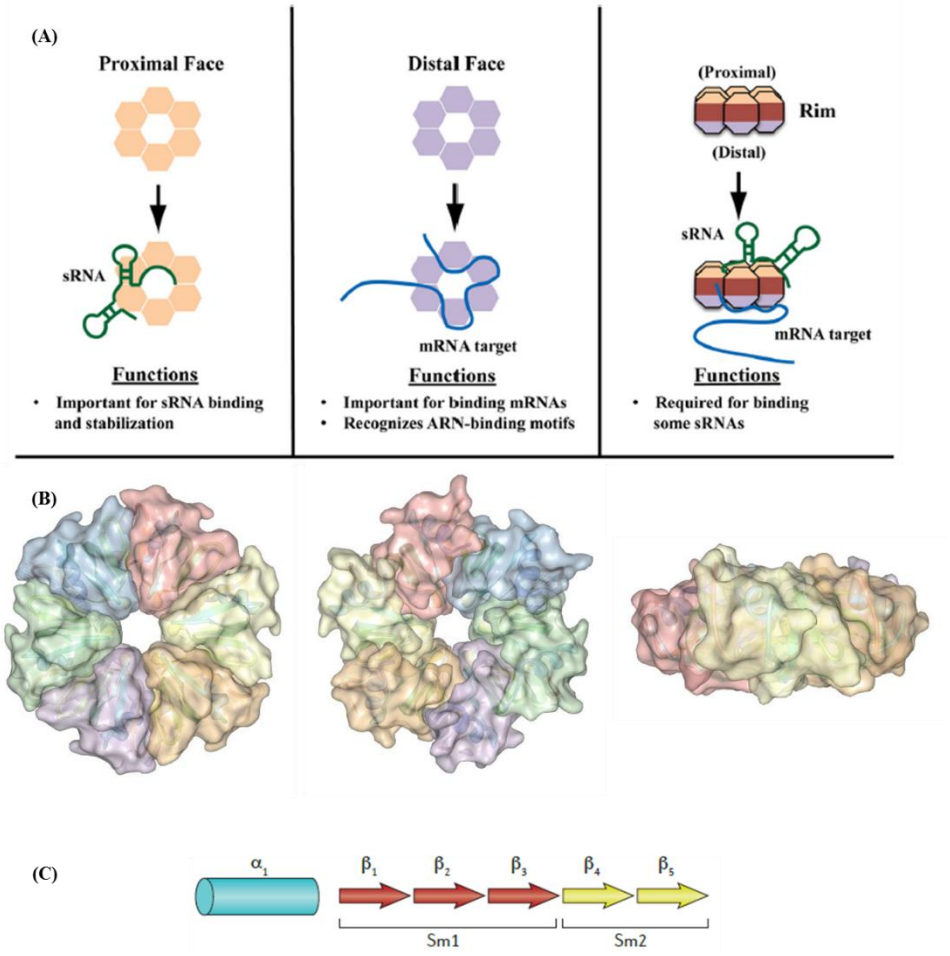

Figure 9 – **Structural features of Hfq.** (A) The three possible ways of RNA binding by Hfq (adapted from [54]). (B) Hfq's hexamer rendering from the crystal structure (PDB code: 3QHS). (C) Hfq monomer structural domain arrangement. (from N to C-terminus): one α-helix, three β-sheets(1-3) (domain Sm1) and two other β-sheets(4-5) (domain Sm2).

2

Materials and Methods

2 Materials and Methods

2.1 Strains and growth conditions

Previous work in our laboratory led to the construction of the strains used during this Thesis. Strains were constructed in *E. coli* K-12 wild-type strain MG1693 (WT), maintaining an isogenic background. A knockout *rnr* mutant (henceforth referred as ΔR) bearing a Kanamycin (Kan) resistance gene, a knockout *hfq* mutant (referred as ΔH) bearing a Chloramphenicol (Cam) resistance gene, and a knockout mutant in both *rnr* and *hfq* (referred as ΔHR) bearing both resistance genes. Whenever relevant additional strains were used as supplementary evidence of the obtained results. These included knockout single mutants for the *rnb* and the *pnp* genes (ΔII and ΔP) – coding two major exoribonucleases, RNase II and PNPase respectively –, a double knockout mutant for both *hfq* and *rnb* genes (ΔHII) and another double knockout mutant for both *hfq* and *pnp* genes (ΔHP). Wild-type strains overexpressing the *hfq* (WT+pHFQ) as well as the *rnr* gene (WT+pRNR) were also used in some experiments.

Strains were grown on an orbital shaker at 180RPM at 37ºC in Luria-Bertani Broth (LB) medium supplemented with Thymine (50µg/ml) and, whenever necessary, with the correct antibiotic (Kan: 30µg/ml; Cam: 30µg/ml). Cells were grown to stationary phase, unless stated otherwise, culture density was check through Optical Density readings at 600nm (OD_{600}≈6 for WT and ΔR; OD_{600}≈2.7 for ΔH and OD_{600}≈2.3 for ΔHR), and then stored at -80ºC after being pelleted.

Table 1 – **LB medium recepie.**

Luria-Bertani Broth
1% Tryptone
0.5% Yeast extract
170mM NaCl

2.2 Protein quantification

Protein quantifications were carried out so that the same amount could be analyzed and compared between strains or conditions. Two colorimetric assays were used for this purpose: Bradford protein assay and Lowry protein assay.

2.2.1 Bradford protein assay

This assays was performed with 1ml of Quick Star Bradford 1X Dye Reagent (Biorad) for 10µl of protein sample. The incubation period was of 5 minutes and then the readings were made at 595nm. After a comparison with the Bradford standard curve, values for protein quantification were calculated

2.2.2 Lowry protein assay

50µl of the sample diluted in 0.2% SDS were prepared and 750µl of the Solution C was added and mixed. After an incubation period of 10 minutes at room temperature (RT), 150µl of the Solution D was added and mixed. After a new incubation period of 30 minutes, the absorbance of the samples was taken at 750nm and compared with the Lowry standard curve for protein quantification.

Table 2 – **Solutions of Lowry protein quantification method.**

Solution A	*Solution B*	*Solution C*	*Solution D*
2% Na2CO3	**0.5%** CuSO4	**50:1** Sol. A + Sol. B	**5:1** Folin–Ciocalteu phenol reagent
0.1M NaOH	**1%** Na-Citrate	**0.2%** SDS	

2.3 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a widely used technique that offers a mean to separate proteins according to their molecular weight. Two different types of resolving gels with different polyacrylamide concentrations were used: 10% gels for proper RNase R separation and 15% gels for Hfq separation. The stacking gels consisted of 5% polyacrylamide.

Protein Loading Buffer was added to protein samples or cell extracts samples and then denatured in boiling water before applied onto SDS-polyacrylamide gels. Gels ran in the Running Buffer at 200V for approximately 2 hours and dyed with Coomassie Brilliant Blue G-250 for in-gel visualization of the separated proteins. The Mini-PROTEAN

Tetra Cell system (Biorad) was used for the preparation and running of the gels, and Precision Plus (Biorad) protein standards was used as marker.

Table 3 – **Solutions for the SDS-PAGE gels.**

Stacking Gel	*Resolving Gel*	*Running Buffer*	*Coomassie stain*
5% Polyacrylamide	**10%/15%** Polyacrylamide	**25mM** Tris-Base	**0.05%** coomassie blue
175mM Tris-HCl pH=6.8	**380mM** Tris-HCl pH=8.8	**190mM** Glycine	**10%** Acetic acid
0.1% SDS	**0.1%** SDS	**0.1%** SDS	**50%** Methanol
0.1% APS	**0.1%** APS		
0.05% TEMED	**0.05%** TEMED		

2.4 Western blotting

After a SDS-PAGE, the in-gel separated proteins were transferred to a nitrocellulose Hybond ECL membrane (GE Healthcare) in Transfer Buffer during 90 minutes at 100V at 4ºC. Then, for control purposes, the gel was stained with Coomassie dye to guarantee that all proteins were transferred. The membranes were blocked with 5% skim milk in T-TBS and incubated for a minimum of 1 hour at 4ºC with casual shaking to prevent unspecific interactions. Afterwards the skim milk solution was discarded and the primary antibody (α-RNase R or α-Hfq) was added in a 1:10000 dilution in T-TBS. The membranes were left again at 4ºC with casual shaking overnight. Following a washing step of 3x10 minutes with T-TBS, the secondary antibody (α-rabbit IgG) was added in a 1:10000 dilution also in T-TBS and incubated for 1 hour at 4ºC with shaking. Another washing of the membranes was preformed (3x10 minutes) and then covered with Western Lightning Plus ECL (PerkinElmer) reagents during a 1 minute incubation at RT The membranes were then visualized in the ChemiDoc XRS+ system (BioRad).

Table 4 – **Solutions for western blotting.**

T-TBS	*Transfer Buffer*
20mM Tris-Base	**25mM** Tris-Base
137mM NaCl	**190mM** Glycine
0.1% Tween20	**0.1%** SDS
	20% Methanol

2.5 Growth curves

For each strain, single colonies were inoculated in 5ml of LB media and left growing overnight (16 hours) at 37ºC with 180rpm of orbital shaking. After that, 50ml of LB media with Thymine, supplemented whenever necessary, with antibiotic, and inoculated. All strains started growing with an OD_{600}=0.03 at 37ºC and 180rpm. Readings were made hourly to follow growth.

2.6 Serial dilution spotting assay

For this assay, 1ml of stationary phase grown cells from each strain (WT, ΔR, ΔH, ΔHR) was taken during growth at 37ºC and 180rpm. The initial cells (10^0) were serially diluted at a 1:10 ratio for seven times, until we reached 10^{-7} dilution. The various dilutions (10^0-10^{-7}) of each strain were spotted onto Luria-Bertani medium plates and incubated overnight at 37ºC. Photographs of the plate were then documented.

2.7 RNA extraction

After growth, cell were harvested and an equal volume of RNA Stop Buffer was added. At this point the RNA Stop Buffer must be at glacial temperatures. The mixture was then pelleted at 7000rpm during 15 minutes at 4ºC in a JA-20 rotor for the J2-MI Beckman Coulter's centrifuge, and the supernatant discarded. The pelleted cells were re-suspended in RNA Lysis Buffer and two freeze-thaw cycles were made, using liquid nitrogen and a water bath at 50ºC. After the first two cycles, 2.8mM of Acetic Acid and 1% of SDS was added and the freeze-thaw cycles were repeated for five more times. An additional 20U of TurboDNase (Ambion) was added and samples were left to incubate for 60 minutes at 37ºC. Equal volume of phenol was added and centrifuged at 17000g for 10 minutes and 4ºC. The aqueous phase was recovered and the phenolic extraction was repeated. One volume of a chloroform/isoamilic alcohol solution was added and the samples centrifuged as before. Approximately 300mM of Sodium Acetate and two volumes of Ethanol 100% was added and incubated overnight at -20ºC for precipitation. The next day samples were firstly centrifuged for 45 minutes at 17000g and 4ºC, the supernatant

discarded and the pellet washed with Ethanol 75%. Another centrifugation was made for 30 minutes, the pellet was air-dried for 2 minutes and then resuspended in MiliQ water.

Table 5 – **Buffers for RNA isolation.**

RNA Stop Buffer	*RNA Lysis Buffer*
10mM Tris-HCl pH=7.2	**10mM** Tris-HCl pH=7.2
5mM $MgCl_2$	**5mM** $MgCl_2$
25mM NaN_3	**300µg/ml** Lysozyme
500µg/ml Chloramphenicol	**10U** TurboDNase

2.8 Northern blotting with agarose gel electrophoresis

After quantification, 2.5µg (if not stated otherwise) of total RNA samples were prepared, Loading Buffer was added and samples were denatured for 10 minutes at 80ºC using a thermo block. Samples were then loaded onto a 1.2% agarose denaturing gel containing 18% formaldehyde, ran for 2 hours at 90V in MOPS Buffer and transferred onto a Hybond N+ membrane (GE Healthcare) overnight by capillarity with SSC Buffer. For crosslinking the RNA molecules to the membrane, the last one was incubated in the UVC 500 oven (GE Healtcare) for 2.3 minutes at 1200J/cm^2. All probe sequences are presented in the Appendix section (6.2). Probes were radioactively labeled at their 5'-end with [γ-^{32}P] ATP, and then purified with G-25 MicroSpin columns (GE Healthcare) to cleanse all unbound [^{32}P]. Hybridization followed overnight in PerfectHyb Plus Hybridization Buffer (Sigma). Membranes were then washed with diluted (1:10) SSC Buffer until background radiation levels were acceptable. Membranes were then exposed on a phosphor screen (GE Healthcare) and the screen scanned with the STROM 860 Molecular Imager system (GE Healthcare).

Table 6 – **Solutions for RNA Northern blot analysis.**

MOPS Buffer	*SSC Buffer*
40mM MOPS	**3M** NaCl
10mM Sodium Acetate	**0.3M** Sodium Citrate
1mM EDTA	

2.9 Northern blotting with polyacrylamide gel electrophoresis

RNA samples were quantified, denatured (10 minutes at 80ºC) and 2.5µg were loaded onto 8% polyacrylamide/7M Urea gels. A pre-run was made to the system prior to sample loading, for 90 minutes at 420V, after which the loaded samples ran in TBE Buffer for 2 hours at 420V. Transfer was made electrophoretically onto a Hybond N+ membrane (GE Healthcare) for 90 minutes at 24V in TAE Buffer at 4ºC. RNA molecules were transferred to Hybond N+ membranes (GE Healthcare) and UV cross-linked for 2.3 minutes at 1200J/cm^2. Again, nucleic acid sequences used for northern blotting can be found in 6.2. Probes were radioactively labeled with [γ-^{32}P] and purified. Hybridization and washing steps were as described above, as well as imaging.

Table 7 – **Polyacrylamide Northern blotting buffers.**

TBE Buffer	*TAE Buffer*
89mM Tris Base	**40mM** Tris Base
89mM Boric Acid	**20mM** Acetic Acid
2mM EDTA	**1mM** EDTA

2.10 Ribosome extraction

Cells were pelleted during stationary phase of growth, after a 16 hour-growth, and disrupted in the French Press system (10000psi) with Buffer A. Next, cell lysates were incubated with DNase (12U of TURBO DNase – Ambion) for 45 minutes at 4ºC, and then centrifuged 2 times for 15 minutes at 20000g at 4ºC for clarification, the supernatant was kept. For each Beckman Coulter's 90Ti ultracentrifuge tube 7ml of Buffer B were added and 3ml of the clarified cell lysate was carefully laid on top of that, so that the interface was not disturbed. Ultracentrifugation was made for 16 hours at 44000rpm at 4ºC on a Beckman Coulter's Ultracentrifuge XL-100 with the type 90 Ti Rotor. After discarding the supernatant, the remaining pellet was washed 2-3 times with Buffer C in order to remove de remaining excess of EDTA. After washing, the pellets were resus-pended in 800µl of Buffer B by gently vortexing. The samples were then clarified for 10 minutes at 14000g at 4ºC, the supernatant was recovered and its absorbance at 260nm measured in Nanodrop 1000, for ribosome quantification.

Table 8 – **Buffers for Ribosome Extraction protocol.**

Buffer A	*Buffer B*	*Buffer C*
50mM Tris-Cl pH=7.5	**50mM** Tris-Cl pH=7.5	**50mM** Tris-Cl pH=7.5
10mM $MgCl_2$	**10mM** $MgCl_2$	**10mM** $MgCl_2$
100mM NH_4Cl	**1M** NH_4Cl	**100mM** NH_4Cl
6mM β-mercaptoethanol	**6mM** β-mercaptoethanol	**6mM** β-mercaptoethanol
0.5mM EDTA	**0.5mM** EDTA	
Roche's Protease Inhibitor Cocktail	**18%** sucrose	

2.11 Ribosome profiling

2.11.1 Associative conditions for 70S analysis

Ribosome profiling analysis under associative conditions was carried out on 15-50% sucrose gradients, prepared in Gradient Buffer on Beckman Coulter's Polyallomer ultracentrifuge tubes appropriate for the SW28 rotor. The gradients were laid "by hand" after which they were left to diffuse in 4ºC for a minimum of 4 hours. After that time, 400µg (if not stated otherwise) of the ribosomal extracts were loaded on top of the gradient, and Associative Gradient Buffer was added to fill the tube. These were then ultracentrifuged in a SW28 rotor at 24000rpm for 16 hours at 4ºC on a Beckman Coulter's Ultracentrifuge XL-100. Gradients were then fractionated in 1ml samples and the absorbance at 260nm was read.

Table 9 – **Associative gradient buffer**.

Associative Gradient Buffer
50mM Tris-Cl pH=7.5
10mM $MgCl_2$
100mM NH_4Cl
6mM β-mercaptoethanol

2.11.2 Dissociative conditions for subunit analysis

Dissociative profiles were performed on 10-40% and 10-30% sucrose gradients, also prepared as stated above (2.11.1) on Polyallomer tubes for SW28 rotor. The same amount of ribosomes was used for these gradients but both the gradients and samples were prepared in Dissociative Gradient Buffer, with a lower amount of $MgCl_2$. Samples were ultracentrifuged on a Beckman Coulter's Ultracentrifuge XL-100 in a SW28 rotor at 24000rpm for 16 hours at 4ºC, fractionated, and absorbance readings at 260nm plotted.

Table 10 – **Dissociative gradient buffer.**

Dissociative Gradient Buffer
50mM Tris-Cl pH=7.5
0.1mM $MgCl_2$
100mM NH_4Cl
6mM β-mercaptoethanol

2.12 Protein isolation from ribosomal fractions

Protein precipitation was made by adding equal volume of TCA to the lysate, mixed and left in -20ºC for 60 minutes. Then samples were centrifuged for 15 minutes at 20000g and 4ºC. After removing all supernatant cold acetone was added and then vortexed. Samples were left on -20ºC overnight. Another centrifugation was made and the supernatant removed. The samples were left open to air dry and the pallet resuspended in the Protein Resuspension Buffer.

Table 11 – **Buffer used for resuspension of protein precipitation samples.**

Protein Resuspension Buffer
20mM Tris-Cl pH=7.5
0.2% SDS

2.13 Far-western

This Western blotting derived method was implemented in order to investigate if there was any protein-protein interaction between Hfq and RNase R. Far-Western analysis was made using purified RNase R (His6-RNase R) as the prey protein immobilized in a nitrocellulose membrane, and purified Hfq (His6-Hfq) as the bait protein.

The bait protein was loaded onto a 10% SDS-polyacrylamide gel and after a typical Western Blot transfer, the nitrocellulose membrane was stained with Ponceau reagent. After distaining, the proteins present in the membrane were denatured and renatured in AC Buffer with gradual decreasing of the denaturing agent, Guanidine-HCl. The membrane was left overnight at 4ºC with the last of the AC Buffers that contained no Guanidine, allowing for correct refolding of the proteins. The membrane was then washed 3 times with T-TBS and blocked with 5% skim milk in T-TBS for 3 hours at RT with casual shaking. After blocking followed an overnight incubation at 4ºC with 5µg of the bait protein in Binding Buffer. The above described Western blotting protocol was carried out, using α-Hfq as the primary antibody.

Table 12 – **Far-western solutions.**

Ponceau stain	*AC Buffer*	*Binding Buffer*
0.1% Ponceau S	**10%** Glycerol	**10%** Glycerol
1% Glacial acetic acid	**100mM** NaCl	**100mM** NaCl
	20mM Tris-HCl pH=7.5	**20mM** Tris-Hcl pH=7.5
	1mM EDTA	**0.5mM** EDTA
	6M/3M/1M/0.1M/0M Guanidine-HCl	**0.1%** Tween20
	2% Skim milk	**2%** Skim milk
	1mM DTT	**1mM** DTT

2.14 Pulldown

For the pulldown protocol RNase R was used as bait. Cells were disrupted in Pulldown Lysis Buffer at 10000psi in the French Press system. The lysates were then treated with Benzonase (125U from Sigma) for 60 minutes at 4ºC and clarified for 15 minutes at 20000g at 4ºC. The extracts were quantified through Bradford Assay (2.2.1). Ni-NTA beads were washed 3 times with Beads/prey Binding Buffer and then incubated at 4ºC with casual shaking for no less than 1 hour with 0.3mg of the lysate from the overexpressing strain WT+pRNR containing His6-RNase R. After this time, the mixture, now containing the bait protein bound to the beads, was washed 4 times with the same buffer and incubated in the same previous conditions but now with 1mg of cell lysates at 4ºC. Five Washing steps with Washing Buffer followed to remove unspecific interactions and then a 3 minute centrifugation at 5800g and 4ºC was made. Elution Buffer was added, gently

mixed, spined and the supernatant now containing our interaction proteins recovered were loaded onto a 15% for Western blot analysis as described above using α-Hfq antibody.

Table 13 – **Buffers for the Pulldown protocol.**

Pulldown Lysis Buffer	*Beads/prey Binding Buffer*	*Washing Buffer*	*Elution Buffer*
50mM Tris-HCl pH=8.0	**50mM** Tris-HCl pH=8.0	**50mM** Tris-HCl pH8.0	**50mM** Tris-HCl pH8.0
125mM NaCl	**300mM** NaCl	**300mM** NaCl	**300mM** NaCl
10% Glycerol	**10%** Glycerol	**10mM** Imidazole	**300mM** Imidazole
0.1% TritonX-100	**0.06%** TritonX-100		
1mM PMSF			

2.15 Co-immunoprecipitation (Co-IP)

The Co-IP assay was carried out using the Pierce Crosslink Immunoprecipitation Kit (#26147 – Thermo Scientific), following label instructions. Cell disruption was made in Co-IP Lysis/Wash Buffer using the French Press system at 1000psi. 1mg of cell lysates were pre-cleared using the Control Agarose Resin, incubated for 60 minutes at 4ºC with gentle mixing. ΔR and ΔH strains were used as controls, while the Wild-type, WT+pHFQ and WT+pRNR were used to test the interaction. All resin centrifugation steps were made at 1000g and 4ºC for 1 minute, including all washing steps. The mixture was then centrifuged and the flowthrough was recovered. For the binding of the antibody, Protein A/G Plus agarose was prepared in spin columns and pre-washed with Coupling Buffer 1X. A sample containing only the beads with no antibody was prepared for control purposes. 40µg of anti-RNase R antibody (α-RNase R) was added to the resin in the column, and incubated for 60 minutes at R.T. with gentle mixing. Three washing steps were made with Coupling Buffer 1X and the flowthrough was discarded. To crosslink the α-RNase R antibody to Protein A/G, 2.5mM of DSS in Coupling Buffer 1X was added and incubated for 60 minutes at RT. Two washings were made with Elution Buffer and another two followed with cold Co-IP Lysis/Wash Buffer. For immunoprecipitation, cleared cell lysates were added to the crosslinked α-RNase R-Protein A/G resin, and incubated for 2 hours at 4ºC. After that, columns were centrifuged and the flowthrough saved for control. Three washing steps were performed with Co-IP Lysis/Wash Buffer, and an additional

one with Conditioning Buffer 1X. To elute the immunocomplex, Elution Buffer was added to the column and after a 5 minute incubation at RT a centrifugation was made and the flowthrough collected. Western blot analysis of the eluted immunocomplexes was performed using α-Hfq antibody.

3

Results

3 Results

3.1 *Characterization of the double mutant Δ*hfq *Δ*rnr

Work performed at this laboratory suggested that Hfq and RNase R could share common RNA degradation pathways. While single mutants of *hfq* and *rnr* have been published and are well characterized [4,17], the double mutant strain Δ*hfq* Δ*rnr* (ΔHR) has not been studied. As stated above (2.1), the mutant strains used were preciously constructed. The work presented herein, started with the confirmation that the newly generated double mutant Δ*hfq* Δ*rnr* strain did not express both Hfq and RNase R.

Total protein extracts from the ΔHR strain were analyzed by Western blotting, using antibodies against RNase R (α-RNase R) or against Hfq (α-Hfq) (Figure 10A). Wild-type (WT) and the single mutants cell extracts were loaded as controls. Results confirm that Hfq and RNase R are not expressed in the double mutant strain. Given the molecular weight of these proteins – RNase R has 92kDa and Hfq 11kDa – and the fact that Hfq antibody revealed a strong cross-hybridization with a lower weight protein (previously reported in [111] and identified as nonspecific in Figure 10A), several attempts were made until the Western blotting protocol could be fully optimized. Although a 4-15% gradient SDS-PAGE revealed to be effective to identify both proteins, increasing resolution was obtained through the use of separate gels to detect RNase R (10% SDS-PAGE) and Hfq (15% SDS-PAGE). Western blotting results were in line with early PCR analysis that verified disruption of both genes (data not shown). Overall, these results validated the construction of the ΔHR mutant strain.

Subsequently we analyzed the growth behavior of the ΔHR strain in liquid LB medium (Figure 10B). The WT and single mutant strains were grown in the same conditions and used for comparison. Whereas the WT and ΔR strains did not show significant differences, the ΔH strain grew much slower and reached lower cell densities than the WT, as expected [100]. The double mutant also presented growth defects but the phenotype was even more severe as the ΔHR mutant grew even slower than ΔH strain (a noticeable feature observed in Figure 10B). Nonetheless, ΔHR and ΔH strains could reach similar cell densities at the stationary phase of growth. Given the more pronounced importance of RNase R and Hfq in stationary phase cells [2,36], we focused our studies on this growth stage. Stationary phase cultures were then serially diluted and spotted on LB

plates (Figure 10C). A slightly decrease in cell density was observed in the ΔR strain when compared to the WT, and the ΔHR strain revealed even lower cell densities than the ΔH strain. Taken together, these results indicate that the combined inactivation of RNase R and Hfq has a stronger impact on cell physiology than the single disruption of each one of them.

Our primarily goal was to analyze the impact of inactivating both RNase R and Hfq on RNA degradation pathways. Following RNA isolation from these strains, total RNA was analysed on a agarose gel stained with ethidium bromide (EtBr) and detected under UV light (Figure 10D). In all strains, the major forms of rRNA (23S, 16S and 5S) are well identified. Strikingly, the ΔHR strain was found to accumulate other RNA fragments that were not detected in the WT or in the single mutants. This strongly support our hypothesis that RNase R and Hfq share common substrates.

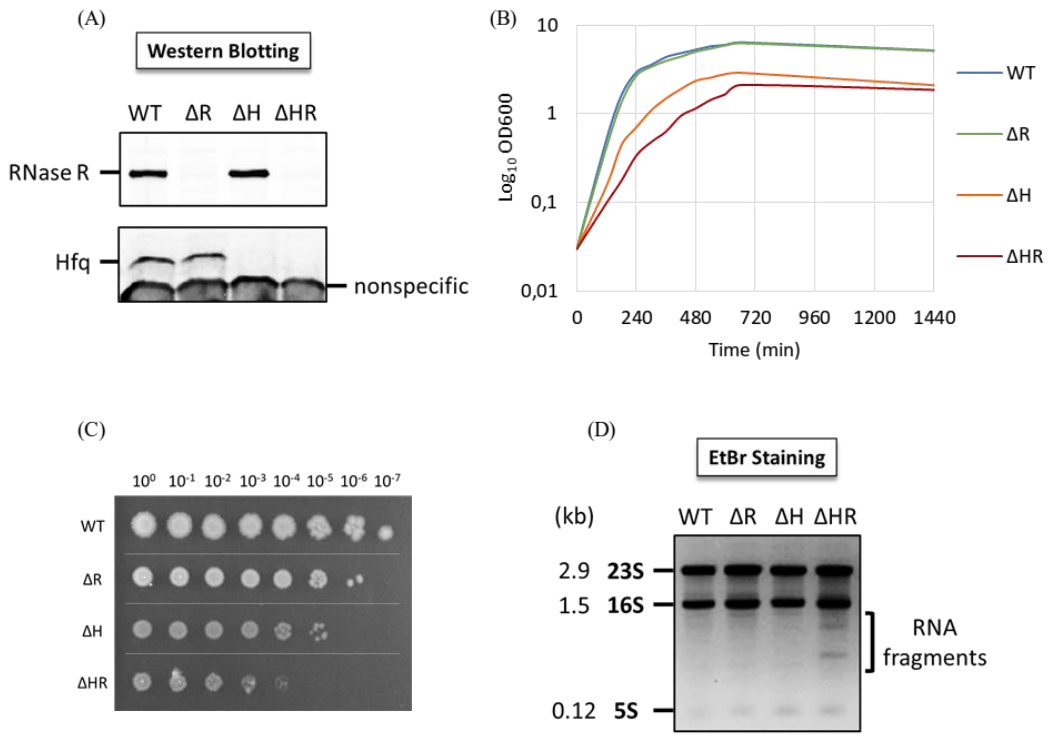

Figure 10 – **(A) Confirmation of the Δhfq Δrnr mutant.** Western blotting analysis of the total protein extracts samples from the WT, ΔR, ΔH and ΔHR strains. Samples were analyzed on a 10% and 15% SDS-polyacrylamide gel and checked with anti-RNase R (gel on the top) and anti-Hfq antibodies (gel on the bottom). **(B) Growth curves.** Growth curves for the ΔHR strain is provided, alongside with the WT and single mutant strains for comparison. Strains were tested in the same conditions (see 2.5 for details). **(C) Serial dilution spotting assay.** Stationary phase cultures were serially diluted as indicated and spotted on LB plate. **(D) RNA integrity.** Total RNA was fracionated in an agarose gel ran in TBE 1x buffer and stained with EtBr. On the left, ribosomal RNA forms are indicated together with their molecular weights.

3.2 The Δhfq Δrnr mutant accumulates 16S and 23S ribosomal RNA fragments

The RNA fragments that were found to accumulate in the double Δ*hfq* Δ*rnr* strain were evident upon EtBr-staining of the agarose gel. This was a clear indication that these RNAs must be abundant in the cell, as only abundant RNAs (as ribosomal RNAs) can be detected with this method. Accordingly, we hypothesized that these RNAs would correspond to ribosomal RNA fragments. We then analyzed by Northern blotting if these fragments could originate from the 16S and/or the 23S rRNA.

Total RNA was fractionated on a denaturing formaldehyde-agarose gel and transferred onto a nylon membrane that was then hybridized with a [^{32}P] 5'-end labeled antisense probe. Specific antisense oligonucleotides were designed to detect either the 16S rRNA the 23S rRNA. A smaller 16S-originating fragment was detected at very low levels in the single Δ*rnr* and Δ*hfq* mutants while it was strongly accumulated in the double Δ*hfq* Δ*rnr* mutant. Furthermore, a higher molecular weight band is only detected in the ΔHR strain. In the wild-type none of these RNA species were detected. When the specific 23S antisense probe was used, smaller 23S-derived fragments were also found in the single Δ*rnr* mutant and these were slightly more abundant in the double Δ*hfq* Δ*rnr* mutant. The same RNAs were then analysed on polyacrylamide/urea gels. This allowed us to better estimate the size of the fragments that were accumulated in the ΔHR strain as this technique provides a higher resolution for small fragments. Using the aforementioned antisense oligonucleotides, we were able to detect two 16S-originating fragments around 350 and 200 nucleotides (nt) respectively and a smaller 23S RNA species with approximately 300nt (Figure 11).

The RNA fragments found to accumulate in the ΔHR strain and visible with EtBr-staining (Figure 10D) were actually found to correspond to a mixture of both 16S and 23S smaller RNA species (Figure 11), as identified by Northern blotting. Some of these fragments were also detected to low levels in the single mutants (namely in the Δ*rnr* mutant), however, they were clearly shown to accumulate to higher levels in the double ΔHR mutant. These intermediary fragments probably build up as they are not efficiently degraded in the cell. Accordingly, our experiments suggests that RNase R and Hfq are required for the correct degradation of such smaller rRNA species.

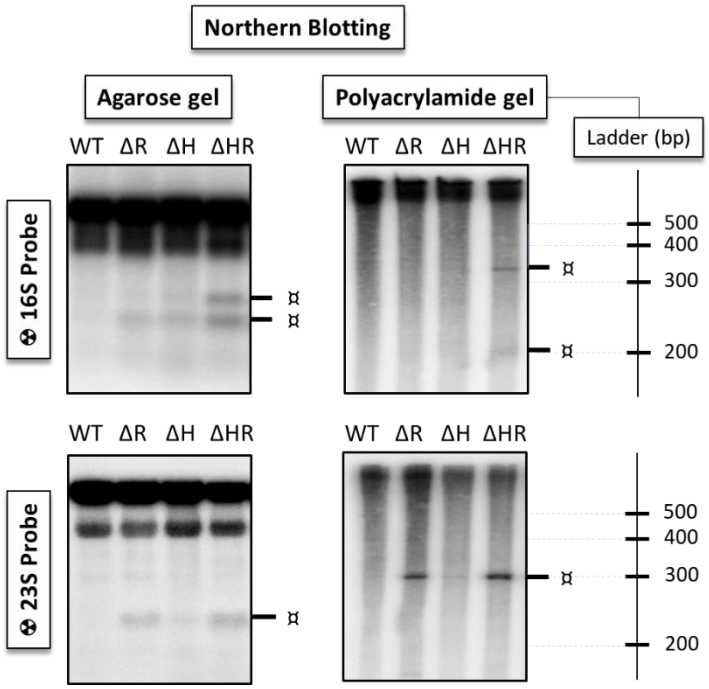

Figure 11 – **Northern blot analysis of rRNA fragments.** Total cellular RNA was isolated, loaded onto 1.2% agarose/formaldehyde gels (left panels) and onto 8% polyacrylamyde/urea gels (right panels). Northern blot analysis was carried out and membranes were probed with radioactively labeled [^{32}P] antisense oligonucleotides of the 16S rRNA (on the top) and of the 23S rRNA (on the bottom). The sign (¤) indicates accumulated fragments. Ladder information (in base pairs) for the polyacrylamide gels is provided on the far right of the image.

3.3 RNase R is specifically required for 16S and 23S fragment removal in the absence of Hfq.

Hfq is an RNA-binding protein that is known to protect the 3'-end small non-coding RNAs from exonucleolytic activity [4]. However, up to now it has never been implicated in the protection of rRNAs. In the absence of RNase R and Hfq, rRNA fragments accumulate in the cell as if they are not efficiently cleared out. Together with RNase R, PNPase and RNase II the are other highly processive exoribonucleases involved in the elimination of a vast repertoire of substrates, including rRNA [7]. We next compared the impact of PNPase and RNase II in the degradation of rRNA, in the presence or absence of Hfq (Figure 12).

Northern blot analysis was carried out to test RNA extracted from a new set of strains: single mutant strains lacking RNase II (Δ*rnb*/ΔII) or PNPase (Δ*pnp*/ΔP) and the respective double mutant strains also not expressing Hfq (Δ*hfq* Δ*rnb*/ΔHII and Δ*hfq* Δ*pnp*/ΔHP, respectively). In the presence of Hfq, no 16S rRNA fragments were detected

in any of the exoribonuclease mutants analyzed. On the other hand, the two fragments which strongly accumulated in the ΔHR double mutant were not visible in the ΔHII or ΔHP strains. A 23S rRNA fragment is readily observed in the single Δ*rnr* and Δ*hfq* mutants. This accumulation is higher when these mutations were combined, as clearly depicted in the ΔHR strain. In contrast, no fragments were detected in cells deficient in RNase II or PNPase, regardless of Hfq presence.

The absence of Hfq in mutants lacking another exoribonuclease that not RNase R, did not show any defects in rRNA degradation. This indicates that RNase R is the major exoribonuclease involved in the removal of aberrant rRNA fragments in the absence of Hfq, in a pathway that is not compensated by RNase II or PNPase.

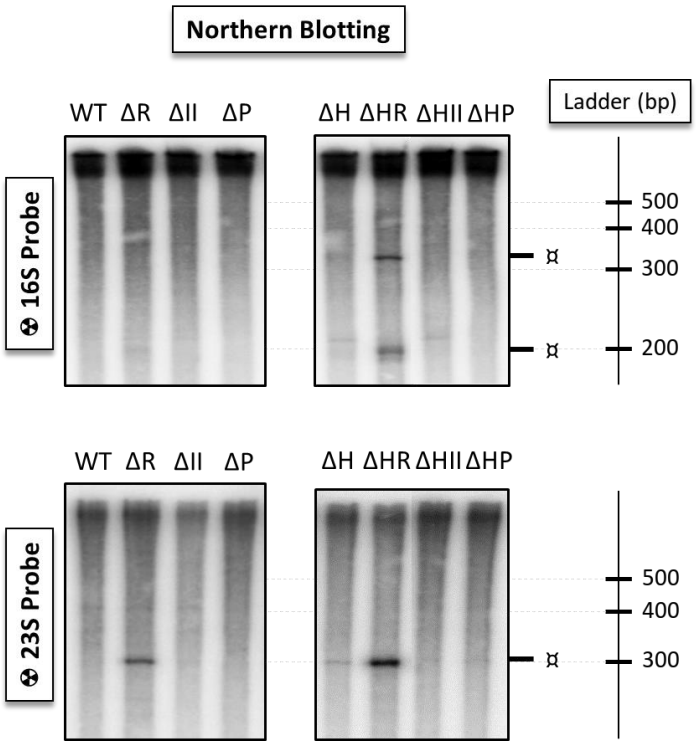

Figure 12 – **Northern blot analysis of the rRNA fragments and comparison other *E. coli* exoribonucleases.** Total RNA isolation was carried out on stationary phase cultures. Samples were loaded onto an 8% polyacrylamide/urea gel. Northern blotting followed on the two separate gels. The 16S (top panels) 23S probes (bottom panels) were used to check fragment accumulation. The sign (¤) reffers to accumulated fragments.

3.4 The 23S fragment originates from a central region of the 23S rRNA

We next focused our attention on the 23S RNA defects found on the double Δ*hfq* Δ*rnr* mutant that were also observed to a lesser extent in the single Δ*rnr* and Δ*hfq* strains. Using an antisense oligonucleotide (23S probe A) that hybridizes with a central region of the 23S rRNA (1458-1479nt), we could detect a fragment of approximately 300nt. In order to further map this fragment, two additional probes were designed: the 23S probe B (complementary to 1269-1289nts of 23S rRNA) that binds upstream the 23S probe A; and the 23S probe C (complementary to 1641-1661nts of 23S rRNA) that binds downstream the 23S probe A (see scheme on Figure 13A).

The 23S rRNA fragment was only detected when using 23S probe A (Figure 13B, upper panels). The 23S probe B and the 23S probe C could detect the 23S rRNA mature form but not this fragment. This allowed us to roughly identify the 23S rRNA region between 1289-1641nt (see scheme on Figure 13A) that is shown to originate the rRNA fragment whose degradation is dependent on both RNase R and Hfq. The ~300nt fragment arises from the central region of the 23S rRNA and is likely to result from endonucleolytic processing. This is in agreement with the current model of RNA degradation in which endoribonucleolytic cleavages can initiate the RNA degradation providing the substrates for exoribonucleases [7,18].

Accumulation of this fragment was always observed in RNA extracted from stationary phase cultures. In order to elucidate if this could be a growth phase-dependent event or a more generalized phenomenon we also tested the exponential phase (Figure 13B, lower panels). Using the same three oligonucleotides results were similar to the ones obtained with stationary phase cultures. Again, we could only detect the ~300nt fragment when using the 23S probe A. Accordingly, the mechanism behind the RNA degradation pathway of this 23S rRNA aberrant fragment, that is dependent on RNase R and Hfq, seems to be independent of growth phase.

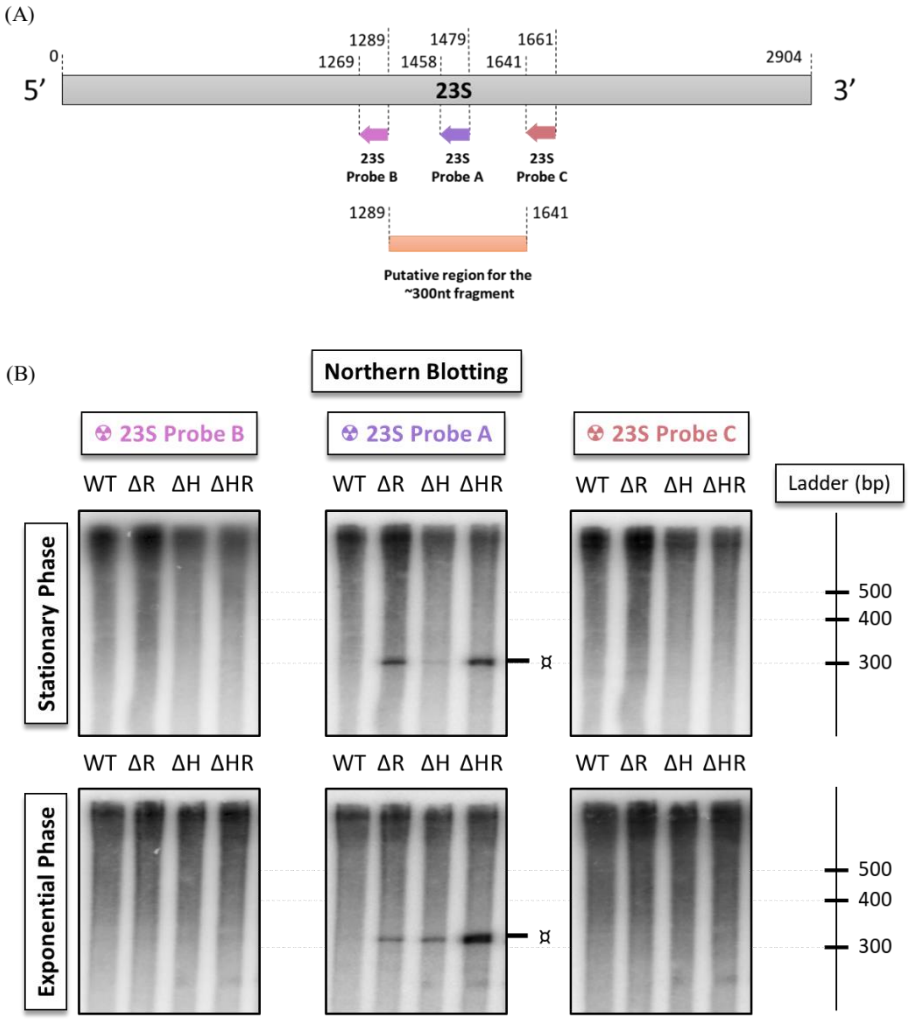

Figure 13 – **(A) Schematization of designed 23S probes and their complementary relative localization.** Arrows indicate the designed probes. 23S Probe A is the same 23S probe used in previous northern blottings. A putative region for the ~300nt 23S rRNA fragment. **(B) Northern blot analysis of the ~300nt 23S rRNA fragment and its mapping.** The indicated strains were both grown into exponential (OD600≈0.5) and stationary phase. Total RNA was isolated and loaded onto an 8% polyacrylamide/urea gel. Stationary phase analysis (top panels) and exponential phase analysis (bottom panels) are presented. In the center there is the 23S/23S probe A image. After membrane stripping, the 23S probe B was used and results are presented on the left. The 23S probe C hybridization results are presented on the right. The sign (¤) refers to the rRNA fragment.

3.5 RNase R and PNPase are involved in the maturation of the 5S rRNA

In *E. coli*, the rRNAs are co-transcribed as a single precursor molecule that is converted to the three mature RNAs (23S, 16S, and 5S RNAs) in a multistep process [50]. In the results above it was shown that RNase R and Hfq are critical for the removal of defective 16S and 23S ribosomal RNA. We next analyzed if similar defects could be found with the 5S RNA.

Total RNA extracted from the ΔHR strain was tested alongside with the WT and single mutants as controls (Figure 14). Stationary phase and exponential phase cultures were compared. While no defects were clearly evident in stationary phase cells, we found a different scenario when analyzing fast-growing cells. In the ΔHR mutant we could observe a smear above the mature 5S rRNA. Depending on the contrast applied to the image, a sharp band could even be detected. This was not observed in the single mutants or the wild-type strain. Curiously, this seems to be a growth-phase specific event, only observed in exponentially growing cells. A similar and even more pronounced result was obtained with the Δ*pnp* mutant, this way implying PNPase in the processing of the 5S rRNA. The Δ*hfq* Δ*pnp* mutant showed a stronger smear with longer 5S rRNA molecules being accumulated. This provided the first indication that PNPase and Hfq together can be involved in the degradation of rRNA.

High molecular weight smearing represents extra nucleotides that elongate the the 3'-end of the 5S rRNA. This nucleotides are preferably degraded by PNPase and RNase R with the assistance of Hfq and are not efficiently trimmed by any of the remaining exoribonucleases in the cell. Similar defects have been reported with an RNase T exoribonuclease mutant [56]. RNase T also processes other rRNA substrates that are not well degraded by any other exoribonucleases. Overall and for the first time, Hfq along with RNase R and PNPase are implicated in the correct maturation of the 5S rRNA.

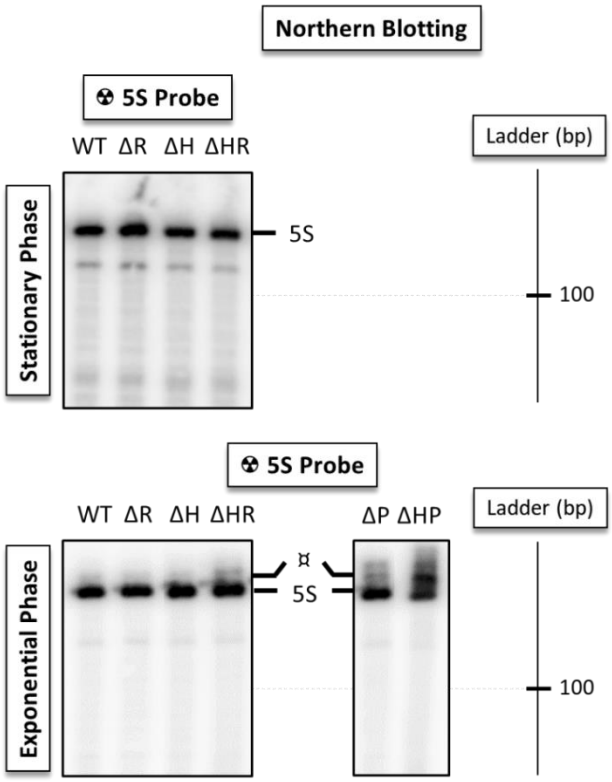

Figure 14 – **Northern blot analysis of the 5S rRNA.** Total RNA from the indicated strains was loaded onto an 8% polyacrylamide/urea gel. Stationary phase analysis (top panels) and exponential phase analysis (bottom panels) are presented. A 5S probe was used to assess the state of this rRNA. The sign (¤) indicates rRNA fragment accumulation of larger sizes than the 5S rRNA.

3.6 RNase R and Hfq participate in the maturation of 17S and pre-23S

The primary transcript of rRNA precursor needs to be processed in order to generate functional matured rRNA molecules. Incorrect processing result in aberrant rRNA molecules that may create a hazardous imbalance to the cells ribosomal population [55]. RNase R and Hfq were shown to be important for rRNA quality control removing defective rRNA fragments that strongly accumulate in the Δ*hfq* Δ*rnr* mutant. We envisaged that these proteins could also affect the correct processing of the precursor rRNA species.

We first checked for defects in the 16S rRNA precursor. This is termed 17S rRNA and has an extra 155nt at the 5'-end and 33nt at the 3'-end (Figure 15A). Total RNA samples from the ΔHR strain were loaded onto a 4% polyacrylamide/urea gel and ran overnight at lower voltages in order to achieve good separation and resolution of the close-migrating 17S and 16S rRNAs. WT and single mutant strains were also loaded for control purposes. The gel was then incubated with EtBr and visualized under UV light. A

well-defined band just above the 16S rRNA could be detected (particularly in the ΔHR mutant). Northern blotting analysis using probes with complementarity to regions only present in immature 16S (17S probe) confirmed that this band corresponded to the 17S rRNA (Figure 15B). This precursor was detected in low levels in the wild-type and single mutant mutants but was greatly accumulated in the double ΔHR mutant (Figure 15B).

A similar approach was applied to the study of the 23S precursor. The pre-23S rRNA contains only an extra 7nt at the 5'-end and 9nt at the 3'-end of the mature 23S rRNA (Figure 15A). EtBr-staining of the gel did not identify major differences between the strains. However, Northern blotting with a specific probe for the pre-23S rRNA showed that this precursor is noticeably more abundant in the ΔHR mutant while no significant differences were found between the wild-type and both the single mutants.

Our results clearly showed that RNase R and Hfq are required for the correct processing of both precursors of the 16S rRNA and the 23S rRNA. In cells not expressing Hfq and RNase R, the levels of these precursors are strongly accumulated which implies that less matured rRNA molecules are available. This may disturb correct ribosome biogenesis, resulting in a defective ribosomal population with lethal consequences to the cell.

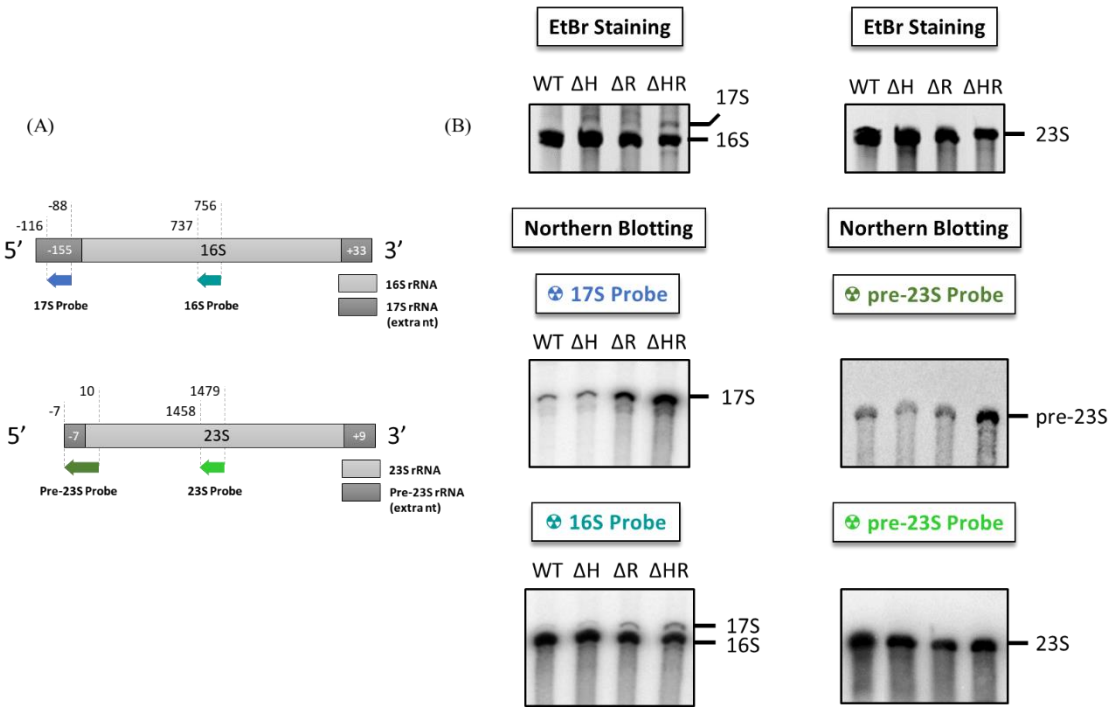

Figure 15 – **(A) Schematic of the hybridization regions of the probes with its targets.** Probes used for this analysis are represented with colored arrows, with their corresponding complementarity to rRNA targets. **(B) Northern blot analysis of the 17S rRNA.** Total RNA from the indicated samples was loaded onto an 8% polyacrylamide/urea gel and blotted. Ribosomal RNA species are indicated.

3.7 Analysis of the ΔHR mutant by Ultracentrifugation ribosomal profiles

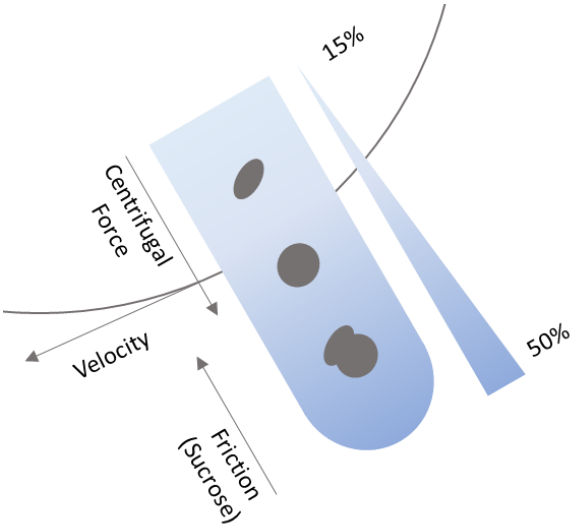

Figure 16 – Representation of ultracentrifugation principle. Schematization of the forces in action during an ultracentrifugal run. Velocity is represented to indicate that a high gravitational field is generated.

Results so far have demonstrated that RNase R and Hfq mediate a previously unknown quality control process that normally removes defective rRNAs. Accumulation of these aberrant rRNA fragments can affect the normal metabolism of the cell and ultimately be lethal to the cell. Interestingly, the newly generated Δ*hfq* Δ*rnr* mutant strain exhibited severe growth defects. Ribosomal RNA associates with ribosomal proteins (r-proteins) to form ribonucleoproteins (RNP) that make the ribosome. The bacterial ribosome consists of two different subunits, a larger 50S subunit and the smaller 30S subunit. Together they form the functional 70S ribosomal complex. The 30S subunit is formed by the 16S rRNA associated with 21 r-proteins (S1-S21) whereas the 50S subunit is formed by the 23S and 5S rRNAs associated with 33 r-proteins (L1-L36). Taken the defects found on rRNA, our experiments suggest that RNase R and Hfq could also affect ribosomal biogenesis.

An overlook of the cellular ribosomal population can be accomplished by isolating and profiling the ribosomes. The most common technique used to trace a ribosomal profile dates back to the 60's and it consists in separating the 30S and the 50S subunits, as well as the 70S particle, through a sucrose density gradient ultracentrifugation [14]. The sucrose gradient starts from the lowest concentration to the highest and it provides a gradual friction increment as the centrifugal force pushes the sample downwards (Figure 16). The smaller particles (like the 30S subunit) sediment at lower densities because less friction is required to balance the opposite forces, whereas larger particles (like the 70S ribosome) sediment at higher densities. However this is only achieved by ultracentrifugation techniques that subject the sample to a high gravitational field. This method was used for both ribosome isolation from cell extracts and for ribosome profiling.

3.7.1 RNase R and Hfq localize in free ribosomal subunit associated fractions

First, wild-type ribosomes isolated under associative conditions (that promote the isolation of the 70S particle) were obtained by standardized methods [15,24]. Ribosome extracts were then subject to sucrose gradient centrifugation to trace a ribosome profile of the WT strain. Upon ultracentrifugation, the gradients were collected from the top in 1ml fractions and 260nm absorbance was read on the Nanodrop spectrophotometer. The graphic observed in Figure 17 revealed a typical ribosomal profile under the conditions used, where the 30S and 50S subunits are present in much lower amounts than the 70S ribosome.

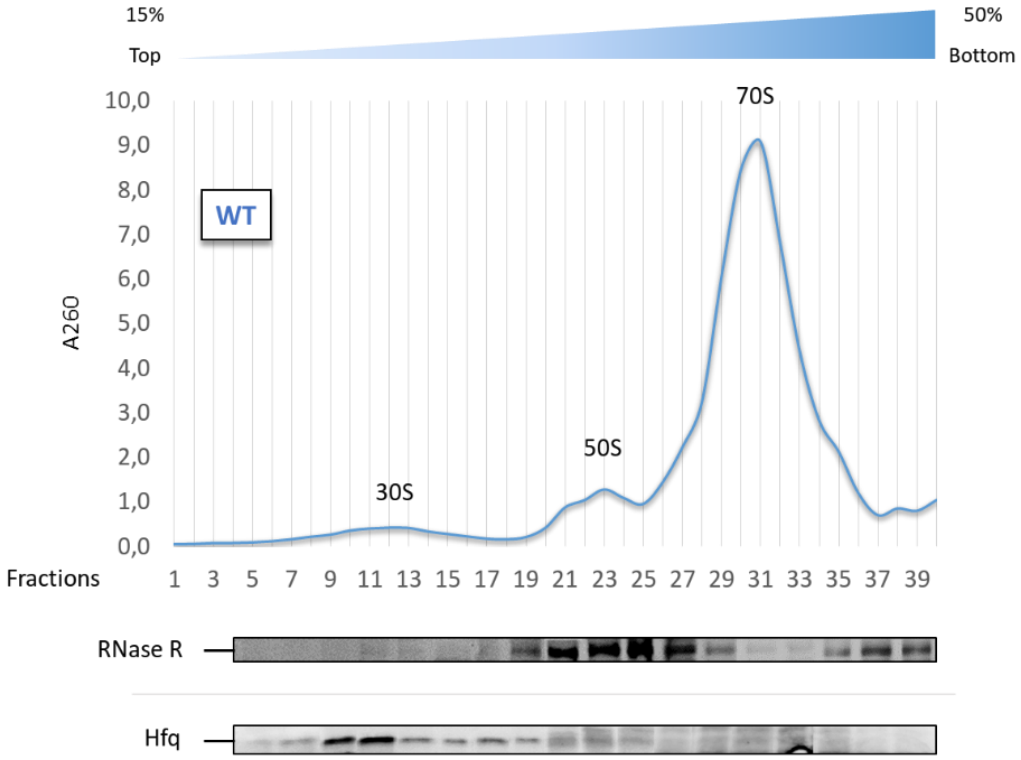

Figure 17 – **Ribosomal profiling of the WT strain and co-localization of both RNase R and Hfq with the ribossomal subunits.** 2mg of ribosomal units were loaded onto a 15-50% sucrose gradient (represented on the top) and subjected to ultracentrifugation. 1ml fractions were made (xx axis) and absorbance readings at 260nm (yy axis) were made and plotted as shown. The absorbance peak of each subunit and of the ribosomal particle are tagged. After protein precipitation of each fractions, 5µg of protein samples were loaded onto a 10% SDS-polyacrylamide gel for RNase R visualization, and onto a 15% SDS-polyacrylamide gel for Hfq visualization. Western blot analysis was carried out on both gels (on the bottom).

To check if RNase R and Hfq could be associated with the ribosome or ribosomal subunits, each of the 1ml fractions was TCA precipitated and proteins were checked by Western blotting. Both proteins were found to associate with the free subunits, but not with the 70S ribosome. RNase R was strongly abundant in the 50S fractions but also

present at lower levels in the 30S fractions. Conversely, Hfq was more abundant in fractions preceding and in the 30S fractions, although it could also be detected at lower levels in the 50S subunit. Results show that RNase R and Hfq co-localize in the free subunits from ribosome isolated pellets.

3.7.2 Ribosomal profile of the ΔHR mutant: accumulation of free 30S and 50S ribosomal subunits

We next analyzed the ribosomal profile of the ΔHR mutant, which had the most severe defects in ribosomal RNA. Control profiles were also traced using ribosomal extracts from the wild-type and single mutant strains. 15-50% sucrose gradients were used to separate the ribosomal forms and the A260 readings were plotted (Figure 18). Under these conditions no significant differences were observed between the WT and Δ*rnr* mutant profiles. However, the single absence of the Hfq proteins leads to a great reduction in the proportion of 70S particles with the concomitant accumulation of free ribosomal subunits, namely the 50S subunits. Remarkably, the combined absence of RNase R and

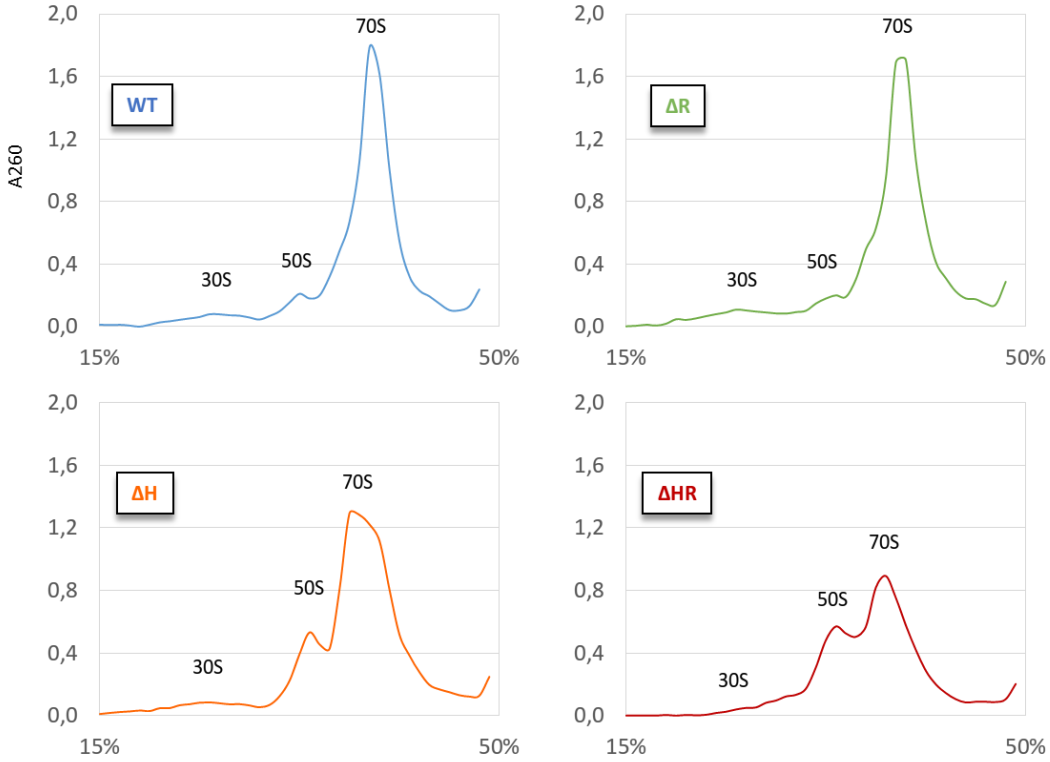

Figure 18 – **Ribosomal profile comparison of the WT, ΔR, ΔH and ΔHR strains.** After ribosome extraction,, 400µg of ribosomal units were loaded onto 15-50% sucrose gradients and submited to ultracentrifugation. Absorbance at 260nm (yy axis) was read and plotted against the corresponded relative percentage of sucrose (xx axis). Peaks are tagged with the ribosomal molecule form that they represent.

Hfq leads to an even more pronounced reduction of the functional 70S ribosome, and a subsequent greater accumulation of the 50S subunit (Figure 18). The lack of both RNase R and Hfq interfere with the ribosome biogenesis process, resulting in a strong reduction of functional 70S ribosome. The simultaneous increase found in the free 50S subunit population suggest that ribosomal assembly of the 70S may be defective. Our results indicate that the rRNA quality control errors detected in the Δ*hfq* Δ*rnr* double mutant the can actually affect ribosomal biogenesis.

A question remained, if the rRNA fragments were being incorporated into ribosomal subunits. To answer this, RNA extraction from the 70S peak fractions was carried out on the ΔR and ΔHR strains (Figure 19). Equal amounts of RNA from each fraction were loaded then on an agarose gel stained with Ethidium Bromide. The relative ratio of 16S and 23S rRNAs was normal and no significant fragment accumulation could be detected on the ΔHR mutant. Results suggest that the aberrant rRNA are not being associated into the 70S ribosome.

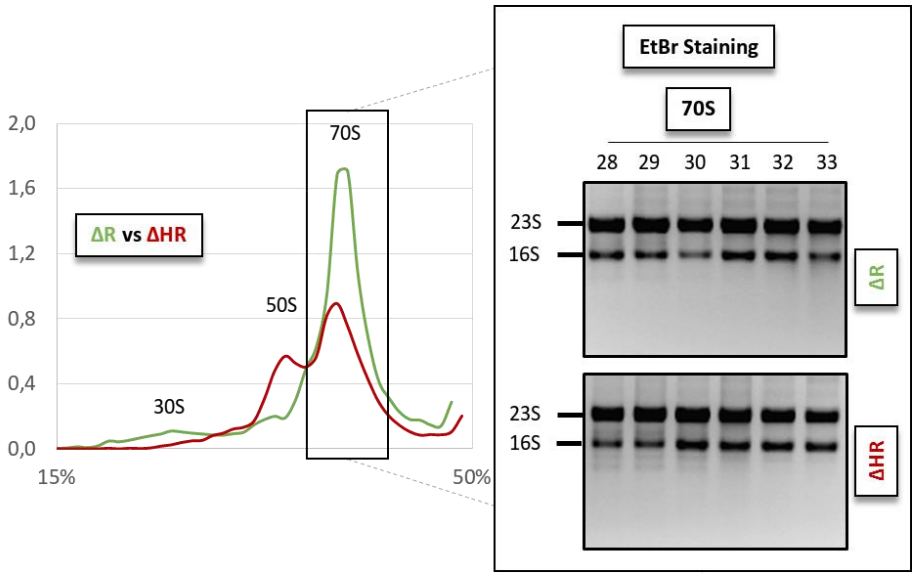

Figure 19 – **Comparative analysis of the ΔR and ΔHR strains ribosomal profiles and rRNA.** Ribosomal profiles (on the left) of each strain correspond to the ones shown above, but are here overlapped to evidence the great effect that the lack of the Hfq protein has on the ribosomal population of the cell. 1ml fractions that contained the 70S ribosomal particle peak (28-33) were treated to isolate RNA and equal volume of each fraction was loaded onto a 1.2% agarose/EtBr gel (on the right). The rRNA molecules are identified.

3.7.3 Dissociative ribosome analysis profile and RNase R localization

Sucrose gradients specifically aimed to favor the dissociation of the 70S particle were used to increase resolution of the free ribosomal subunits. Ribosome dissociative conditions are achieved by lowering the concentration of the Magnesium ion (Mg^{2+}) in the sucrose gradient buffer, which is essential to maintain both subunits associated [82,112]. Mg^{2+} ions are used at 10mM when isolating ribosome particles under associative conditions [77]. In dissociative conditions, only 0.1mM of $MgCl_2$ salt is used to promote subunit dissociation (Figure 20). As expected, WT ribosomes analyzed under this conditions revealed no 70S peak while good resolution of the free 30S and 50S subunits was obtained. As RNase R was observed to co-localize with the free ribosomal subunits (Figure 17) we decided to imply the same methodology to analyze RNase R localization in dissociative ribosomes. Protein precipitation was carried out on these fractions and Western blot analysis followed using the α-RNase R antibody. With a better resolution of the ribosomal subunits we were confirmed that RNase R protein was localized with the 30S and more strongly with the 50S associated fractions.

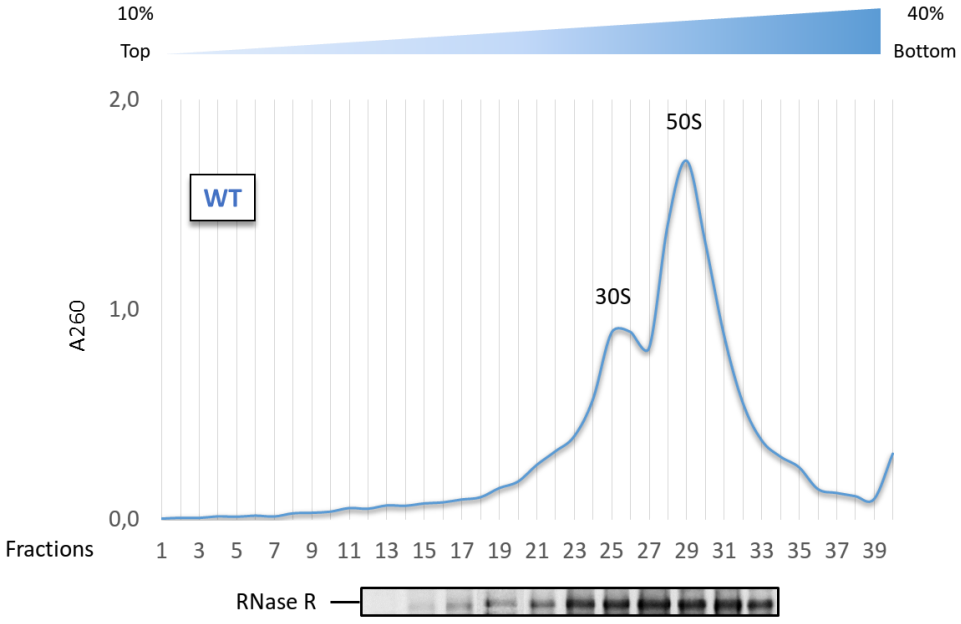

Figure 20 – **Dissociative ribosomal profiling of the WT strain and co-localization of the RNase R protein with the ribosomal subunits.** After ribosome extraction, 400µg were loaded onto a 10-40% sucrose gradient (represented on the top) and ultracentrifuged. Absorbance readings at 260nm (yy axis) were taken and plotted against the corresponded fractions (xx axis). After protein precipitation of fractionated gradient, equal volume of each sample was loaded onto a 10% SDS-polyacrylamide gel and western blot analysis was carried out for RNase R visualization.

3.7.4 The ΔHR mutant assembles less ribosomal subunits

The protocol that promotes dissociation of the 70S ribosome into free subunits was fully optimized with the wild-type strain, we then proceeded to similar studies with the ΔH and ΔHR strains (Figure 21). When compared to WT, the ΔH strain revealed a different ribosomal subunit profiling with lower peaks corresponding to the 30S and 50S. This is in agreement with what we previously observed once ΔH showed a marked reduction in the 70S peak when compared to wild-type (Figure 18). Interestingly, the ΔHR mutant shows even a greater reduction in the 30S and 50S free populations. The lack of RNase R when combined with the absence of Hfq leads to severer defects in ribosome biogenesis. This fact points to deficiencies in the assembly process of the ribosomal subunits, as both RNase R and Hfq seem to arise as important factors in ribosomal biogenesis.

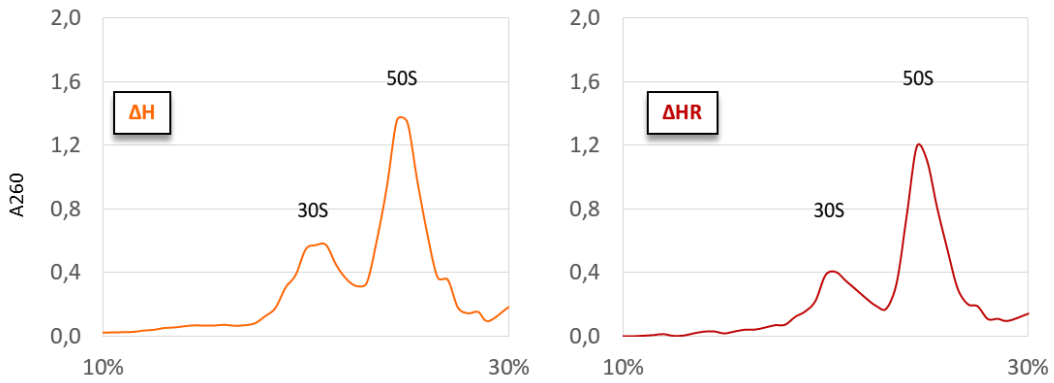

Figure 21 – **Dissociative ribosomal profile comparison between ΔH and ΔHR strain.** 400µg of ribosomal units were treated with low salt concentrations (0.1mM $MgCl_2$) for dissociation of the 70S particle and then loaded onto a 10-30% sucrose gradient. The gradient was ultracentrifuged, fracctionated and measurements of absorbance at 260nm were taken. Absorbance values (yy axis) were plotted against their relative sucrose percentage (xx axis). Peaks are tagged for the corresponded free subunit.

3.8 RNase R and Hfq direct protein-protein interaction

Our results showed that RNase R and Hfq are critical for rRNA quality control and ribosome biogenesis. Hfq is widely known for its function as an RNA-binding protein but there are an increasing number of reports on its protein-binding ability. Namely, Hfq was found to associate with other RNA-binding proteins, such as PNPase or RNase E [70,71]. This prompted us to further investigate the interesting hypothesis that Hfq and RNase R could also interact.

We used Far Western blotting in our initial analysis to test for the possible interaction between Hfq and RNase R. Each of the proteins were expressed by the pET system and carry a His6-tag at the N-terminus. Purified His6-RNase R was used as bait and increasing amounts were loaded on a 10% SDS-PAGE. BSA protein was used as negative control. Proteins were transferred to a nitrocellulose membrane and renatured *in situ* using Guanidine-HCl. Purified His6-Hfq was then used as prey and incubated with the membrane. The subsequent steps followed a typical Western blotting analysis and Hfq antibody was used to detect His6-Hfq bound to the nitrocellulose membrane (Figure 22A). Remarkably, a prominent band which matches the position of RNase R (as determined on the Ponceau red staining of the membrane) was clearly identified when using the α-Hfq. Furthermore, the signal became stronger as increasing amounts of RNase R were loaded on the gel. Hfq could even be detected when very low amounts of RNase R (10ng) were loaded, thereby suggesting a strong interaction between these two proteins.

At this point, we wanted to test if Hfq and RNase R were binding directly to each other or if this interaction was mediated by a third partner. Both Hfq and RNase R are RNA-binding proteins and RNA molecules are known effectors of protein-protein interactions [39]. To analyze if RNA could in fact be responsible for this association, purified His6-RNase was treated with Micrococcal Nuclease (MNase), a non-specific endo-exonuclease. This was compared to an RNase R sample not digested with MNase (Figure 22B). Following the Far-western protocol (in which the prey His6-Hfq was also treated with MNase) we observed that α-Hfq could detect Hfq bound to RNase R, regardless of MNase treatment. This strongly suggests that Hfq and RNase R can physically interact directly and do not require an RNA molecule as a third partner.

In order to validate this interaction, we also tested the Hfq-RNase R association through a protein pulldown assay (Figure 22C). Total protein extracts from the WT (hfq^+) or the ΔH (hfq^-) strains were incubated with purified His6-RNase R bound to Ni-NTA beads. Beads incubated with binding buffer without any cell lysate were used as blank control. His-RNase R and its interacting factors were eluted from the beads with imidazole and loaded on a SDS-PAGE. The levels of His6-RNase R were checked by Western blotting to assure correct binding to Ni-NTA beads. Hfq was also detected by Western blotting and was only visible when the WT protein extract was used. As expected, no Hfq

was detected within the ΔH protein extract. Using His6-RNase R as bait we could successfully pulldown native Hfq from cell lysates. This confirmed that Hfq and RNase R indeed interact.

We then decided to carry out immunoprecipitation experiments as an alternative approach to confirm if Hfq-RNase R interaction could in fact be detected *in vivo*. RNase R polyclonal antibodies were bound to Protein A/G beads – which bind to the Fc region of IgG antibodies – and then used to pulldown RNase R and associated proteins from different cell lysates. The immunopurified complexes were eluted with sample loading buffer, separated by SDS-PAGE and the presence of Hfq was analysed by Western blotting. As observed in Figure 22D, Hfq was immunoprecipiated with RNase R being detected when wild-type lysate was used but not in the ΔH sample. Furthermore, increasing the amount of Hfq in the cell (with a plasmid overexpressing Hfq) greatly increased the

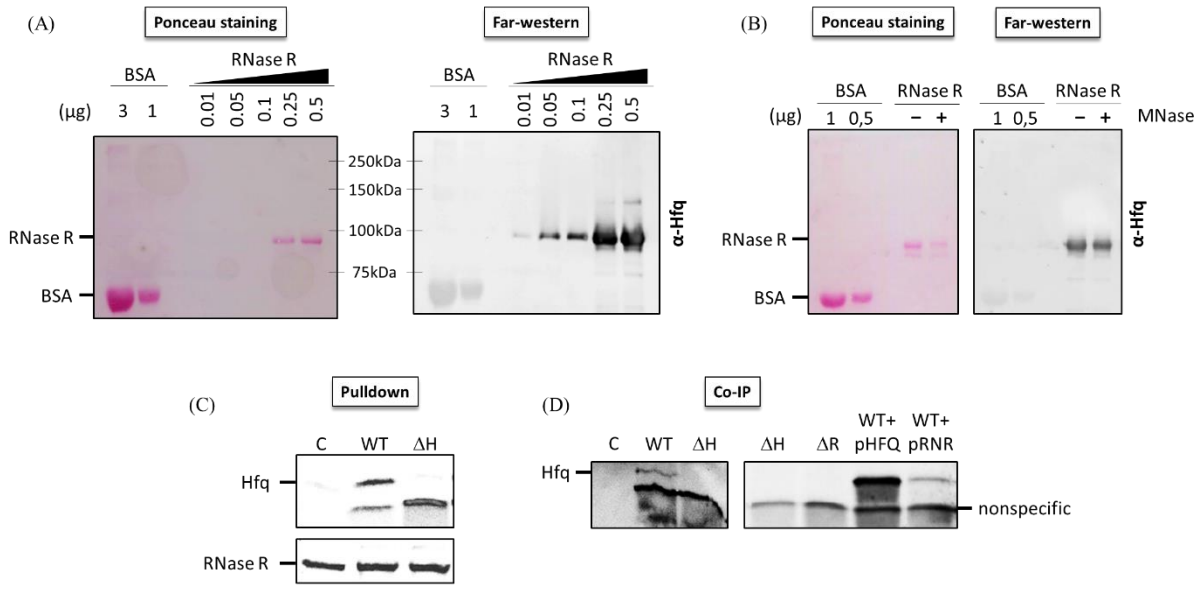

Figure 22 – **(A) Far-western analysis of RNaseR-Hfq interaction**. 3µg and 1µg of BSA protein was loaded for negative control purposes. Growing amounts of the bait RNase R (0.01µg, 0.05µg, 0.1µg, 0.25µg and 0.5µg) were used to check interaction. 5µg of the purified prey protein were used to incubate the membrane. On the left side the pounceu stained gel is presented as a control before renaturing and to check the relative quantity of loaded proteins. On the right, the image obteined through with the use of α-Hfq antibody. **(B) Far-western analysis of direct interaction**. 1µg and 0.5µg of BSA were again used as negative control. 0.25µg of the bait protein RNase R were used. The – sign indicates RNase R not treated with MNase and the + sign indicates MNase treated RNase R. 5µg of the MNase treated prey Hfq were used. Again, on the left, tha ponceu stained gel is presented, and on the right, the image obteneid using α-Hfq antibody. **(C) Hfq pulldown through RNase R interaction**. Sample from beads/cell lysate incubation was loaded as negative control. The WT cell lysate was incubated with beads bound to RNase R to check interaction, and the ΔH cell lysate was incubated with beads bound to RNase R for control purposes. Both samples were then loaded. The top image refers to the membrane checked with α-Hfq antibody, as for the bottom image it refers to the same membrane checked for the presence of the bait RNase R with α-RNase R antibody, after stripping. **(D) Co-immunoprecipitation of RNaseR-Hfq complex using α-RNase R antibody**. Two different gels were made. On the left, free beads were incubated with the cell lysate providing a negative control. Then the WT and ΔH cell lysate was incubated with the beads bound to α-RNase R antibody and loaded. Samples were then loaded, blotted and checked with α-Hfq antibody. The gel on the right is similar, but with cell lysates from other strains, namely the ΔR, WT+pHfq and WT+pRNR, also checked with α-Hfq antibody.

amount of Hfq found to co-immunoprecipitate with RNase R. In the same line of thought, increasing the amount of RNase R in the cell extracts (with a plasmid overexpressing RNase R) also resulted in higher levels of immunoprecipitated Hfq. As expected, no signal was detected when using cell extracts in which Hfq or RNase R were deleted. This set of results support that Hfq and RNase R can form protein complexes *in vivo*.

4

Discussion and Conclusions

4 Discussion and conclusions

In this work we have demonstrated that both RNase R and Hfq are necessary for correct rRNA quality control. Taken together our results also indicate that both RNase R and Hfq are crucial for ribosome biogenesis. Ribosomal profiles demonstrated that in cells lacking both RNase R and Hfq the pool of 70S ribosomal particles was greatly reduced. Furthermore, we have found that the ΔHR mutant also exhibits an increased number of free subunits. Ribosome biogenesis factors – such as proteins that facilitate the assembly of the ribosome [55,66] or even RNases involved in the rRNA maturation [29,96] – are known to alter the ribosomal profile, yielding less associated 70S ribosomes and more free subunits. This pattern reveals defects in the ribosomal biogenesis process [88,97]. Moreover, our results revealed a co-localization of RNase R and Hfq in gradient fractions associated to the 30S subunit. This is in line with recently published work that independently identified RNase R and Hfq as possible interaction partners of the S12 ribosomal protein, one of the r-proteins that forms the 30S subunit [95]. Nevertheless, RNase R and Hfq are proteins known to localize in the cytoplasm. [7,103].

The drastic effect observed in the ribosomal population of the ΔH strain when compared to the wild-type may indicate an important role for Hfq as a ribosome assembly factor. RNA chaperones have already been hypothesized to facilitate ribosome assembly [50]. A model where RNA chaperones would rescue kinetically trapped rRNAs from misfolding events was put forward to elucidate a possible role for these proteins in the assembly process [108]. Hfq is widely known as an RNA chaperone capable of not only of binding RNA molecules but also of modifying their secondary structures [85]. Its great importance for the post-transcriptional regulation mechanism of small RNAs comes precisely from Hfq ability to facilitate the base-pairing of two RNA molecules with partial complementarity [103]. Our observations of the affected ribosomal profiles of cells lacking Hfq suggest that this protein could in fact be a ribosome assembly factor. Its ability to rearrange complex secondary RNA structures can easily fit the proposed model, thereby facilitating rRNA folding. This would nicely expand the role of known functions of Hfq in the cell. However, further investigation is needed in order to confirm this hypothesis.

The presence of well-defined lower size bands in the agarose gels performed to check the integrity of the RNA of the double mutant prompted us to investigate their possible origin. Through Northern blotting analysis we have demonstrated that in the Δ*hfq* Δ*rnr* double mutant, intermediates of 16S and 23S rRNA degradation greatly accumulate. Our results confirm that both proteins are needed for the correct removal of these fragments. Their accumulation could be potentially lethal to the cell [24]. Although RNase R role in rRNA quality control was a well-known fact [5,7,102], the implication of Hfq in this degradation pathways had not yet been reported.

RNase R is one of the three major processive 3'-5' exoribonucleases present in *E. coli* [63], along with RNase II and PNPase. Although its unique features make it suitable for the degradation of highly structured RNA molecules – as ribosomal RNAs – a conjugated action of RNase R and PNPase in the rRNA quality control was reported [24], whereas RNase R and RNase II were implicated in rRNA fragment removal during cell starvation [5,13]. The cooperation between these three major exoribonucleases was tested to see if either PNPase or RNase II were also necessary for the removal of the identified rRNA fragments. As it was point out, none of the additional strains lacking PNPase or RNase II accumulate specific 16S and 23S rRNA fragments, even with simultaneous absence of Hfq. Focusing on the 23S-originating fragment (~300nt), accumulation was visible in the ΔR strain, although less strongly than in the ΔHR. This degradation seems to be exclusively carried out by RNase R, since none the *E. coli* RNases were able to remove it. Although overlapping roles have been described for both RNase R and PNPase in rRNA degradation [24] our results indicate that PNPase cannot substitute RNase R in fragment removal when Hfq is not present. Furthermore, the single lack of Hfq also lead to a minor accumulation of the ~300nt fragment, reinforcing the idea that both proteins are specifically necessary for the correct degradation of rRNA. Finally we could conclude that when Hfq was absent, neither RNase II nor PNPase were sufficient for the elimination of these rRNA fragments, which indicates a specific action performed by RNase R.

The synthesis and assembly of ribosomal particles comes with a great expense to the cellular resources [108]. However, when cells find themselves in conditions that reduce rapid growth – such as entering in stationary growth phase – ribosomes become less and less necessary, and so they may be targeted for degradation in order to recycle its constituents [74]. Nutrient depletion lowers the *E. coli* cells' translational activity, and

the mounting number of idle ribosomal subunits triggers ribosome degradation by the existing ribonucleases [113]. A model for stable ribosomal degradation was proposed by Kaplan and Apirion which suggests that endoribonucleolytic rRNA cleavages start off the process that is further concluded by exoribonucleases [51]. RNase R (along with PNPase) is part of this process that removes the rRNA fragments originated by the initial endoribonucleolytic cleavages. Our rough mapping of the 23S-originate fragment (1289-1641nt of the 23S rRNA) suggests that this may arise from endonucleolytic activity. Some endoribonucleases can be put forward as responsible for these primary cuts on the rRNA, namely RNase III, YbeY and RNase E, which are also known to act on rRNA maturation [29,50]. RNase E was also shown to act (although not exclusively) on the ribosomal subunits during starvation [13]. There may be more than one specific endoribonuclease mediating these endoribonucleolytic cleavages. However, a strong genetic relation was observed between the *rnr* and the *ybey* genes, as cells with both genes disrupted were shown to accumulate not only 16S precursor RNAs but also 16S degradation products [44]. RNase R is strongly induced in stationary phase [2] and *trans*-encoded sRNA levels are also elevated during this growth phase [91]. Given the role of RNase R and Hfq our previous results tested the accumulation of fragments specifically during stationary phase. This prompted us to investigate if this accumulation in the ΔHR strain was a growth stage-specific event. Our results from exponential phase analysis revealed that the 23S-derived fragment was also strongly accumulated in this mutant, revealing that RNase R-Hfq mediated rRNA degradation pathway is independent of the growth phase of the cells.

The activity of both RNase R and Hfq towards ribosomal RNA is not limited quality control degradation. We have also observed during this work that both RNase R and Hfq are needed for the correct maturation of the 16S and 23S rRNA precursors. The role of RNase R (combined with PNPase, RNase II and RNase PH) in the maturation of the 16S rRNA precursor (17S rRNA) had already been reported [96]. However, it seems that in cells where both Hfq and RNase R were absent, none of the other 3'-5' exoribonucleases present were able to carry on with maturation of this precursor. On the other hand, new information comes from the fact that also the pre-23S precursor accumulates in the Δ*hfq* Δ*rnr* double mutant, which for the first time indicates that both RNase R and Hfq are needed for the maturation process of the 23S rRNA. It would be interesting in the future to ascertain if the lack of Hfq combined with the absence of any of these processive

3’-5’ exoribonucleases would also lead to accumulation of precursor rRNAs, or if it is a specific feature of RNase R. Defects in rRNA maturation are severely harmful to cell [88]. Immature ribosomal RNAs are not functional and if this forms persist without being maturated or degraded, their accumulation creates stress to the bacterial cell. This intermediate molecules sustain binding sites for both assembly factors and ribosomal proteins. Therefore immature molecules would probably compete with the matured ones for these essential proteins in the formation of the ribosomal subunits.

Studies on the 5S rRNA were also conducted since precise 23S/5S interactions are crucial for subunit formation [54], and consequently, correct maturation of the rRNAs are necessary. We have observed that an exponential phase-dependent accumulation of 5S precursor forms arise when both Hfq and RNase R are absent. The presence of higher weight smear above the 5S rRNA band was noticeable, which would probably relate to immature forms of this molecule. This effect is even more visible both in the single Δ*pnp* mutant and in the double Δ*hfq* Δ*pnp* mutant. A similar accumulation pattern was already reported in an RNase T mutant, where extra nucleotides accumulate in the 3’-end of the 5S rRNA due to trimming defects of precursor molecules. Another interesting hypothesis, is that the longer precursor forms would arise from post-transcriptional polyadenylation. RNA polyadenylation occurs essentially during exponential phase of growth [4]. In the future it would be interesting to determine if whether or not the 5S rRNA is polyadenylated by PAP I in this context. Nonetheless, this is the first time that PNPase and RNase R, along with Hfq, are implicated in the maturation of 5S rRNA. The defects in rRNA maturation observed could possibly link to the slow-growth phenotype of the ΔHR mutant. In exponential growth is when it is more noticeable that these strains grow slower than the wild-type. This is the period when there are more ribosomes and cells are translationally more active.[50,55].

E. coli has about twenty ribonucleases which are part of the cellular RNA degradation machinery. Although a global mechanism is widely accepted every substrate is different. Therefore, there is no universal mechanism by which RNA is degraded [5]. Here we have shown that in Δ*hfq* Δ*rnr* mutants, immature rRNA forms and fragments from rRNA degradation accumulate, which may point to the necessary conjunctional activity of these two proteins to eliminate such fragments. This set of results implicate RNase R and Hfq in a previously unknown ribosomal RNA degradation pathway. Our

results support that RNase R and Hfq can directly interact. This is a new interaction that was not previously described and brings to light a new set of hypothesis for further investigation.

In the future it would be of the utmost importance to further characterize this interaction by knowing how and which domains of each proteins are implicated in the interaction. Furthermore, it would also be interesting to understand how that affects the function and activity of both proteins towards the shared substrates we identified through the course of this work. Ribosomal biogenesis is a crucial process for the bacterial cell that has evolved to higher precision and complexity levels. Due to its importance to the global functioning of the cell and its impact on cellular resources it is also target of tight regulation [27,108]. Our results allowed us to connect two well characterized proteins in newly cellular functions related to ribosome biogenesis. This comprises new information regarding the activity of RNase R and Hfq on ribosomal RNAs, in both their maturation and quality control. Hopefully future work will enlighten this newly discovered pathway and the close relation between the exoribonuclease RNase R and the RNA chaperone Hfq.

5

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5 References

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6

Appendix

6 Appendix

6.1 Strains and relevant genotype

Table 14 – **Strains used in this work.**

Strain	*Abbreviation*	*Genotype*	*Reference*
Wild-type (MG1693)	WT	*thy* $^-$	[9]
HM104	ΔR	*thy* $^-$ *rnr* $^-$ Kan^R	[2]
CMA1101	ΔH	*thy* $^-$ *hfq* $^-$ Cam^R	This work
CMA1102	ΔHR	*thy* $^-$ *hfq* $^-$ *rnr* $^-$ Kan^R Cam^R	This work
MG1693 + pRNR	WT+pRNR	*thy* $^-$ Amp^R	[2]
MG1693 + pHFQ	WT+pHFQ	*thy* $^-$ Amp^R	[4]
CMA201	ΔII	*thy* $^-$ *rnb* $^-$ Tet^R	[17]
SK5691	ΔP	*thy* $^-$ *pnp7*	[17]
CMA1103	ΔHII	*thy* $^-$ *hfq* $^-$ *rnb* $^-$ Cam^R Tet^R	This work
CMA1104	ΔHP	*thy* $^-$ *hfq* $^-$ *pnp7* Cam^R	This work

6.2 Nucleic acid sequences

Table 15 – **Nucleic acid sequences used in Northern blotting analysis.**

Probe	*Sequence (5' – 3')*
23S/23S Probe A	CCT ACA CGC TTA AAC CGG GAC
16S	GCG TCA GTC TTC GTC CAG GG
23S Probe B	GCT TTT CAC CCG CTT TAT CG
23S Probe C	CCC GAG TTC TCT CAA GCG CC
5S	CAT CGG CGC TAC GGC GTT TCA CTT C
17S	TTA AGA ATC CGT ATC TTC GAG TGC CCA CA
Pre-23S	CGC TTA ACC TCA CAA C