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Ribosome Degradation in Living Bacteria

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Supervisors: Ülo Maiväli, PhD and Tanel Tenson, PhD. Institute of Technology, University of Tartu, Estonia

Opponent: Tracy Nissan, PhD. University of Umea, Sweden

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LIST OF ORIGINAL PUBLICATIONS

The current dissertation is based on the following original publications, referred to by their numerals.

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II. Maiväli, Ü., **Paier, A.** and T. Tenson (2013). ''When stable RNA becomes unstable: The degradation of ribosomes in bacteria and beyond." Biol Chem. **394**(7):845–55.

III. **Paier, A**., Leppik, M., Soosaar, A., Tenson, T. and Ü. Maiväli (2015). "The effects of disruptions in ribosomal active sites and in intersubunit contacts on ribosomal degradation in Escherichia coli." Scientific Reports **5**: 7712.

IV. Mets, T., Lippus, M., Schryer, D., Liiv, A., Kasari, V., **Paier, A.**, Maiväli, Ü., Remme, J., Tenson, T. and N. Kaldalu (2017). "Toxins MazF and MqsR cleave Escherichia coli rRNA precursors at multiple sites." RNA Biol **14**(1):124–135.

Author's contributions: In paper I, I performed part of the DNA cloning and experimental work. In paper II, I participated in writing of the manuscript. In paper III, I performed most of the cloning and experimental work, and participated in the drafting of the manuscript. In paper IV, I participated in the analysis of ribosome profiles.

LIST OF ABBREVIATIONS

CAM	Chloramphenicol
CP	Central Protuberance
DC	Decoding Center
EF	Elongation Factor
FRET	Fluorescence Resonance Energy Transfer
GAC	GTPase-Associated Center
GTP	Guanosine Triphosphate
H69	Helix 69
IF	Initiation Factor
LSU	Large ribosomal Subunit
mRNA	messenger Ribonucleic Acid
NRD	Nonfunctional rRNA Decay
ORF	Open Reading Frame
Ψ	Pseudouridine
post PT QC	post-Peptidyl-Transfer-Quality-Control
PTC	Peptydil Transferase Center
RBD	RNA Binding Domain
RNase	Ribonuclease
ROS	Oxygen Reactive Species
RRF	Ribosome Recycling Factor
rRNA	ribosomal Ribonucleic Acid
S	Svedberg
SSU	Small ribosomal Subunit
TLD	tRNA-like domain
tmRNA	transfer-messenger Ribonucleic Acid
TOR	target of rapamycin
TRAMP	Trf4p/Air/Mtr4p
tRNA	transfer Ribonucleic Acid
Å	Angstrom

INTRODUCTION

Ribosomes are a major component of prokaryotic and eukaryotic cells. They produce the cell proteins, and are very expensive in term of 'building material' and energy. The traditional view is that ribosomes are stable. In the past, ribosomal degradation in bacteria has been studied in stress conditions like nutrients starvation, presence of damaging chemicals, or antibiotics treatment. The ribosome stability has, until recently, not been tested in bacteria actively growing in nutrient-rich media. One aim of my work is to shed light on this issue.

The contribution of this thesis to the understanding of ribosome degradation in bacteria is two-fold. First, by developing a test system that allows direct measurement of ribosomal stability in growing *E. coli* cultures, we could show that wild-type ribosomes are degraded during the slowing of growth that precedes entry into the stationary phase, presumably facilitating recycling of the cellular components no longer needed by the cell. Second, we showed that certain mutations that are known to have deleterious effects on the cellular phenotype induce degradation of the ribosomes. We also showed a role for two RNases, YbeY and RNase R, in the degradation of both WT and mutant ribosomes in growing cells.

REVIEW OF LITERATURE

1. Historical overview

Ribosomes are the universally conserved protein factories of all prokaryotic and eukaryotic cells, and are also their most abundant component. The very first scientific observations of ribosomes were made by the Frenchman, G. Garnier, in 1897–99. He called them 'ergastoplasms' (Siekevitz and Zamecnik 1981). In the early 1940s' Caspersson and Brachet noticed the correlation between the amount of RNA in various cells and their protein-synthesis capacity. G. Palade microscope studies led him to describe the ribosomes as particles either attached to the endoplasmic reticulum or free in the cytoplasm, and to postulate that the attached particles could account for the high RNA content of the reticulum. He defined them as 'small particulated components' (Palade 1955). The term ribosome was coined in 1958 by Dick Roberts. In the mid-1950s, after significant advancements in electron microscopy techniques, rRNA (ribosomal RNA) was recognized as a component of the ribosome. The role of ribosome as protein 'builders' was firmly established by 1960 (Hill et al. 1990). However, early studies on ribosomes were based on the assumption that rRNA carries the information for the synthesis of proteins. The discovery of mRNA disposed of this notion in the early 60s. Already a few years earlier Zamecnik and Hoagland had noticed *in vitro* that a certain fraction of RNA was able to transfer amino acids to microsomal protein *in vitro*. By 1953 Zameknink had succeeded in making the first cell-free system capable of carrying out net peptide bond formation using ^{14}C-amino acids. Using this system, Zamecnik and Hoagland noticed that the RNA in a particular cytoplasmic fraction became labeled with ^{14}C-amino acids and that the labeled RNA was subsequently able to transfer the amino acids to microsomal protein. The transfer was dependent upon guanosine triphosphate. They had discovered the transfer RNA (tRNA) (Hoagland et al. 1958, Kresge et al. 2005, Zamecnik 2005). By the late 60s the cyclic mechanism of subunit dissociation and re-association during ribosome function was established (Hill et al. 1990). Alexander Rich, known also for his pioneer studies on DNA crystal structures, advanced our understanding of translation with the discovery of polysomes, ribosome clusters that read a strand of mRNA simultaneously (Warner et al. 1963). In the same period, studies on ribosomal proteins and their chemical characteristics were carried on by Traut, Moore and Noller (Traut et al. 1967, Moore et al. 1968). Traub and Nomura succeeded in reconstituting ribosomes *in vitro*. The composition of the peptidyl transferase center was determined by Nierhaus and his collaborators in the early 80s (Nierhaus 1980, Schulze and Nierhaus 1982). Since the 1960s, ribosome footprinting analysis has become an established method to identify the position of active ribosomes bound to target mRNA, thanks to the pioneering work of Steitz and Kozak (Jackson and Standart 2015). Thermal neutron scattering, irradiation of free low energy neutrons, was introduced in the 70s by Engelman and Moore to study the three-dimensional structure of the ribosome (Moore et

al. 1975). Improved X-ray crystallographic techniques made possible to obtain high-resolution atomic structures of the prokaryotic large and small ribosomal subunits by 2000, when the structure of the large ribosomal subunit (from the archaeon *Haloarcula marismortui*) was revealed (Ban et al. 2000). The structure of the small ribosomal subunit was described soon after (Schluenzen et al. 2000). Advances in crystallography techniques made possible further progress in recent years (Moore and Steitz 2003, Sanbonmatsu 2012, Zhou et al. 2012, Jenner et al. 2012). In the past decades investigation of ribosomal structure has also been carried on by chemical and enzymatic probing (Vaughn et al. 1984, Green and Noller 1997, Lauber and Reilly 2011). Recently advances in electron cryo-microscopy have made it possible to study ribosomes in solution at near-atomic resolution (Bai et al. 2013). Atomic mutagenesis experiments on ribosomes are used to study protein biosynthesis (Erlacher et al. 2011). Recent advances in FRET allow studying the dynamic changes in single ribosomes in the range of 10–90 angstroms and a millisecond time-resolution (Wang and Xiao 2012, Wang et al. 2014).

2. Structure and function of the prokaryotic ribosome

The bacterial ribosome weights about 2.5 MDa and sediments as a 70S particle (S stands for Svedberg, a unit indicating the sedimentation velocity of a particle when subjected to a centrifugal force). It consists of a large and a small subunit (Figure 1). The large subunit is about 250 Å in diameter, with an approximately hemispheric shape, and it sediments as a 50S particle. It is composed of two rRNA molecules (23S and 5S) and of 33 L-proteins. Its structural landmarks are a central protuberance (CP) in the middle and two stalks (L1 and L7/L12) protruding at either side of the CP. Below the CP, at roughly the center of the intersubunit face is the peptdyl-transferase center (PTC). It comprises parts of the A and P tRNA binding sites and the LSU RNA nucleotides directly participating in the catalysis of making of the peptide bond. Also, during termination of protein synthesis, the PTC catalyzes the transfer of the peptide moiety to water (Amort et al. 2007) The exit tunnel starts close to the P site and leads to the opposite side of the 50S subunit. The GTPase-associated center (GAC) is situated at the base of the L7/L12 stalk. The A site is the point of entry for the aminoacyl tRNA but the first aminoacyl tRNA enters at the P site, where the peptidyl tRNA is formed in the ribosome. The E site is the exit site of the tRNA after it gives its amino acid to the growing peptide chain.

The small (30S) subunit is composed of 16S rRNA and 21 S-proteins (S1–S21). It has a more modular shape with a moveable head, which sports a beaklike structure over a shoulder. On the opposite side from the beak the head rests on a protruding platform, with a tapering body underneath, which ends in a spur. The A, P and E sites are located at the base of the head.

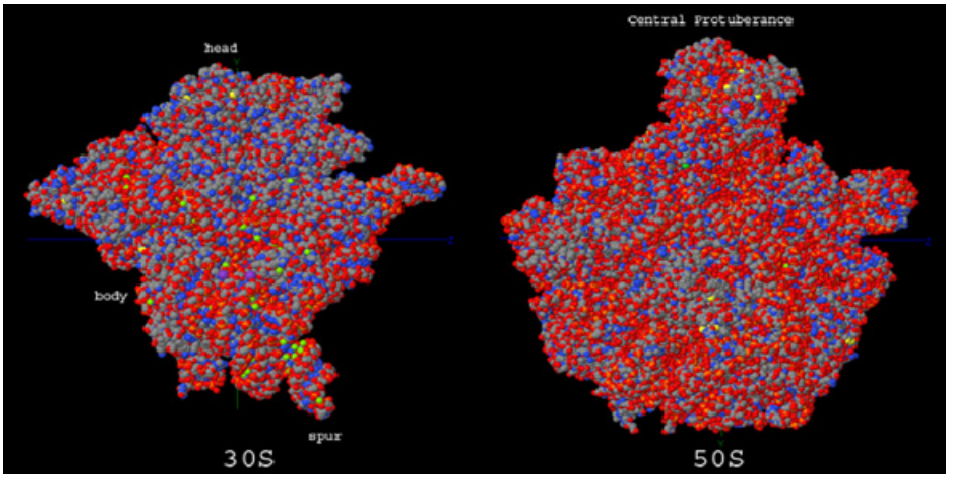

Fig 1. Structure of prokaryotic ribosomal subunits (Jmol 14.6, from RCSB PDB Protein Data Bank). The major landmarks of the subunits are labelled (head, body, spur, and the central protuberance) and atom identities are presented in color code.

The two ribosomal subunits accomplish various and different functions in protein synthesis (Bashan and Yonath 2008, Steitz 2008, Schmeing and Ramakrishan 2009). The 30S subunit associates with messenger RNA (mRNA) during translation initiation. In the decoding center (DC) of 30S interactions between codons in the mRNA and anticodons in transfer RNA (tRNA) determine which amino acid will be incorporated into the polypeptide chain. The PTC of the 50S subunit acts as a catalyst for two key reactions. It performs the peptide bond formation between the amino acid attached to the tRNA in the A-site (aminoacyl-tRNA) and the emerging peptide chain attached to the tRNA in P-site (peptidyl-tRNA) during translation elongation. And it's involved in the hydrolysis of nascent peptide from P-site tRNA during translation termination. The polypeptide exit tunnel that begins below the PTC provides the nascent peptides with a stable path through the 50S subunit.

Codons are three-nucleotide sequences in the mRNA that are matched to complementary sequences in the tRNA, the anticodon. Thus tRNA acts as the link between the mRNA transcripts and the amino acid sequences of the proteins translated in the ribosome. The first codon of an mRNA translated by the ribosome is called start codon, and it always codes for Methionine in eukaryotes or for a modified form, formyl-Methionin, in prokaryotes (Figure 2). The most common start codon is AUG, but there are alternate start codons: GUG, UUG and AUU (Blattner et al. 1997, Sacerdot et al. 1982).

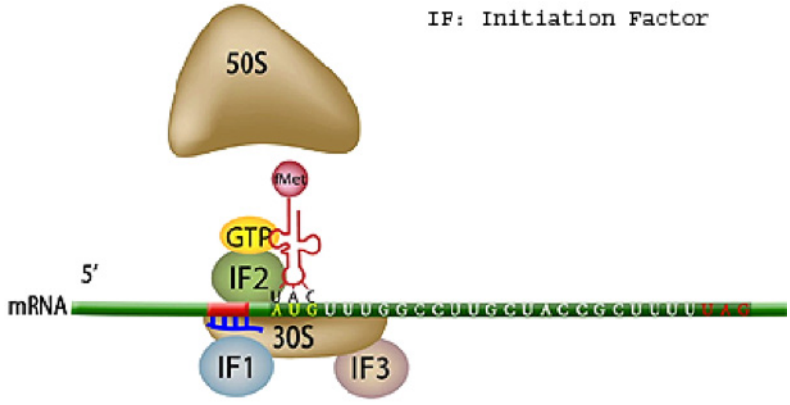

Fig 2. Prokaryotic translation initiation (Modified from Alila Medical Media). mRNA is in green, the ribosomal subunits are in grey, the Shine-Dalgarno sequence in the 16S rRNA is in blue, the fMet-tRNA is in red, and the GTP is in yellow. The initiation takes place in the AUG start codon (yellow), where the fMet-tRNA-GTP-IF2 ternary complex is bound to the ribosomal P site. The IF1 facilitates this process and the IF3 prevents any premature binding of the large subunit to the initiation complex.

The two subunits associate upon translation initiation, connecting through protruding structures called inter-subunit bridges (Yusupov et al. 2001, Maiväli and Remme 2004, Hennely et al. 2005, Schuwirth et al. 2005, Pulk et al. 2006, Kietrys et al. 2009). Some of the inter-subunit bridges are composed entirely of RNA, and they are mostly located in the central region of the subunit interface. The inter-subunit bridges that have protein components are mostly located peripherally (Yusupov et al. 2001, Schuwirth et al. 2005, Korostelev et al. 2006, Selmer et al. 2006). In prokaryotic ribosomes only one of the 13 bridges, B1b consists solely of protein-protein interactions (Kietrys et al. 2009). Rearrangement of inter-subunit bridges during elongation is part of the coordinated action between the DC of the 30S and the PTC of the 50S, and of the translocation of the mRNA/tRNA complex – exactly one codon at the time relative to the ribosome (Yusupov et al. 2001, Frank et al. 2007, Zhang et al. 2009, Jenner et al. 2010). The subunits remain together during the translation process and separate when the finished polypeptide is released (Bashan and Yonath 2008, Steitz 2008, Schmeing and Ramakrishnan 2009). In bacteria, translocation is induced by the elongation factor G (EF-G). EF-G is also involved in ribosome recycling, i.e. disassembly of the ribosome into subunits after the termination of protein synthesis. Recycling also requires ribosome recycling factor (RRF) and GTP hydrolysis by EF-G (Salsi et al. 2014).

When a ribosome reaches the end of an mRNA, a release factor frees the ribosome from it. There are three known release factors in bacteria. RF1, which recognises stop codons UAA and UAG, RF2, which recognizes stop codons UAA and RF3, which promotes termination by either factor and participates in

quality control during translation elongation in *E. coli* through post-peptidyl-transfer-quality-control (post PT QC) (Zaher and Green 2011). Release factors induce termination by having a conformation that mimics that of tRNA molecules (Ito et al. 1996, Baranov et al. 2002, Zaher and Green 2011), by fitting to the sites of tRNA binding. If the mRNA is defective by lacking the stop codon the release factor cannot bind and the ribosome is 'trapped' on the mRNA. Bacterial cells contain a species of RNA called tmRNA, which mimics both tRNA and mRNA. tmRNA is composed of a tRNA-like domain (TLD), a short ORF encoding the ssrA peptide, and four pseudoknot structures. When it meets a trapped ribosome it binds alongside the defective mRNA in the A-site, allowing protein synthesis to proceed and, as tmRNA contains a stop codon, allowing the release factor to bind and disassemble the ribosome (Janssen and Hayes 2013). The defective proteins thus made are subsequently degraded and the ribosome is freed to once again participate in protein synthesis. Thus bacteria possess a regulatory mechanism that rescues ribosomal function from a potentially harmful mRNAs mutation (Madigan et al. 2012).

rRNA is the key component of the ribosome, which is constituted by almost two thirds of it by weight. Ribosomal proteins stabilize rRNA tertiary structure and assist in various steps of translation such as mRNA binding (S1), decoding, as well as in ensuring the optimal fidelity of translation (S4, S5, S12) (Klein et al. 2004, Korobeinikova et al. 2012). Some r-proteins are essential for the biogenesis of ribosomal subunits, but their function becomes dispensable once the ribosome is fully assembled (Wilson and Nierhaus 2005). Assembly and processing of ribosomal components into functional ribosomes is a complex and energy-intensive process. rRNA genes are organized into operons (Deutscher 2009), seven of which are present in *E. coli*. Each operon contains genes for each of the three rRNA species – 16S, 23S and 5S rRNA. One or more tRNA genes are located between the 16S and 23S sequences (Kaczanowska and Ryden-Aulin 2007, Deutscher 2009). Ribosome assembly *in vivo* is a rather slow process, which takes minutes to go to completion (Lindahl 1975). Ribosome assembly is initiated before rRNA transcription is completed. While transcription is taking place, secondary structure motifs start to form, creating binding sites for r-proteins. R-proteins stabilize the rRNA and assist in its folding. Some of them may also act also as chaperones to protect rRNA from misfolding (Semrad et al. 2004). Assembly proceeds through multiple pathways that generate an array of intermediate particles (Sykes et al. 2010). The three rRNA species are transcribed together in a single transcript, the pre-rRNA (Kaczanowska and Ryden-Aulin 2007, Deutscher 2009, Shajani et al. 2011). Ribonuclease III (RNase III) is the main rRNA processing enzyme, even if surprisingly it is not essential for bacterial survival (Takiff et al. 1992, Court 1993). It starts cleaving the pre-RNA before its transcription is completed. In *E. coli*, both 16S and 23S rRNA are synthesized as one large transcript that contains precursor sequences, and RNase III produces the separate 16S and 23S rRNA precursors (King and Schlessinger 1983). The pre-5S rRNA is processed by RNase E and T. The endonucleases RNase E and RNase G are required for

the final processing of the 5' end of the 16S rRNA, while the exonuclease RNase T is involved in the final processing of the 23S rRNA end, by trimming the extra 3' residues (Li et al. 1999b). YbeY (Davies et al 2010) and other still unidentified RNases are also involved in pre-rRNA processing. Four 3' to 5'-exoribonucleases, RNases II, R, and PH, and polynucleotide phosphorylase (PNPase), participate in maturation of the 3' end of 16S rRNA. The presence of any of these four RNases is enough to allow maturation to occur, but with different efficiencies (Sulthana and Deutscher 2013). Final processing of the LSU rRNAs is completed during formation of the translation initiation complex or during the first cycles of translation (Kaczanowska and Ryden-Aulin 2007, Deutscher 2009). The final processing of the 5S rRNA is inhibited by the antibiotic chloramphenicol, which inhibits protein synthesis (Deutscher 2009).

Ribosome assembly is assisted by a number of assembly factors, such as RNA helicases, ribosome-dependent GTPases, heat-shock proteins, and RNA chaperones. Most assembly factors are not essential for cell growth, except under stress conditions.

Specific ribonucleotides in rRNA are covalently modified during ribosome biogenesis, mostly by methylations and of pseudouridylations (Ψs). 36 different naturally occurring nucleoside modifications are present in *E. coli*. Modified nucleotides (MN) are concentrated around the actives sites of the ribosomes, such as the mRNAs and tRNA binding sites on the 30S subunit, the PTC and the entrance of the polypeptide exit tunnel on the 50S subunit and the inter-subunit bridges. This suggests that nucleotide modifications play important roles in ribosome stability and functioning. Accordingly, there are three conserved MNs in the stem-loop 69 of 23S rRNA, which is a universally conserved secondary structure in the 50S subunit, that is involved in several steps of initiation elongation and ribosome recycling (Agrawal et al. 2004, Wilson et al. 2005, Ali et al. 2006). MNs in bacteria are made by specific modification enzymes, such as pseudouridine synthases and methyltransferases. Different rRNA modifications take place at different phases of ribosome assembly, so that defects in the latter can likely lead to undermodification of rRNAs. Modifications also take place in r-proteins, eleven of which are known to be post-translationally modified in *E. coli* (Arnold and Reilly 1999, Polevoda and Sherman 2007). R-protein modifications consist mostly of acetylations and methylations.

Finally, let us mention the regulation mechanisms of ribosome synthesis. Ribosomes constitute as much as 50% of the cell dry mass, which makes ribosome biogenesis very expensive energetically (Condon et al. 1995, Dennis et al. 2006). Obviously ribosome synthesis must be a highly coordinated and efficient process. A complex network of regulatory mechanisms control ribosome synthesis in bacteria. rRNA synthesis is regulated by the stringent response, by growth rate control, by transcriptional antitermination, by upstream activation, and by feedback control (Wagner 1994). In this context, mechanisms that ensure quality control and prevent the formation of defective ribosomes are very important, and we need to elaborate further on this subject.

3. Ribonucleases

As mentioned above, ribonucleases (RNases) are the main actors involved in rRNA degradation. They catalyze the degradation of RNA into smaller components. Ribonucleases can be divided into endoribonucleases and exoribonucleases. Exonucleases hydrolyze nucleotides at the ends where a free 3' or 5' hydroxyl group is present in the polynucleotide chain. Endonucleases do not require a free 3' or 5' hydroxyl group at the end of the polynucleotide chain; they attack internal 3' or 5' linkages. Importantly, *E. coli* does not have 5'-to-3' exonucleases so it can only degrade RNAs starting from their 3'ends (either mature or those generated by endonuclease cleavages). RNases are present in both prokaryotic and eukaryotic cells, resulting in very short life-span for RNAs not protected from their action. Cells have a variety of mechanisms, like ribonuclease inhibitors, to protect their RNA from the action of RNases. Other protection mechanisms include 5' end capping, 3' end polyadenylation and association with proteins, that shield RNAs from RNase action. Proteins conjugated with RNA form Ribonucleoproteins (RNPs), which are involved in many cellular processes, including catalyzing RNA chemical modifications, and participation to pre-mRNA splicing (Bleichert and Baserga 2010).

Here follows a summary of the major RNases involved in ribosome processing, function and degradation (see also Table 1).

- **RNase I** is an endoribonuclease that cleaves the 3'-end of ssRNA at essentially random phosphodiester bonds, even at the dinucleotide level. It remains latent upon association with the ribosome in *E. coli* in *vitro* (Datta and Burma 1972, Raziuddin et al. 1979), but is active on the 50S of other prokaryotes, like *S. typhimurium* (Datta and Burma 1972). It accounts for the majority of the RNase activity in *E. coli*. Most of the enzyme resides in the periplasmic space (Subarrayan and Deutscher 2001). RNase M is a mutated form of RNase I present in *E. coli* strain MRE600 (Subbarayan and Deutscher 2001).
- **RNase III** is an endoribonuclease whose active form is a homodimer (Court 1993). In bacteria it cleaves pre-16S rRNA and pre-23S rRNA from transcribed polycistronic RNA operons. In particular, it is involved in the processing of the 3' terminus of immature 23S rRNA (King et al. 1983). RNase III also participates in the processing of pre-microRNA (Lee et al. 2003). It is regulated posttranscriptionally, by autoregulatory processes in which RNase III cleaves its own mRNA making it vulnerable to degradation by other RNases (Court et al. 2013).
- **RNase E** is a endoribonuclease that cleaves single-stranded RNA in A- and U-rich regions, with the most common site of cleavage close to an AU dinucleotide (Mackie 1992, Mackie et al. 1997). It functions as part of a large macromolecular complex known as the RNA degradosome and evidence suggests that this complex associates with the inner membrane of bacteria (Mackie 2013). Its action is modulated by stem-loops regions, which limit cleavage at potentially susceptible sites (McDowall et al. 1995).

It participates in the processing of the 9S precursor to 5S rRNA and shortens the 17S precursor of 16S rRNA, facilitating 5′ maturation by RNase G (Mackie 2013). It is also involved in tRNA processing and maturation (Deutscher 2003). It is known that a single endonucleolytic cleavage after residue 1942 initiated degradation of 23S rRNA (Basturea et al. 2011), and a recent study indicates that RNaseE is responsible for this excision, both during quality control and during starvation (Sulthana et al. 2016).

- **RNase G** is an endoribonuclease that cleaves the 5' end of the 16S rRNA during maturation. RNase G and E are both required in this process (Li et al. 1999). It is homologous to the catalytic domain of RNase E but its functions and importance are more limited (Deana and Belasco 2004). It participates inefficiently to the degradation of 9S rRNA and isn't involved in the degradation of tRNA (Ow et al. 2003).
- **RNase P** is a type of endoribonuclease that is a ribozyme, like the ribosome itself, and is composed by RNA plus one or more proteins. Its function is to cleave off precursor sequences on tRNA molecules, and furthermore it acts as a transcription factor for pol III (Jarrous and Reiner 2007). One type of RNase P in animal mitochondria, mtRNase P, lacks RNA (Holzmann et al. 2008).
- **MazF** is a single-strand specific endonuclease that participates in ribosomal degradation by cleaving the 3'-end of S16 rRNA (Vesper et al. 2011, Moll and Engelberg-Kulka 2012). MazF is the toxic component of a toxin-antitoxin (TA) module and its endoribonuclease activity is inhibited by the cognate antitoxin MazE. Various stress conditions can lead to programmed cell death by MazF activation through MazE degradation (Engelberg-Kulka et al. 2005). MazF governs a post-transcriptional stress response mechanism in *E coli.* During stringent response, as well as oxidative stress and heat shock, 43 nucleotides can be cleaved from the 3′-end of the mature 16S rRNA by the endonuclease MazF, possibly in fully assembled ribosomes (Vesper et al. 2011, Moll and Engelberg-Kulka 2012). This results in the accumulation of specific stress ribosomes that lack anti-SD sequences. The stress ribosomes in turn reprogram global translation patterns giving preference to leaderless mRNAs lacking SD sequences (Vesper et al. 2011) – although the new "pattern" of translation seems not to give preference to any type of cellular functionality (Sauert et al. 2016). Many of these mRNAs are also processed by MazF, providing a regulatory layer with both specialised ribosomes and specialized mRNAs, which are selectively translated by them. Moreover the RNA ligase RtcB can catalyze the re-ligation of the truncated 16S rRNA in *E. coli*, thus reversing the MazF-induced heterogeneity (Temmel et al. 2016).

  In addition to its role in cleaving the 5' UTRs of specific mRNAs, MazF cleaves single-stranded mRNAs at ACA sequences thus inhibiting protein synthesis (Zhang et al. 2003, Zhang et al. 2005). Recently, it has been suggested that MazF may cleave preferentially the ACA sites that are located in the open reading frames of mRNAs, while out-of-frame ACAs are resilient to cleavage (Oron-Gottesman et al. 2016).

- **MqsR** is a toxin that is induced in E. coli biofilms (Ren et al. 2004) and in association with the antitoxin MqsA is involved in regulation of cell motility (Barrios et al. 2006). MqsR is a member of the RelE/YoeB family of bacterial RNase toxins, and likely functions as a ribosome-dependent RNase, playing a role in bacterial persistence by inhibition of translation and, in turn, cell growth (Brown et al. 2009). MqsR toxicity is dependent on the activities of proteins CspD, ClpX, ClpP, Lon, YfjZ, and is assisted by RelB, RelE and HokA (Kim et al. 2010).
- **RNase BN** is an RNase Z homologue that has dual endo- and exonuclease activity (Dutta and Deutscher 2009). It participates in the processing of tRNA precursors. The removal of its exonuclease activity in cells lacking other processing RNases leads to slower growth and affects the maturation of several tRNA precursors (Dutta et al. 2012).
- **RNase II** was one of the first exonucleases studied in bacteria. It is a 3'–5' ribonuclease, which is very active in cell-free extracts, but possesses enzymatic activity even when attached to ribosomes (Spahr 1964). It degrades single-stranded RNA (Deutscher 2009) and participates in the maturation of the 3'end of 16S rRNA (Sulthana and Deutscher 2013).
- **RNase D** is a 3'–5' exoribonuclease involved in the 3' processing of various stable RNA molecules (Zuo et al. 2005). One of its functions is to add the 3' CCA sequence to tRNA in prokaryotic tRNA processing. Cells lacking RNase D have a reduced ability to synthesize extracellular matrix, show reduced motility, and take longer to recover from prolonged starvation (Taylor 2014).
- **RNase T** is an exoribonuclease that is involved in the metabolism of stable RNA, including tRNA end turnover and the maturation of the 23S rRNA by removing the last residues of the 3' terminus after the initial cleavage by RNase III. RNase T is the only enzyme that can efficiently remove residues near the double-stranded (ds) stem present in most stable RNAs (Zuo and Deutscher 1999).
- **RNase PH** is a 3'–5' exoribonuclease. It is involved in the processing of tRNA and of the 3'end of 16S rRNA (Sulthana and Deutscher 2013). It is a phosphorolytic enzyme, which uses phosphate as a cofactor to cleave nucleotide-nucleotide bonds, releasing diphosphate nucleotides. It can digest RNA duplexes that are resistant to the action of RNase II and RNase T. It has also been found to play a role in initiating the degradation of rRNA upon starvation of *E. coli* cultures (Basturea et al. 2011, Jain 2012). Degradation of 16S rRNA during starvation occurs after RNase PH initiates the removal of nucleotides from the 3′ end. The entire 3' minor domain is removed, altering RNA packing in the two ribosomal subunit, thus exposing the 16S and 23S rRNA to the action of RNaseE (Sulthana et al. 2016). RNase PH has also been found to play a role in initiating the degradation of rRNA in *E. coli* upon starvation (Basturea et al. 2011).
- **Polynucleotide Phosphorylase (PNPase)** is a 3'–5' exoribonuclease that is involved in phosphorolytic degradation of mRNA and rRNA in bacteria

(Condon 2003). It has a minor role in the processing of the 3'end of 16S rRNA (Sulthana and Deutscher 2013). It is also involved in the processing of CRISPRs, a class of bacterial non-coding RNA that confers immunity against bacteriophages (Sesto et al. 2014).

Table 1. Ribonucleases involved in ribosome processing and degradation (adapted from Mackie 2013).

Enzyme	Gene	Essential	Substrates			
			mRNA	rRNA	tRNA	sRNA
Endoribonucleases						
YbeY	*ybey*	Yes	+	+	?	+
RNase E	*rne*	Yes	++	++	++	+
RNase G	*rng*	No	+	++	?	?
RNase P	*rnpA*, *rnpB*	Yes	+	–	++	?
RNase BN	*rbn*	No	?	–	+	?
RNase I	*rna*	No	–	–	–	–
RNase III	*rnc*	No	+	++	++	?
Exoribonucleases						
RNase D	*rnd*	No	?	–	+	?
RNase PH	*rph*	No	?	–	++	?
RNase R	*rnr*	Synthetic lethality§	+	?	?	+
RNase T	*rnt*	No	?	++	++	?
PNPase	*pnp*	Synthetic lethality§	++	?	+	+
RNase II	*rnb*	Synthetic lethality§	++	–	+	?
Toxin–antitoxin systems						
MazF	*mazF*	No	+	+	–	–
RelE	*relE*	No	+	–	–	–
RNase LS	*rnlA*	No	+	–	–	–
YhaV	*yhaV*	No	+	–	–	–
YoeB	*yoeB*	No	+	–	–	–
ChpB	*chpB*	No	+	–	–	–
MqsR	*mqsR*	No	+	+	?	+

*++, the enzyme activity is required for the majority of that class of RNA; +, the enzyme activity may be needed for some RNA species in the class; –, there is no evidence for participation of the enzyme activity in processing; ?, there is no definitive evidence for cleavage of the indicated substrate. §, synthetic lethality with another RNase.

4. YbeY and RNase R

YbeY and RNase R are two RNases whose role in ribosomal degradation – as evidenced by our recent study (see next chapter) – appears to be so important that they deserve a chapter of their own.

YbeY is an endonuclease involved in degradation of defective 70S ribosomes and in the maturation of 16S rRNA (Jacob et al. 2013) and of the 23S rRNA and 5S rRNA 5' termini (Davies et al. 2010). YbeY influences the maturation of 5S, 16S and 23S rRNA (Davies et al. 2010) but its role in the removal of 3'-residues from pre-16S is debated (Sulthana and Deutscher 2013). Deletion of YbeY in *E. coli* results in decreased growth rate and sensitivity to diverse stress conditions, such as temperature variation, β-lactam antibiotics, detergents, etc. (Davies et al. 2010). YbeY also plays a major role in bacterial sRNA regulation. Studies suggest that it participates in both Hfq-dependent and Hfq-independent sRNAs-mediated interactions in *E. coli* (Pandey et al. 2014). It has also a role in the adaptation of pathogens during infection of host cells. YbeY is an essential RNase in the pathogen *Vibrio cholerae*, playing an important role for cell fitness and general stress tolerance. It is crucial for 16 S rRNA 3′ end maturation, assembly of functional 70 S ribosomes and ribosome quality control, and regulates virulence-associated small RNAs (Vercruysse et al. 2014). YbeY is recruited to the ribosomes through interaction with ribosomal proteins S11 and Era, thus participating in the maturation and stress regulation of 16S rRNA (Vercruysse et al. 2016). In *vitro* studies show that YbeY, together with RNase R, act as a mechanism of ribosome quality control by removing defective non-translating and translating 70S ribosomes in *E. coli*. This process is mediated by the defective 30S ribosomal subunit, while in a ribosome containing a mutant 50S and a WT 30S the degradation does not occur (Jacob et al. 2013). YbeY also participates in programmed cell death a.k.a. apoptosis-like death (ALD) in *E. coli*. ALD is the consequence of a response to DNA damage, and YbeY is involved through its rRNA degrading action (Erental et al. 2014)

RNase R is a homolog of RNase II that can act on essentially all RNAs inclusive those with extensive secondary structure. Both enzymes are non-specific processive ribonucleases that release 5′-nucleotide mono-phosphates and leave a short undigested oligonucleotide core. RNase R shortens RNA processively to di- and trinucleotides. RNase R is encoded by the *rnr* gene (Cheng and Deutscher 2002). Together with PNPase it removes defective rRNAs as soon as they are generated. *E. coli* cells lacking both RNases are inviable (Cheng and Deutscher 2003). In presence of altered environmental conditions, like nutrients starvation and heat shock, and in response to entry into stationary phase the level of RNase R is dramatically increased, up to 10-fold (Chen and Deutscher 2005). RNase R requires a 3' single-stranded overhang with a length of at least five nucleotides to degrade through a double RNA strand (Deutscher 2009). Part of RNase R is bound to ribosomes in growing cells, where it is stable and is involved in trans-translation. The free form is very unstable;

inhibition of its binding with ribosomes results in greatly increased RNA degradation (Liang and Deutscher 2013). In stationary phase, the enzyme is stabilized, leading to its accumulation (Chen and Deutscher 2010). Its stability is regulated by acetylation of Lys544, which occurs in exponential growth phase but not in stationary phase (Liang et al. 2011). The acetylating enzyme, protein lysine acetyltransferase, Pka (YfiQ), is absent from late exponential and stationary phase cells (Liang and Deutscher 2012). The helicase activity of RNase R is essential for its function in *vivo* (Hossain and Deutscher 2016). RNase R acts with YbeY to degrade defective 70S ribosome by a process mediated by the 30S ribosomal subunit (Jacob et al. 2013). RNase R is involved in the maturation of the 3'end of 16S rRNA (Sulthana and Deutscher 2013). It also participates in mRNA decay (Cheng and Deutscher 2005).

5. The Degradosome

The degradosome is a protein complex involved in the degradation of mRNA. The turnover of mRNA is a necessary aspect of the cell normal lifecycle and its genetic regulation. The principal components of the *E. coli* degradosome are RNase E, PNPase, RhlB helicase, and the glycolytic enzyme, enolase (Carpousis 2007). The non-catalytic portion of RNase E forms a scaffold for the physical association of the other enzymes in the degradosome. The degradosome scaffolding domain also includes RNA binding domains (RBD and AR2), and a membrane targeting sequence (Katarzyna et al. 2013). RNase E and enolase are the most crucial elements. The removal of the scaffold region of RNase E suppresses the rapid degradation of ptsG mRNA in response to the metabolic stress without affecting the expression of ptsG mRNA under normal conditions, while the depletion of enolase but not the disruption of pnp or rhlB eliminates the rapid degradation of ptsG mRNA (Morita et al. 2004). The degradation is the result of the combined action of endo- and exoribonucleases. Two parallel pathways for RNA degradation through RNase E have been identified: one requiring the 5′ end monophosphate and one depending on the RNA fold (Katarzyna et al. 2013). Two heat shock proteins, GroEL and DnaK, and polyphosphate kinase (Ppk) also are associated with degradosomes in substoichiometric amounts (Bernstein et al. 2003). Several studies suggest that there are alternative forms of the RNA degradosome depending on growth conditions or other factors. These alternative forms appear to regulate RNase E activity in the degradation of mRNA (Carpousis 2007). The degradosome complex mediates the decay of some transcripts, whereas other transcripts are likely to be degraded independently of the complex. Different categories of mRNA are marked for degradation by structural features, biochemical factors and the function of the protein encoded by the transcripts (Bernstein et al. 2004).

6. Ribosome degradation in bacteria under stress conditions

Since ribosomes are so abundant in cells, their production is very costly in terms of energy and resources. An efficient regulation of ribosome production is therefore essential for cell survival. Cessation of growth implies that excess ribosomes become redundant. Cells respond either by storing ribosomes for future use or by degrading and recycling them.

Upon entry into stationary phase, bacterial ribosomes can be inactivated and stored as 100S dimers (Wada et al. 2000). Ribosomes can also be stored by inactivation at the 70S level, or by dissociation into subunits (Häuser et al. 2012). Tests on *E. coli* in batch culture found over half of the ribosomes as 100S at the onset of the stationary phase (Wada et al. 2000). The proteins RMF and HPF are necessary to 100S formation, blocking the anti-Shine-Dalgarno:Shine-Dalgarno interaction and the tRNA, IF1 and IF3 binding sites (Polikanov et al. 2012). The 100S dimer is dissociated after a few hours from the onset of stationary phase, and after several more days the final ribosomal degradation occurs. At this point cell viability is lost (Wada et al. 2000). The storage of ribosomes at the 70S level can be achieved through inactivation by binding of the YfiA protein, or through the action of proteins like YqiD, which tie them to the membrane in *E. coli* (Yoshida et al. 2012). Another strategy is binding of YbeY to the 50S with resulting subunits dissociation (Häuser et al. 2012).

Under various stress conditions – like starvation, heat shock or in presence of antibiotics – recycling of ribosomes becomes a key survival strategy for bacteria. Several mechanisms therefore lead to ribosomal degradation under those conditions. This kind of stress- induced degradation has been the focus of most studies on ribosomal degradation over the past decades.

The work on ribosomal degradation has focused mostly on starvation conditions in *E. coli*: starvation of Mg^{2+}, phosphate, nitrogen, carbon, and growth in a minimal sea-salt medium (Aronson and McCarthy 1961, Ben-Hamida and Schlessinger 1966, Jacobson and Gillespie 1968, Kaplan and Apirion 1975, Davis et al. 1986, Zundel et al. 2009). Studies have also been done on *E. coli* cells with increased membrane permeability (Yuan and Shen 1975), during antibiotic treatment (Shen and Bremer 1977), and under protein over expression (Dong et al. 1995). These studies focus generally on non-growing stressed cells, where RNase I can efficiently move to the cytoplasm and degrade rRNA (Deutscher 2009). Under normal conditions *E. coli* has a mechanism protecting its rRNA from the action of RNase I, which sequesters it to helix 41 of the 16S rRNA and thus renders it inactive (Kitahara and Miyazaki, 2011).

RNA degradation during starvation is largely confined to rRNA, and there is some evidence that tRNA and ribosomal proteins are stable in these conditions (Deutscher 2003). This is important for cell recovery as ribosome production may be able to restart as soon as new rRNA is synthesized. Ribosomal degradation can be very extensive, exceeding 95% in some studies (Deutscher 2003). There is also evidence that at all growth rates there is an excess of rRNA

production relative to ribosomal proteins, and at the very slow growth rates occurring during starvation this excess rRNA is degraded (Deutscher 2003). Under starvation conditions, the exoribonucleases RNase PH, RNase II and RNase R participate in the removal of rRNA fragments. RNase II and R both contribute to rRNA degradation during starvation (Basturea et al. 2011). 23S rRNA cleavage in the region of H71 occurs during starvation, while for 23S rRNA degradation begins with shortening of its 3' end by RNase PH (Basturea et al 2011)

Degrading ribosomes could be a survival strategy, where cells utilize the material released by ribosome breakdown to allow a small population of surviving cells to resume growth, as it has been shown in *E. coli* (Basturea et al. 2012). However, as it will become apparent in the light of our research (see next chapter), ribosomal degradation does not necessarily depend on stress conditions or on the presence of non-functional ribosomes: growing wild type *E. coli* cells exhibit ribosomal degradation during growth before the onset of the stationary phase (Piir et al. 2011). This kind of rRNA degradation is independent of the stringent response. During early to mid-exponential phase RNase R, one of the main rRNA degrading enzymes (see above), is inactivated by ribosome binding but is active in late exponential phase, as acetylation decreases (Liang and Deutscher 2012, Liang and Deutscher 2013). Only a small population of ribosomes, which have bound nonstop mRNA and undergo *trans*-translation, binds RNase R, providing an interesting example of RNase regulation ((Liang and Deutscher 2011, Liang 2013). In persister cells ribosomes are found largely as inactive subunits, while most rRNAs and tRNAs are degraded – only a small fraction of the ribosomes remain mostly intact, except for reduced amounts of seven ribosomal proteins (Cho et al. 2015).

Apart from nutrient starvation, membrane damaging compounds such as toluene, dodecyldiethanolamine and Hg^{2+} ions promote RNA degradation (Deutscher 2003). These agents lead to alteration of membrane permeability, causing loss of ions, including Mg^{2+}, which likely leads to alterations in the ribosome structure, rendering the rRNA more accessible to degrading RNases. Degradation in these conditions may be due to the actions of the non-specific endoribonuclease, RNase I (Beppu et al. 1969, Lambert et al. 1975). As Mg^{2+} is a known RNase I inhibitor, magnesium loss increases its action on rRNA. In the case of degradation due to Hg^{2+}, it occurs only in cells during exponential growth, but not during the stationary phase. This is probably due to changes in the cell membrane that occur during stationary phase and affect the entry of RNase I into the cytoplasm (Deutscher 2003).

The action of several classes of antibiotics (Table 2) can also lead to rRNA degradation (Deutscher 2003). The majority of known antibiotics target the elongation phase of translation (Wilson 2014). However, one effect of compounds such as streptomycin, mitomycin C, and polymixin E is promotion of degradation of the ribosomes, likely by affecting membrane permeability, which results in the loss of ions such as Mg^{2+}, which, as mentioned above, alter ribosome structure and render rRNA accessible to RNases. However, the physio-

logical and clinical importance of the action of antibiotics on ribosomal degradation is presently unknown.

Ribosome-targeting antibiotics also act in other ways to inhibit cell growth and/or survival (Lambert 2012, Wilson 2014, Hong et al. 2014). Ribosomal antibiotics can target the decoding site, the peptidyl-transferase center, the protein exit tunnel, and other elements involved in protein biosynthesis. Their modes of action include promoting miscoding, interfering with tRNA and translation factor binding, and blocking the protein exit tunnel (Yonath 2005). In general, ribosomal antibiotics can be divided into 30S subunit inhibitors and 50S subunit inhibitors (Lambert 2012).

Table 2. Main antibiotics classes (modified from Lambert 2012).

30S ribosomal subunit inhibitors		**50S ribosomal subunit inhibitors**	
Main antibiotic classes	**Main mode of action**	**Main antibiotic classes**	**Main mode of action**
Amino-glycosides	Mistranslation through binding to 16S rRNA	Macrolides, lincosamides and streptogramins (MLS)	Block peptide chain extension
Tetra-cyclines	Impairing of binding of tRNA to the ribosomal A-site	Pleuromutilins	Block peptide formation
		Orthosomycins	Inhibition of translation
		Phenicols	Inhibition of peptydil transferase activity
		Fusidic acid	Inhibition of protein synthesis by preventing the turnover of elongation factor G (EF-G)
		Oxazolidinones	Stop formation of the initiation complex for protein synthesis

Crystallographic studies on the structure of naturally produced antibiotics and their semi-synthetic derivatives bound to ribosomal particles have provided new understanding of their mechanisms of action. For instance, crystal structures of neomycin and gentamicin interacting with the 70S ribosome have revealed a second binding site of these antibiotics within helix 69 (H69) of the 23S rRNA (which interacts with H44 of the 16S rRNA to form the B2a intersubunit

bridge). Crystallographic studies show that this second binding site restricts the movement of H44 relative to H69, thus likely impairing translocation and ribosome recycling (Wilson 2014). As will become apparent in the results section of this thesis, the 23S rRNA H69 is intimately connected to controlled ribosomal degradation, suggesting a further link between antibiotics and regulating cellular ribosome concentrations.

7. Ribosome degradation in yeasts

Ribosomal RNA quality control and degradation in yeast was largely ignored until the first decade of the present century – with some notable exceptions (Frank and Mills 1978). A landmark study (LaRiviere et al. 2006) analysed the effect on rRNA stability in mature ribosomes affected by deleterious mutations. The study established that rRNAs containing functionally those mutations are significantly down-regulated in *S. cerevisiae*. This down-regulation occurs almost entirely through decreased stability of the mature mutant rRNAs. This indicates that budding yeast contains a quality control mechanism that targets the RNA component of mature ribosomal subunits ("nonfunctional rRNA decay" or "NRD"). The mutations analyzed were chosen based on their known negative consequences on translation and viability in bacteria (A2451G or U2585A in the 23S rRNA PTC and G530U and A1492C in the 16S rRNA DCC: *E. coli* nomenclature). The dominant lethality caused by expression of these mutants in bacteria indicates that such rRNAs are assembled into mature ribosomes. Several quality-control pathways that eliminate erroneously processed pre-rRNAs and/or improperly assembled pre-ribosomes have been described in *S. cerevisiae*. Dim1p, for example, blocks pre-rRNA processing steps required for maturation of 18S rRNA in absence of pre-rRNA dimethylation, (Lafontaine et al. 1998). Also, the nuclear exosome degrades other incorrectly processed pre-rRNAs (Allmang et al. 2000, Zanchin and Goldfarb, 1999). In some cases Trf4p/Air/Mtr4p (TRAMP) complex-mediated pre-rRNA polyadenylation is involved in the degradation process (Dez et al. 2006). However it is likely that NRD is separate from these known nuclear pre-rRNA surveillance pathways, as it acts on mature rRNAs and likely occurs in the cytoplasm. Because mature 18S and 25S rRNAs derive from a single pre-rRNA transcript, the mutations in one rRNA (18S or 25S) did not affect the level of the other co-transcribed wild-type rRNA (25S or 18S, respectively). This indicates that reduction of the deleterious rRNA species must occur after both transcription and cleavage of 32S pre-rRNA into the 20S and 27S intermediates. In budding yeast, conversion of 20S pre-rRNA to 18S rRNA is a wholly cytoplasmic event (Venema and Tollervey 1999), and since the defective mutant 18S rRNAs were found in 80S particles, 18S NRD is likely to occur in the cytoplasm. Even if 25S rRNA maturation occurs entirely in the nucleus the study suggests that 25S NRD is cytoplasmic. Nucleic acids within cells are subject to damage by a number of factors, such as alkylating agents, oxidative

stress, and UV irradiation, and there is evidence that not only DNA, but RNA too, is subject to insult (Bregeon and Sarasin 2005). In *S. cerevisiae*, RNA nucleotides vastly outnumber DNA nucleotides, and rRNA constitutes 80% of total RNA (Warner 1999). Thus rRNA is very likely affected by damaging agents. It is therefore likely that NRD serves to clear from cells aging rRNAs that become increasingly dysfunctional over time.

Ribosomal degradation has been shown to occur also in yeast exposed to stimuli known to induce apoptosis, such as exposure to ROS. This process is most likely endonucleolytic, is correlated with stress response, and is dependent on the mitochondrial respiratory status, whereas rRNA of cells with functional defence against oxidative stress is less susceptible to degradation (Mroczek and Kufel 2008). Another pathway for ribosomal regulation in eukaryotes is the target of rapamycin (TOR), which plays a central role in regulating cellular responses to nutrient availability and mitogenic signals (Arsham and Neufeld 2006, Wullschleger et al. 2006, Wei and Zheng 2011). Rapamycin is a TOR inhibitor that inhibits cell proliferation and growth (Barbet et al. 1996, Zaragoza et al. 1998). TOR-mediated control of cell growth and proliferation inhibits the synthesis of new ribosomes (Powers and Walter 1999). A recent study (Pestov and Shcherbik 2012) suggests that TOR control of the cellular ribosome content extends beyond the regulation of new ribosome synthesis. The study found that the number of existing ribosomes present in a *S. cerevisiae* culture during growth in rich medium rapidly decreases by 40 to 60% when the cells are treated with rapamycin. It was found that the exosome contributes to the efficient processing of 25S rRNA degradation intermediates in yeast cells. The process involves the exosomic cofactors Ski7 and the SKI complex. Furthermore, the 25S rRNA decay occurring in yeast culture upon nutrient depletion also involves the Ski-exosome system, and is independent from NRD. The study indicates that TOR-mediated control of the ribosome content can affect both ribosome synthesis and the degradation of mature ribosomes. One intriguing hypothesis underlined by the above mechanisms is that yeast cells may have a system that recognizes such idle subunits and targets them for degradation.

Ribosomal proteins in yeast are degraded by a selective autophagic process termed ‘ribophagy’. This process requires the catalytic activity of the Ubp3p/ Bre5p ubiquitin protease (Kraft et al. 2008, Heinrichs 2008).

2. AIMS OF THE STUDY

The purpose of this dissertation is to shed light on the fate of ribosomes in growing bacteria. Ribosomes constitute a large part of the cell mass and are expensive to make in terms of energy and materials. So far, almost all studies on ribosome degradation in bacteria have focused on non-growing cells under stress conditions. In contrast, we wanted to find the physiologically relevant growth conditions where mature ribosomes are degraded. In addition, we aimed to gain knowledge on the trigger mechanisms and molecular pathways of the degradation, and to compare the process of mature ribosome degradation in Escherichia coli with the corresponding process in yeast.

3. MATERIALS AND METHODS

MG1655 cells transformed with pBAD-23t-ara, pBAD-16t-ara, or pBAD-hybrid plasmid were grown in batch at 37 °C in MOPS or LB medium (as specified in individual experiments) supplemented with 0.2–0.3% arabinose and ampicillin for plasmid selection. At the indicated optical density, 5,6-[^{3}H]-uridine was inoculated to 2-ml culture, which was then grown for a further 30–45 min at 37 °C. Cells were subsequently pelleted and re-suspended in 2 ml fresh MOPS medium supplemented with 0.4–0.5% glucose and 1 mM non-radioactive uridine, and growth was resumed at 37 °C. At the indicated time points, 200 µl aliquots were collected and stored at −85 °C. In control experiments, [^{3}H]-uridine was added 10 min after shifting the cells to glucose-containing media. Before lysis, each aliquot was mixed with 20 ml of culture grown under inducing conditions in non-radioactive medium. This was done to minimize inter-sample variability during cell lysis and affinity-purification of tagged rRNAs. For the turbidostat experiments, the induction of tagged RNAs and pulse labelling with 5,6-[^{3}H]-uridine was performed in batch cultures at an optical density of 0.2 U/ml as described above. After repression in 4–5 ml fresh MOPS or LP glucose, the cultures were transferred into the pre-warmed turbidostat and grown for 5 h and optical density of the culture was kept between 0.4 and 0.45 U/ml. Volumes of medium that were collected for lysis after every 30 min were increased in proportion to the increases in culture volumes as fresh medium was added to keep the culture turbidity constant. Radioactive cells were mixed with normalization cultures. RNA was extracted by incubation of cells re-suspended in 600 µl TEN buffer containing 1% SDS, and 1% Brij in equal volume of phenol pH 5.5 at 65 °C for 15 min (0.6% Na-deoxycholate was added in the experiments for paper 1, but omitted in subsequent ones as it didn't affect the effectiveness of the lysis). This was followed by phenol:chloroform extraction and ethanol precipitation. Purification was carried on as following: 23S rRNAs tagged with streptavidin binding aptamer were incubated overnight with 20 µl Sepharose High Performance Streptavidin sepharose (GE Health-care), washed with LLP, and incubated overnight with 20 µl (5 mM) biotin (Sigma-Aldrich). After centrifugation, the supernatant containing the strep-tavidin-tagged 23S rRNAs was extracted. 16S rRNAs carrying the M2S tag were purified by incubation with 6 µl MS2 protein (titrated for activity) and 150 µl amylose resin (BioLabs), washed with binding buffer (20 mM Tris pH = 7.5, 0.1 M NH_4Cl, 10 mM $MgCl_2$), and placed in a 1.5 ml amylose resin column. After washing with binding buffer the samples were eluted with 300 µl elution buffer (20 mM Tris pH = 7.4, 0.2 M NaCl, 10 mM Maltose).

Concentrations of 23S rRNA and 16S rRNA were measured by absorbance at 260 nm. Radioactivity from incorporated [H^3]-uridine was measured by scintillation counting and normalized for the A_{260} absorbance value of the sample. After RNA extraction and prior to purification, the total radioactivity was measured. The specific activity of each sample was further normalized for

the total radioactivity after RNA extraction to account for differences in extraction efficiency.

In the experiments with co-transformed mutants and "wild type" plasmids (paper 3) an untagged rrnB operon from a pBAD plasmid was cloned into a pBAD33 plasmid. 1919G and 1960G mutations were introduced by cloning from the mutant plasmids. Each construct was co-transformed with the pHyb plasmid using chloramphenicol and ampicillin selection. As a control, a construct with an untagged wild-type rrnB operon cloned in the pBAD33 plasmid was co-transformed with the pHyb plasmid.

For the experiments in the unpublished manuscripts the strains MGJ5987 (Δ10TA), ΔPAP, Δrph, ΔRNase R and ΔYbeY from the Keio collection (Baba et al. 2006) were transformed with pBAD-1919G. ΔYbeY was also transformed with pBAD-1960G.

For the experiments in paper 4, *E. coli* cells were grown in LB and LB plates at 37 °C. Toxin expression was induced in BW25113 and MG1655exo-, harboring plasmids pSC228 and pSC3326 for the induction of MazF and MazE or pTX3 and pAT3 for the induction of MqsR and MqsA. Samples were analyzed by primer extension and 3' RACE. cDNA libraries were constructed and sequenced by Vertis Biotechnologie AG.

For the sucrose analysis performed by me Bacterial pellets were suspended in TNM-10 buffer, lyzozime and Dnase I were added and the cells were disrupted by glass beads in Precellys 24 homogenizer (Bertin Technologies). After centrifugation at 12,000 g for 20 min., 30–50 A260 units of S30 lysate was loaded onto a 10–25% sucrose gradient in TNM-10 buffer, followed by centrifugation at 21,000 rpm for 16 h. fractions containing ribosomal particles were collected and precipitated by addition of 2.5 volumes of ice-cold ethanol. Ribosomal pellets were suspended in TNM-10 buffer and stored at –80 °C.

4. RESULTS AND DISCUSSION

1. rRNA degradation in wild type ribosomes (paper I)

The aim of studies on ribosomal degradation has traditionally been to investigate the ribosomes under conditions of starvation or other stresses, but the stability of wild type ribosomes growing under normal, non-stress conditions has been a subject largely ignored. While ribosome degradation has been suggested to occur in growing *Salmonella* strains (Hsu et al. 1994), it has never been shown in growing *E. coli*. However, rRNA fragments have been found in degradosomes in *E. coli* cells, suggesting either degradation of mature ribosomes or co-assembly degradation of pre-ribosomal ribonucleotide particles (Bessarab et al. 1998). The effect of ribosome-inactivating mutations on ribosome degradation in bacteria was also unknown. Although studies on yeast have shown the presence of a regulatory mechanism to degrade non-functional rRNA (LaRiviere et al. 2006), few such studies exist on bacteria (Liiv et al. 1996, Cheng and Deutscher 2003). One question that arises is at which step of ribosomal biogenesis quality control occurs and what mechanisms are involved. Recent work suggests that cells evolved multiple strategies to recognize specifically, and target for clearance, ribosomes that are structurally and/or functionally deficient, as well as in excess (Lafontaine 2009). The study by La Riviere (LaRiviere et al. 2006), described above, indicates the presence of a quality control mechanism that degrades mature ribosomes in yeasts and suggests that at least in eukaryotes rRNA is subjected to specific mechanisms of surveillance and control (Meenakshi and Parker 2006). A purpose of our research is to shed light on these mechanisms in bacteria.

Growing cells need to synthesize new ribosomes at a fast rate, as active ribosome concentration is rate limiting for growth (Scott et al. 2010). At fast cell division rate there are about 70 000 ribosomes in a single *E. coli* cell (Bremer and Dennis 1996) Modulation of chemical composition and other parameters of the cell by growth rate. (Neidhardt 1996) thus it is inevitable that some errors occur during ribosome synthesis. As ribosome synthesis is a very expensive process in term of energy and building components, it is important for the cell to precisely tailor the ribosome production. Therefore, the question arises whether bacteria growing in non-stress condition normally degrade ribosomes that are defective or in excess.

When a cell divides, the existing ribosomes are distributed to the daughter cells. As we cannot exclude the possibility that any observed reduction in the number of ribosomes per cell is due to dilution of the ribosomes upon cell division, rather than their degradation, it is difficult to directly measure ribosomal degradation in growing cells.

In order to assess the stability of ribosomes in growing cells, we devised a system that can measure ribosomal degradation without the bias due to ribosomal dilution.

We cloned an rRNA operon in the presence of repressible arabinose BAD (araBAD) or rhamnose BAD (rhaBAD) promoters. We then inserted streptavidin-binding (Leonov et al. 2003) and MS2 RNA aptamers (Youngman and Green 2005) to 23S rRNA and 16S rRNA respectively, in order to purify the affinity-tagged ribosomes by affinity chromatography (Figure 3).

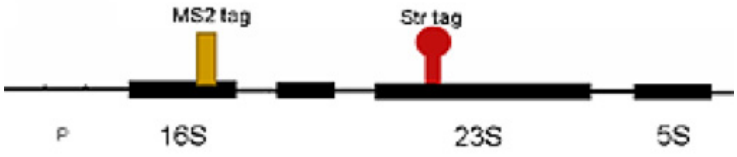

Fig 3. Schematics of the rnnB operon in the pBAD-Hyb plasmid. The placement of the MS2-tag (yellow) and the Streptavidine-tag (red) are shown in the 16S rRNA and 23S rRNA.

The cells were labelled with H^3-Uridine for a limited time interval, after which the culture was switched to a repressible medium. An unlabeled 'cold' culture was also grown in tag-expressing conditions, which was to be added to the samples in order to minimize inter-sample variation. By collecting culture samples at different time points post-repression of tagged ribosome synthesis, we were able to follow the degradation of the ribosomes present during the labelling, while ignoring the fates of the ribosomes synthesized from chromosomally encoded rRNA operons. Specific activity at each time point was calculated by normalizing the radioactive activity with the optical density, which was obtained mostly from the excess unlabeled tagged rRNA derived from cold culture added to each sample in equal amounts (Figure 4).

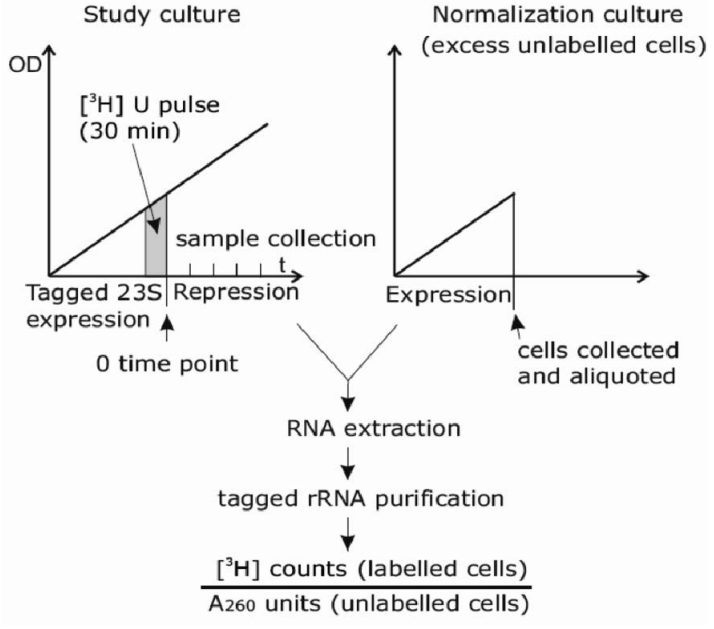

Fig 4. Schematics of the experimental setup.

We performed our study on bacteria growing with constant rate in turbidostat, and in batch culture. The turbidostat is an incubator where fresh medium is constantly added to the culture to keep its density, and thus its growth rate, constant. The optical density in the turbidostat system was kept between 0.4 and 0.45 U/ml, which corresponds to mid- logarithmic growth phase.

Both 23S rRNA and 16S rRNA was found to be stable at constant growth rate (Paper 1, Figure 2). In batch culture, however, both rRNA species were found to be degraded, and the degradation occurred in the transition phase between exponential and stationary culture growth (Paper 1, Figure 3). On the other hand, ribosomes were stable in the stationary phase of the batch cultures. Therefore, degradation seems to be limited to the intermediate-growth stage between logarithmic and stationary growth phase, when complex changes take place in the cellular physiology in preparation to entry into the stationary phase.

2. rRNA degradation in mutant ribosomes (paper III)

After studying degradation of WT ribosomes, we wanted to assess the effect of disrupting mutations in rRNA on ribosomal stability. As described in the previous chapter, LaRiviere et al. convincingly demonstrated that two mutations (A2451G or U2585A in E. coli nomenclature) in the 25S rRNA peptidyl transferase centre (PTC) and two mutations (G530U and A1492C) in the 18S rRNA decoding centre (DCC) lead to rRNA degradation in *S. cerevisiae*, pointing to the existence of a quality control process working on mature ribosomes (LaRiviere et al. 2006). Using the same experimental set-up as in Paper 1, we developed to study degradation in WT ribosomes, we investigated the effect of the corresponding mutations in *E. coli*. In addition, we studied the effect of three mutations in intersubunit bridge B2a (A1912G, A1919G and ΔH69) and one intersubunit bridge B3 (A1960G) in the domain IV of 23S rRNA (Figure 5). These structures, particularly B2a, where H69 (helix 69) is located, are important for subunit association and ribosome recycling (Maivali et al. 2004, Pulk et al. 2006). The mutations A1912G, A1919G, and A1960G have been shown to have a strong effect on growth phenotypes (Liiv et al. 2005, Liiv and O'Connor 2006), while ΔH69 confers a lethal phenotype (Ali et al. 2006). In order to avoid the ribosome degradation that occurs upon slowing of bacterial growth in the batch culture (as described in Paper 1), our experiments were conducted in a turbidostat upon constant growth rate.

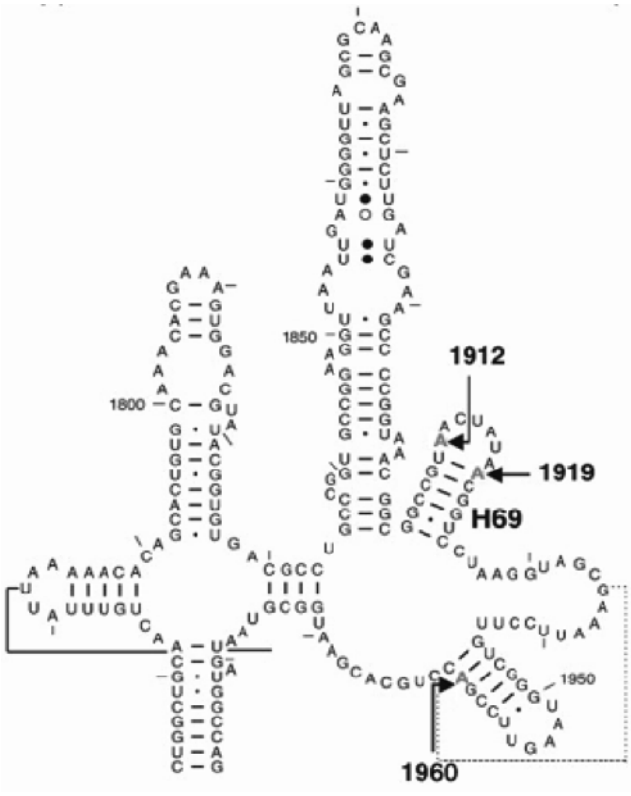

Fig 5. Domain IV of 23S rRNA with the location of helix69 and the positions 1912, 1919 and 1960.

Rather surprisingly, only one of the four mutations that lead to degradation in yeast, U2585A in the 23S PTC, resulted in degradation of *E. coli* ribosomes, even if these mutations are known to confer lethal phenotypes ((Powers and Noller 1990, Thompson et al. 2001). Furthermore, while in yeast the ribosome-inactivating mutations lead to degradation of only the subunit in which they are located, in *E. coli* U2585A leads to degradation of both ribosomal subunits. The extent of degradation is similar for both subunits, about 40–50% over the five-hour experiment. These findings suggest that ribosomal degradation in yeast and in bacteria involve different molecular mechanisms. As the growth rates of the four mutants were similar, it is unlikely that the degradation observed in the presence of the U2585A mutation is due to a different physiological state of the culture.

As for the mutations in the intersubunit bridges, only A1919G in B2a and A1960G in B3 lead to ribosomal degradation. Again, both subunits are degraded, at a similar rate (up to 40–50% in 4 hours). Surprisingly, the deletion of the whole helix 69 doesn't seem to affect ribosomal stability. H69 is a conserved structure, known to be involved not only in subunit association and tRNA

binding, but also in initiation, translocation, translational accuracy, the peptidyl transferase reaction. However, while its deletion confers a lethal phenotype, ΔH69 ribosomes in vitro are able to carry out accurate incorporation of phenylalanine from a poly(U) template, can synthesize a full-length protein using a natural mRNA, and carry out translocation at wild-type rates (Ali et al. 2006).

In addition, we found that the A1919G and A1960G mutations lead not only to degradation of mutant rRNA containing ribosomes, but also to degradation of fully wild-type ribosomes, where neither 16S nor 23S contain the inactivating point mutation. For this experiment we co-transformed untagged plasmids carrying these mutations and tagged WT plasmids, and found that degradation of tagged WT rRNA occurred to a similar extent as in the experiments where the mutant ribosomes were directly tagged (Paper 3, Figure 4). This suggests that the degradation pathway activated by the expression of mutant ribosomes is equally adept at degrading wild-type ribosomes, where neither subunit carries mutations.

As our previous work showed that wild-type ribosomes were stable during constant growth in turbidostat and there was no growth in the stationary phase, we decided to measure the stability of A1919G mutant ribosomes, which induced degradation in both mutant and wild type rRNA, during the stationary growth phase in the batch culture. We found that ribosomes are stable in the stationary phase even in presence of this destabilizing mutation (Paper 3, Figure 5). Next we investigated if interruption of protein synthesis would rescue ribosomes from degradation in the presence of this same mutation. We found that ribosomes grown in presence of cloramphenicol, a protein synthesis inhibitor, become stable (Paper 3, Figure 5).

The results of our experiments on the mutations leading to ribosome degradation in *S. cerevisiae* suggest that a different mechanism than the non-functional decay active in yeast is involved in *E. coli*. *In vitro* studies by Zundel et al. support the theory that ribosomal degradation in *E. coli* is due to exposure of RNA-rich intersubunit surfaces to cellular RNases (Zundel et al. 2009). Our *in vivo* studies point to a more complex model. While the single point mutation in 23S rRNA H69 (A1919G) leads to degradation, the deletion of the whole H69 seems to result in stable ribosomes. We know that when the subunit separates, the tip of H69 is released from contact with the 30S subunit and becomes in contact with the cytoplasm. It is therefore possible that H69 is an entry point for the endonuclease that initiates degradation of the 50S subunit. The wholesale deletion of H69 would thus block degradation by removing the structure needed for entry of the (hypothetical) degradation machinery into the ribosome.

Another surprising finding of our study is that two destabilizing mutations in the intersubunit bridges B2a and B3, which lead to ribosome degradation, also lead to degradation of the tagged ribosomes that do not contain any mutation. In both subunits, the presence of a mutation in one of the subunits leads to the degradation of the other tagged subunit, and the degradation occurs at a similar

rate. However, the co-expression of tagged WT ribosomes with a mutant form of $tRNA^{Trp23}$ does not lead to degradation, suggesting that ribosomal degradation is not simply a generic response to overexpression of RNA. The results of our study suggest a mechanism that is activated by the mutant ribosomes, which is inactive in non-growing cells in stationary phase. A possible explanation is that the degradation is activated by the overexpression of an RNase that cleaves the RNA exposed in the intersubunit surfaces, and doesn't discriminate between mutant and WT subunits, as indicated by our experiments where both WT and mutant ribosomes are co-expressed, and by the dependence of ribosomal degradation on ongoing protein synthesis (the chloramphenicol experiment discussed above).

Our investigations lead to the question of which RNases are involved in ribosomal decay in *E. coli*. Cheng and Deutscher (2003) present evidence of the key role of PNPase and RNase R in controlling ribosomal viability, by eliminating deleterious 23S and 16S fragments. In the absence of PNPase and RNase R activity these fragments accumulate, eventually resulting in loss of viability.

Another candidate for the degradation mechanism implied by our study is YbeY.

YbeY is involved in rRNA processing (Davies et al. 2010, Jacob et al. 2013, Warner et al. 2013, Vercruysse et al. 2016) but it has also an important role, in conjunction with RNase R, in degrading defective rRNA (Jacob et al. 2013, Warner 2013). Quite appropriately this enzyme has been called 'the jealous tailor' (Warner 2013). The role of YbeY and RNase R will be discussed in more detail below.

3. rRNA processing by MazF and MqsR (paper IV)

The role of toxin-antitoxin systems in rRNA processing and regulation in *E. coli* is studied in our fourth paper (Mets et al. 2016). Even if the main targets of the toxin-antitoxin systems is mRNA, some endoribonuclease toxins also target other RNA species, including ribosomal RNA (Moll and Engelberg-Kulka 2012, Sauert et al. 2016, Temmel et al. 2016). In *E. coli* MazF is known to cleave the 3'-end of 16S rRNA and mRNA upstream of of the AUG start codon, producing leaderless mRNAs (lmRNAs). (Amitai et al. 2009, Vesper et al. 2011) Under stress conditions the 3'-terminal 43 nucleotides of the 16S rRNA, and, concomitantly, the 5'-untranslated regions of specific transcripts, are removed (Moll and Engelberg-Kulka 2012). Experiments on overexpression of MazF resulted in large rRNA cleavage fragments (Kasari et al. 2013), suggesting that MazF must cleave rRNA also elsewhere, and/or initiate cleavage by other endoribonucleases.

To further examine rRNA cleavage by toxins MazF and MqsR a version of differential RNA-seq, which allows specific mapping of the toxin-cleaved 5'- and 3'-ends of RNA was adopted.

The MazF and MqsR overexpression experiments resulted in a substantial fragmentation of rRNA including a band corresponding to the 43-nt 3' terminal fragment of 16S rRNA. 5'-ends were mapped by ligation of RNA adapters to the RNA molecules. 3'-ends were mapped by poly(A)tailing of RNA, which requires 3'-OH groups. Transcription, ordinary cellular RNases, and toxin endonucleases produce different RNA ends, which can be resolved by enzymatic treatments. Thus we can from a single biological sample quantify the RNA ends produced by these three sources. The analysis was performed under five different conditions: a log phase culture, cultures where growth was arrested by expression of either MazF or Mqsr, and stationary phase cultures of wt and exo- *E. coli* strains. The exoribonuclease mutant strain (-exo), where the rRNA decay intermediated are preserved, was included in the study because RNA from decayed ribosomes is rapidly recycled in wt *E. coli*.

Several MazF and MqsR specifically cleaved 5'-ends, which were generated in both 16S and 23S rRNA, as identified by RNA-seq. In contrast, only one major direct cleavage site of MqsR was identified in 16S rRNA. However, MqsR-cleaved 5'-ends were verified at C2628 in 23S rRNA. All these cleavage sites were detectable in total RNA but not in the 70S fraction.

Sucrose density gradient ultracentrifugation experiments further revealed that MazF and MqsR induce accumulation of abnormal ribosome subunits (Paper 4, Figure 6). Northern analysis and primer extension confirmed that most of the toxin-induced cleavage takes place in those abnormal subunits, which probably are immature ribosomal particles containing precursor RNAs that cannot be assembled properly. The reason for such imbalanced ribosome synthesis is likely to be imbalanced cellular protein synthesis, which is due to selective cleavage of different mRNAs by overexpressed MazF and MqsR. Consistently with this emerging picture, it was found that expression of MqsR induced the build-up of the unprocessed precursors of 16S and 23S RNAs, suggesting inhibition of synthesis of mature ribosomal particles.

The findings of our study suggest that cleavage of rRNA precursors by toxins could help to remove unproductive ribosomal assembly intermediates in stressed bacteria. Since one of our aims is to identify the ribonucleases involved in the mutant rRNA degradation described in our previous papers, we used a modified MG strain lacking ten toxins, to test the stability of mutant ribosomes transformed into the cells. As the missing toxins included MazF and MqsR, we could test if these toxins are required for the degradation of mature ribosomes in growing cells. The answer turned out to be in the negative (see the next chapter), further emphasizing the differences in the mechanisms of degrading ribosomal precursors and mature ribosomes.

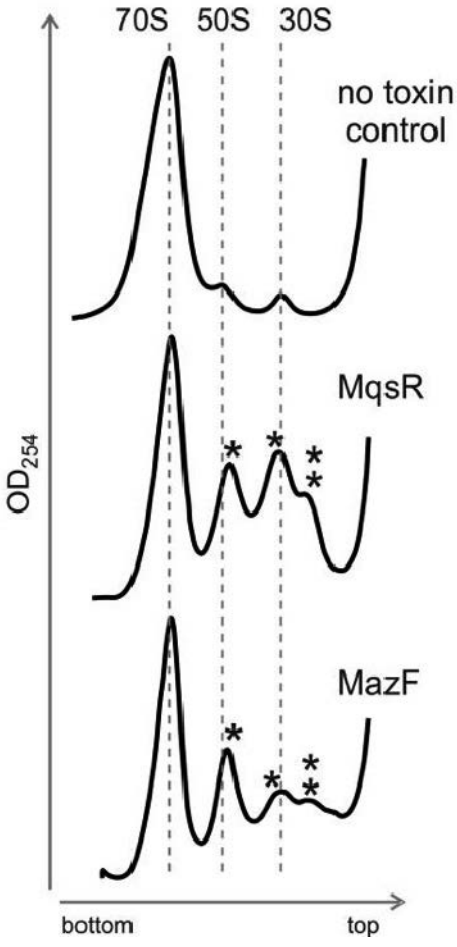

Fig 6. Aberrant ribosomal subunits on the overexpression of MazF and MqsR were visualized by sucrose gradient ultracentrifugation (adapted from Mets et al. 2017). Gradients where collected from the top (the lightest fractions) and optical densities measured continuously at 254 nm. Three representative experiments (no toxin expression, MqsR expression, and MazF expression) are shown. Asterisks denote the aberrant ribosomal subunits.

4. RNases involved in mutant rRNA degradation (unpublished results)

As mentioned earlier, our degradation studies on mutant rRNA (Paier et al. 2014) lead to the question, which nucleases are involved in the degradation processes? To answer this question, we performed a series of experiments with *E. coli* strains transformed with the plasmids containing some of the degradation inducing mutations described above, but lacking the most important endonucleases, known to cleave rRNA.

We tested the MGJ5987 (Δ10TA) strain lacking 10 toxins, a strain lacking PolyA Polymerase (ΔPAP), one lacking RNase PH (Δrph), one lacking RNase R and one lacking YbeY. All the strains were obtained from the Keio collection (Baba et al. 2006). MGJ5987 (Δ10TA) was developed by Maisonneuve *et al* (2011).

The Keio WT strain does not behave differently from MG1655 in regard to rRNA degradation, and its ribosomes are stable during turbidostat growth. The MG Δ10TA, ΔPAP, and Δrph strains, when transformed with pBAD-1919G and grown in turbidostat exhibited a level of degradation similar to that seen in the previous experiments with MG1655 containing the same mutation (data not shown). However, the ΔRNase R and ΔYbeY strains did not show evidence of rRNA degradation with 1919G (Fig. 6). ΔybeY was also tested with the 1960G mutation, which also induced degradation in the wild-type YbeY context, in turbidostat. As with 1919G, there was no rRNA degradation. As a control we repeated the experiments with the MG1655* strain used in Paier *et al* 2014, which was engineered to in addition lack the genes for functional YbeY or RNase R, and transformed with pBAD-1919G. Again no significant rRNA degradation was detected.

We also wanted to test how the lack of YbeY affects the stability of WT ribosomes when grown in batch conditions. As shown in previous experiments, WT ribosomes are degraded in late exponential phase, prior to entry in stationary phase (paper 1). Thus we transformed the MG1655 ΔYbeY strain with the pBAD-Hyb plasmid, and performed the degradation experiments in batch culture. Both 23S and 16S rRNA were stable, indicating that YbeY has a key role in the normal degradation process, which occurs at the end of the exponential growth phase (Figure 7).

We repeated the experiment with the MG1655 Δ RNase R strain, and as above the the ribosomes were stable. This suggests that ribonucleases YbeY and RNase R are both required for the physiological process of rRNA degradation that prepares the cells for the stationary phase, and that neither of them can compensate for the absence of the other. While these two ribonucleases have been previously shown to have a role in rRNA degradation (Jacob et al. 2013, Warner 2013), the absence of effect from the lack of RNase PH was surprising, as this exoribonuclease is known to share a common degradation pathway with RNase R (Jain 2012).

We also know that the translation inhibitor chloramphenicol stabilizes rRNA (Paier et al. 2014). We repeated degradation experiments in batch conditions with MG 1655 transformed with pBAD-1919G. After initial growth in LB with glucose, to inhibit tagged rRNA expression, the culture was labelled with ^{3}H-Uridine. After 10 minutes the medium was switched to LB arabinose and low concentration of CAM, and grown for two hours. Then the medium was switched into LB with glucose and cold uridine, and time points were taken. No degradation was found in either 23S or 16S rRNAs.

The same experimental setup was repeated, but CAM was added also on the second medium switch. Again, no rRNA degradation was found. This confirms the previous findings about the role of Chloramphenicol in rescuing ribosomal stability.

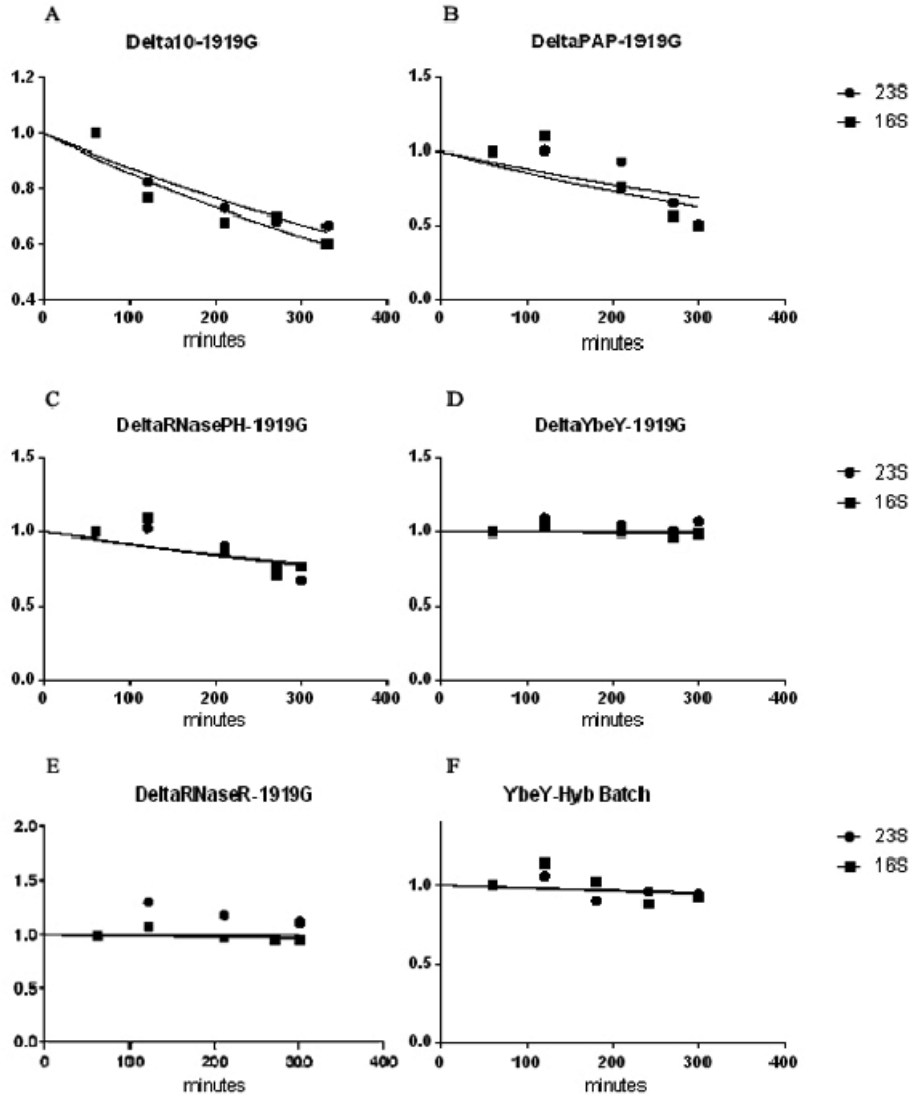

Fig 7. Degradation curves of Δ10-1919G (A), ΔPAP-1919G (B), ΔRNasePH-1919 (C), ΔYbeY-1919G (D), ΔRNase R-1919G (E), and of ΔybeY-Hyb in batch culture. The X-axis denotes time in minutes and the Y-axis denotes specific activity normalized to the first time point. Circles denote 23S rRNA and squares denote 16S rRNA.

The role of YbeY and RNase R in ribosome degradation has been suggested by previous research, notably due to their combined action in degrading mature 70S ribosomes (Jacob et al. 2013). Our research shows that each of these RNases individually is required for the degradation of mature ribosomes prior to entry into stationary growth phase.

CONCLUSIONS

- WT ribosomes are degraded during late exponential growth phase in batch culture, but are stable in early stationary phase. This suggests that the degradation process is part of a mechanism that prepares the cell to enter into stationary phase. Conversely, WT ribosomes are stable during constant-rate exponential growth.
- The ribosome-inactivating 23S rRNA mutation U2585A in the peptidyl transferase center leads to degradation of both the 23S and 16S rRNA during constant cell growth in mid-exponential phase. Because the growth rates of different mutants were similar, it is unlikely that the observed ribosomal degradation in the cells expressing U2585A 23S rRNAs is simply due to a general physiological state of the culture. The reason why ribosomal degradation is triggered in presence of the U2585A mutation, while leaving a different ribosome-inactivating PTC mutation intact, is a question unanswered.
- Mutation A1919G in the intersubunit bridge B2a and A19160G in B3 lead to degradation of both the 23S and 16S rRNA during constant cell growth in mid-exponential phase. These results support the hypothesis that intersubunit bridges are key factors for ribosome stability.
- Surprisingly, deletion of the entire helix 69 did not result in ribosomal degradation. We know that when the subunits separate, the tip of H69 is released from contact with helix 44 of 16S rRNA and becomes fully exposed to the cytoplasm. H69 could be an entry point to the 50S subunit for the endonuclease that initiates the degradation of this subunit. In this case the deletion of H69 would be expected to block degradation.
- Our study provides evidence of differences between ribosome degradation in bacteria and in yeast. In *S. cerevisiae* the degradation is carried out by the NRD pathway and only acts on the RNA species affected by the mutation, while the U2585A mutation in *E. coli* leads to degradation of both the mutant and the wild-type ribosomes, suggesting activation of a pathway that is equally adept at degrading mutant and WT ribosomes. Our results show that regardless of which subunit (50S or 30S) the inactivating mutation is located, the other tagged subunit is degraded at a similar rate. During translation most tagged 50S ribosomes are associated with non-tagged 30S, and *vice versa*, thus the presence of mutant ribosomal subunits causes dissociation and degradation of wild-type ribosomes as well.
- The toxins MazF and MqsR cleave rRNA at multiple sites, very probably during new ribosome assembly, and induce accumulation of abnormal ribosomal subunits. We have no evidence of either toxin cleaving mature ribosomes.
- YbeY and RNase R are involved in the degradation of non-functional rRNA in the mature ribosomes. Furthermore, these RNases are necessary for the degradation of WT rRNA during the phase preceding entry into stationary growth phase.

SUMMARY

Ribosomes are macromolecular complexes that consist of two large and one small RNA and of many different small proteins. The ribosome synthesizes all cellular proteins and the concentration of active ribosomes is rate limiting for cell growth. As synthesis or ribosomal RNA encompasses 80% of cellular RNA synthesis activity and the ribosomal proteins can make up half of the cellular protein mass, it is clear that ribosomal metabolism, including ribosomal degradation, makes a worthy object of study. Nevertheless, during the past half century it has been widely believed that mature ribosomes are quite stable in the cells.

The major goal of this dissertation is to describe the degradation of mature ribosomes in growing E. coli cells and to shed light on the molecular mechanism of degradation. We discovered that while mature ribosomes are indeed degraded in cells growing in batch cultures, this process is limited to the slowing of growth phase, which precedes entry into the stationary phase. We were unable to detect degradation during constant-rate growth and during early stationary phase.

In addition, we found that some, but not all, ribosome-inactivating mutations in 23S rRNA and 16S rRNA led to degradation of both mutant and wild-type ribosomal RNAs. Thus, unlike in yeast, the ribosome degradation in E. coli is a general process that, once initiated, does not discriminate between active and inactive ribosomes. As ribosome degradation is inhibited by the protein synthesis inhibitor chloramphenicol, we further suggest that de novo protein synthesis might be needed for triggering the degradation program.

To pinpoint the enzymes responsible for degradation we tested several strains defective for different RNases. We found two RNases, RNaseR and YbeY, whose deletion saved ribosomes from degradation. RNaseR is a well studied 3' to 5' exonuclease whose role in degrading heavily structured RNAs, including the rRNAs, is well established. In contrast YbeY is a potential endonuclease recently implicated in a late step ribosomal quality control, which could well be the initiating endonuclease, whose cut(s) in rRNA would present substrates for RNaseR to further scavenge into mononucleotides.

SUMMARY IN ESTONIAN

Ribosoomide lagundamine bakterites

Ribosoomid on makromolekulaarsed kompleksid, mis koosnevad kahest suurest ja ühest väikesest RNAst ja paljudest erinevatest valkudest. Ribosoomides sünteesitakse kõik valgud, mida organismis leida võib, ning aktiivsete ribsoomide konsentratsioon (ja seega sünteesi kiirus) limiteerib rakkude kasvu kiirust. Ehk teisisõnu, mida kiiremini sünteesitakse uusi ribosoome, seda kiiremini kasvab ja jaguneb ka rakk. Kuna ribosomaalse RNA süntees hõlmab ca 80% raku RNA sünteesi aktiivsusest ja ribosoomi valgud moodustavad kuni veerandi raku valgumassist on selge, et mitte ainult ribosoomide funktsioon valgusünteesil vaid ka nende metabolism on rakulises majapidamises määrava tähtsusega. Tõepoolest, juba mõnda aega on teada, et aeglaselt kasvavates bakterirakkudes tegeleb enamus raku RNA lagundamise võimekusest värskelt sünteesitud ribosomaalse RNA lagundamisega. Sellegipoolest on viimase 50 aasta vältel üldiselt usutud, et kord juba valmis tehtud ja kokku pakitud ribosoomid on äärmiselt stabiilsed ning, et neid lagundatakse vaid tugeva stressi tingimustes. Samuti on meie teadmised ribosoomide lagundamise molekulaarsetest mehhanismidest bakteris üsnagi piiratud.

Käesoleva doktoritöö eesmärk on kirjeldada ribosoomide lagundamist kasvavates soolekepikese (*Escherichia coli*) rakkudes ja heita valgust ribsoomide lagundamise mehhanismidele, molekulaarsetele radadele ning ensüümidele, mis selles protsessis osalevad. Me avastasime üllatusega, et kuigi ribosoome tõepoolest lagundatakse kasvavates bakterirakkudes, toimub see protsess vaid rakukultuuri kasvu aeglustumise perioodil, mis eelneb statsionaarse kasvufaasi saabumisele. Meil ei õnnestunud tuvastada küpsete ribosoomide lagundamist ei ühtlase kiirusega kasvavates ega ka null-kiirusega kasvavates rakkudes. Võimalik, et ribosoomide lagundamine aitab rakke neid ette valmistades eluks statsionaarses faasis, mil ei vajata suurt valgusünteesi võimekust, küll aga vabu komponente, millest elutingimuste paranedes kiiresti uusi makromolekule tootma hakata.

Lisaks leidsime, et osad (kuid mitte kõik) ribosoomi RNAd inaktiveerivad mutatsioonid viivad samuti ribsoomide lagundamisele, kuid miskipärast lagundatakse siis nii mutantseid ning inaktiivseid kui metsiktüüpi ning aktiivseid ribosoome. Jällegi viitab see, et ribsoomide lagundamise eesmärk võiks olla üldise ribosoomide konsentratsiooni alandamine rakus. Kui me lisasime ribsoomide lagundamise katsesüsteemi valgusünteesi pärssivat antibiootikumi kloramfenikool, päästsime me sellega ribosoomid lagundamisest. Seda tulemust võib tõlgendada viisil, et *de novo* valgusüntees on vajalik ribosoomide lagundamisprogrammi käivitamiseks rakus.

Testides ribosoomide lagundamise võime osas bakteritüvesid, kus puuduvad erinevad RNAd lagundavad ensüümid, leidsime kaks ensüümi, mille puudumise korral ribosoome ei lagundatud. Neist esimene, RNaas R, lõhub RNAsid alates nende tagumisest ehk 3’ otsast ning tunneb erilist lõbu kõrge sekundaar-

struktuuriga RNA-de hävitamisest. RNaas R on ka eelnevalt näidatud osalevat ribosoomide lagundamisel. Teine ensüüm on seevastu suhteliselt vähetuntud endoribonukleeas nimega YbeY, mis lõikab RNAd katki keskelt, mitte ei lagunda seda otstest. See huvitav valk on arvatud osalevat ribsoomide kokkupakkimise kvaliteedikontrollil, kus ta on vajalik kõige viimases etapis, mil tuntakse ära valgusünteesil ebaõnnestuvad ribosoomid ja suunatakse need lagundamisse. Meie katsed viitavad, et seesama valk võib valla päästa ka töökorras olevate ribosoomide lagundamise, tehes ribosoomi RNAsse esimese lõike ning tekitades sellega kaitsetu 3' otsa, mida tunneb ära RNaas R, mis omakorda suudab ribosoomi RNA täielikult lagundada.

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PUBLICATIONS

CURRICULUM VITAE

Name: Anton Paier
Date of birth: 20 October 1964
Country of birth: Italy
Citizenship: Swedish
Mother tongue: Italian

Academic degree:
Master degree in Languages and Literatures, Master degree in Biomedical Laboratory Sciences

Current position:
PhD student (2009–2017), Engineering and Technology, University of Tartu, Estonia
Scientist (February 2017–January 2018), Institute of Gerontology, Kiev, Ukraine

Supervisors: Dr. Ülo Maiväli, Prof Tanel Tenson

Academic Education:
Master degree in Biomedical Laboratory Sciences, Karolinska Institute, Sweden, 2009
Advanced Course in Military History, Stockholm University, Sweden, 2004
Master degree in Languages and Literatures, University of Genoa, Italy, 1994

International conferences:
Annual Meeting of the RNA Society, Quebec City, Quebec, 2014
Annual Meeting of the RNA Society, Ann Harbor, Michigan, 2012

Teaching Experience:
Teacher in Italian language 1996–2004 in several private schools and Studie-förbund in Sweden.

Experience in research outside university programs:
Researcher at K.I., Sweden, 2008–2008
Guest researcher at Tartu University, Institute of Molecular and Cell Biology, 2008–2009

List of publications:

Mets, T., Lippus, M., Schryer, D., Liiv, A., Kasari, V., **Paier, A.**, Maiväli, Ü., Remme, J., Tenson, T. and N. Kaldalu (2017). "Toxins MazF and MqsR cleave Escherichia coli rRNA precursors at multiple sites." RNA Biol **14**(1):124–135.

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Piir, K., **Paier, A.**, Liiv, A., Tenson, T. and. Maiväli (2011). "Ribosome degradation in growing bacteria." EMBO Rep **12**: 458–462.

Paier, A., Agewall, S. and K. Kublickiene (2009). "Expression of heat shock proteins and nitrotyrosine in small arteries from patients with coronary heart disease." Heart Vessels **24**(4): 260–6.

ELULOOKIRJELDUS

Nimi: Anton Paier
Sünnikuupäev: 20.10.1964
Sünnimaa: Itaalia
Kodakondsus: Rootsi
Emakeel: Itaalia keel

Akadeemiline kraad:
Magistrikraad kirjandusteaduses, Magistrikraad: Biomedical Laboratory Sciences

Akadeemiline positsioon:
Teadur: K.I., Sweden, 2008–2008
Külalisteadur Tartu Ülikooli Molekulaar- ja Rakubioloogia Instituudis, 2008–2009
Doktorant Tartu Ülikoolis: Engineering and Technology, 2009–2017
Teadur, Institute of Gerontology, Kiev, Ukraine, 2017

Juhendajad: Dr. Ülo Maiväli, Prof Tanel Tenson

Hariduskäik:
Master degree in Biomedical Laboratory Sciences, Karolinska Institute, Sweden, 2009
Advanced Course in Military History, Stockholm University, Sweden, 2004
Master degree in Languages and Literatures, University of Genoa, Italy, 1994

Rahvusvahelised konverentsid:
Annual Meeting of the RNA Society, Quebec City, Quebec, 2014
Annual Meeting of the RNA Society, Ann Harbor, Michigan, 2012

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