DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
167

DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
167

LAURI PEIL

Ribosome assembly factors in *Escherichia coli*

TARTU UNIVERSITY
PRESS

Institute of Molecular and Cell Biology, University of Tartu, Estonia

The dissertation is accepted for the commencement of the degree of *Doctor philosophiae* in molecular biology at the University of Tartu on July 13, 2009 by the Council of the Institute of Molecular and Cell Biology, University of Tartu.

Supervisor: Prof Jaanus Remme
Department of Molecular Biology, Institute of Molecular and Cell Biology
University of Tartu, Estonia.

Opponent: Dr Daniel N. Wilson
Gene Center Munich,
Ludwig-Maximilians-Universität München; Germany

Commencement: September 3, 2009.

The publication of this dissertation is granted by the University of Tartu

ISSN 1024–6479
ISBN 978–9949–19–183–3 (trükis)
ISBN 978–9949–19–184–0 (pdf)

Tartu Ülikooli Kirjastus
www.tyk.ee
Tellimus nr. 286

TABLE OF CONTENTS

LIST OF ORIGINAL PAPERS

Current dissertation is based on the following original publications which will be referred to in the text by their Roman numerals:

I **Ofengand, J; Malhotra, A; Remme, J; Gutgsell, NS; Del Campo, M; Jean-Charles, S; Peil, L; Kaya, Y:** Pseudouridines and pseudouridine synthases of the ribosome. Cold Spring Harb Symp Quant Biol 2001, 66:147–59.

II **Leppik, M; Peil, L; Kipper, K; Liiv, A; Remme, J:** Substrate specificity of the pseudouridine synthase RluD in *Escherichia coli*. FEBS J 2007, 274:5759–66.

III **Ero, R; Peil, L; Liiv, A; Remme, J**: Identification of pseudouridine methyltransferase in *Escherichia coli*. RNA 2008, 14:2223–33.

IV **Peil, L; Virumäe, K; Remme, J**: Ribosome assembly in *Escherichia coli* strains lacking the RNA helicase DeaD/CsdA or DbpA. FEBS J 2008, 275:3772–82.

The journal articles are reprinted with the permission from the copyright owners.

My contribution to the articles is as follows:

Ref. I – I conducted the ribosome profile experiments, prepared Figure 6 and participated in writing of the manuscript.

Ref. II – I constructed the *rluD*-minus strain, conducted P1 transduction, did initial pseudouridine determination on RNA from *deaD*-minus cells and participated in writing of the manuscript.

Ref. III – I conducted the bioinformatics analysis and participated in writing of the manuscript.

Ref. IV – I designed and conducted this study (except for the peptidyl-transferase assays), analyzed the data and drafted the manuscript.

LIST OF ABBREVIATIONS

aa-tRNA – aminoacyl-tRNA
ATPase – adenosine triphosphate hydrolase
dsRNA – double-stranded RNA
GDP – guanosine diphosphate
GDPNP – non-hydrolyzable analog of GTP
G-protein – GTP-binding protein
GTPase – guanosine triphosphate hydrolase
GTP – guanosine triphosphate
L-proteins – ribosome large subunit proteins
PCR – polymerase chain reaction
nt – nucleotide
PR – pseudorevertant
RNase – ribonuclease
RNP – ribonucleoprotein
r-proteins – ribosomal proteins
rRNA – ribosomal RNA
S-AdoMet – S-adenosylmethionine
snoRNA – small nucleolar RNA
snRNA – small nuclear RNA
S-proteins – ribosome small subunit proteins
tmRNA – transfer-messenger RNA
tRNA – transfer RNA
wt – wild-type
Ψ – pseudouridine

INTRODUCTION

Assembly of ribosomes is a complex process which is initiated during ribosomal RNA transcription and that ends with functional ribosomal subunits capable of participating in translation (Kaczanowska and Rydén-Aulin, 2007; Nierhaus, 1991). Each ribosome consists of two subunits (large and a small), whereas each subunit consists of ribosomal RNA(s) and ribosomal proteins, few of them modified to some extent (Kaczanowska and Rydén-Aulin, 2007). Since ribosome is a complex ribonucleoprotein particle, its assembly process involves a number of ribosomal and extra-ribosomal factors (Kaczanowska and Rydén-Aulin, 2007; Wilson and Nierhaus, 2007), many of which are still incompletely characterized. The biogenesis of translational apparatus has been studied extensively for decades, using both *in vivo* and i*n vitro* approaches but there are still many unanswered questions.

Extra-ribosomal factors needed for the effective and correct assembly of ribosomes contain many classes of functionally different proteins – RNA modification enzymes, RNA helicases, RNA chaperones, heat-shock proteins and ribosome-dependent GTPases, to name few (Kaczanowska and Rydén-Aulin, 2007; Wilson and Nierhaus, 2007). So far there is evidence for the direct involvement of extra-ribosomal proteins in the ribosome assembly process only for few; for example most of the rRNA modification enzymes are indispensable for the ribosome assembly and cell viability. In all kingdoms of life, ribosomal RNAs undergo specific post-transcriptional modifications that are thought to play an important role in structure stabilization among other functions (Ofengand and Del Campo, 2004). Of those modifications, pseudouridylation is the most common single modification found (Ofengand, 2002), followed by different species of methylated nucleotides (Ofengand and Del Campo, 2004). Despite the fact that there are many modified nucleotides in ribosomal RNA (total number ranging from 1 to more than 100 modifications per ribosomal RNA molecule) and that they mostly cluster in functionally important regions, no clear function has been assigned to most of them yet. In *Escherchia coli*, all enzymes responsible for pseudouridine synthesis in ribosomal RNA have been identified (Ofengand, 2002) while there are still few methyltransferases missing (Kaczanowska and Rydén-Aulin, 2007). At the same time, many other extra-ribosomal assembly factors mentioned above have been shown to participate in a number of different cellular processes beside ribosome biogenesis, ranging from translation to the heat-shock response.

One of the major unanswered question in ribosome biogenesis has been whether the accumulated abnormal ribosomal particles can mature into 70S ribosomes or not. Ribosome assembly was previously thought to happen via certain pre-determined pathways that were mostly based on *in vitro* results (Nierhaus, 1991). However, some recent studies have shown the heterogeneous nature of such abnormal particles (Charollais et al., 2004; Charollais et al., 2003; Hager et al., 2002; Hwang and Inouye, 2006; Jiang et al., 2006), indicating that there is no singular distinct pathway for ribosome assembly and

that it may be time to re-evaluate ribosome assembly mechanisms. This suggestion is also well supported by the work of James Williamson group, where they have analyzed the assembly map and pathways of ribosome small subunit in detail (Talkington et al., 2005).

Current thesis focuses on the characterization of different extra-ribosomal factors involved in ribosome assembly, with the special focus on RNA modification enzymes and RNA helicases.

REVIEW OF LITERATURE

The ribosome is a large, complex and dynamic ribonucleoprotein particle consisting of a large and small subunit. In *Escherichia coli*, the large (50S) subunit contains two rRNA molecules (23S rRNA, 2904 nt, and 5S rRNA, 120 nt) and 33 ribosomal proteins (r-proteins), whereas the small (30S) subunit contains one rRNA molecule (16S rRNA, 1542 nt) and 21 r-proteins (Kaczanowska and Rydén-Aulin, 2007; Wilson and Nierhaus, 2005). Assembly of ribosomes is a complex and highly coordinated process, which is initiated during rRNA transcription (Lewicki et al., 1993) and involves processing, modification and folding of rRNA and r-proteins, as well as their association to form functional ribosomal subunits. 16S rRNA contains 11 modified nucleotides (10 methylations and 1 pseudouridine) and 23S rRNA contains 25 known modifications (14 methylations, 9 pseudouridines, one methylated pseudouridine and one unknown modification) (Table 1) whereas there are 11 known post-translationally modified ribosomal proteins (Table 2).

Table 1. Modified nucleotides in *Escherichia coli* rRNAs and their modifying enzymes. Table adapted from (Kaczanowska and Rydén-Aulin, 2007) and (Wilson and Nierhaus, 2007) according to current knowledge. Unknown modification is indicated with *.

Modifying enzyme			
Nucleotide	**Modification**	**Name**	**Synonym**
16S rRNA			
516	Ψ	RsuA	YejD
527	m^7G	RsmG	GidB
966	m^2G	RsmD	YhhF
967	m^5C	RsmB	Fmu, YhdB
1207	m^2G	RsmC	YjjT
1402	m^4Cm	RsmH; RsmI	*hypothetical*
1407	m^5C	RsmF	YebU
1498	m^3U	RsmE	YggJ
1516	m^2G	RsmJ	*hypothetical*
1518	$m^6{}_2A$	RsmA	KsgA
1519	$m^6{}_2A$	RsmA	KsgA
23S rRNA			
745	m^1G	$RlmA^I$	RrmA,YebH
746	Ψ	RluA	YabO
747	m^5U	RlmC	YbjF, RumB
955	Ψ	RluC	YceC
1618	m^6A	RlmF	YbiN
1835	m^2G	RlmG	YgjO
1911	Ψ	RluD	YfiI, SfhB
1915	$m^3Ψ$	RluD RlmH	YfiI, SfhB YbeA

Modifying enzyme			
Nucleotide	**Modification**	**Name**	**Synonym**
1917	Ψ	RluD	YfiI, SfhB
1939	m^5U	RlmD	YgcA, RumA
1962	m^5C	RlmI	YccW
2030	m^6A	RlmJ	*hypothetical*
2069	m^7G	RlmK	*hypothetical*
2251	Gm	RlmB	YjfH
2445	m^2G	RlmL	YcbY
2449	D	RldA	*hypothetical*
2457	Ψ	RluE	YmfC
2498	Cm	RlmM	YgdE
2501	*Cc	RltA	*hypothetical*
2503	m^2A	RlmN	YfgB
2504	Ψ	RluC	YceC
2552	Um	RlmE	FtsJ, RrmJ
2580	Ψ	RluC	YceC
2604	Ψ	RluF	YjbC
2605	Ψ	RluB	YciL

Table 2. Modifications of *Escherichia coli* ribosomal proteins. Table adapted from (Arnold and Reilly, 1999) and (Kaczanowska and Rydén-Aulin, 2007) according to current knowledge.

Protein	**Modification**	**Position**
S5	Acetylation	N-terminus (A1)
S6	Glutamic acid residues	C-terminus, up to four E residues are added
S11	Monomethylation; partial modification with isoaspartate	N-terminus (A1); unknown
S12	Methylthio-aspartate	D88
S18	Acetylation	N-terminus (A1)
L3	Monomethylation	Q150
L7/L12	Monomethylation	K81
L12	Acetylation	N-terminus (S1)
L11	Three trimethylations	N-terminus (A1), K3, K39
L16	Monomethylation; unknown	N-terminus (M1); R81
L33	Monomethylation	N-terminus (A1)

It is known that many extra-ribosomal factors are involved in the ribosome assembly process, especially in eukaryotes. For example, there are more than 200 known ribosome assembly factors in *Saccharomyces cerevisiae*, both proteins and small nucleolar RNAs (Hage and Tollervey, 2004). In bacteria, the number of extra-ribosomal components so far identified as being involved in

ribosome assembly is more than ten-fold smaller (Kaczanowska and Rydén-Aulin, 2007; Wilson and Nierhaus, 2007).

Extra-ribosomal assembly factors can be divided into different groups, based on their primary activity or other unique properties. One can classify them on many different ways but for the sake of unification, same classification as proposed by (Kaczanowska and Rydén-Aulin, 2007) and (Wilson and Nierhaus, 2007) will be used here. According to them, extra-ribosomal factors involved in ribosome biogenesis (only some proteins are listed; ribonucleases involved in rRNA maturation have been excluded) are classified as:

1. RNA modification enzymes (RluD (Gutgsell et al., 2005) and RlmE (Bügl et al., 2000; Caldas et al., 2000a; Caldas et al., 2000b),
2. RNA helicases (DeaD/CsdA (Charollais et al., 2004), SrmB (Charollais et al., 2003), DbpA (Elles and Uhlenbeck, 2008; Fuller-Pace et al., 1993) and RhlE (Jain, 2008)),
3. heat-shock proteins (DnaK/DnaJ (Al Refaii and Alix, 2009; Alix and Guérin, 1993) and GroEL (El Hage et al., 2001)),
4. ribosome-dependent GTPases (Era (Inoue et al., 2003; Inoue et al., 2006), RsgA (Campbell and Brown, 2008; Himeno et al., 2004), $CgtA_E$/ObgE (Jiang et al., 2006; Sato et al., 2005) and EngA/Der (Hwang and Inouye, 2006, 2008)),
5. RNA chaperones (RimM (Bylund et al., 1998) and RbfA (Bylund et al., 1997; Bylund et al., 1998; Inoue et al., 2003; Inoue et al., 2006)).

The role of above-mentioned proteins in ribosome assembly and biogenesis in *E. coli* will be described in more detail below.

1. Ribosomal RNA synthesis and processing

In *Escherichia coli*, each rRNA operon is transcribed as a primary transcript molecule (also called 30S RNA) that contains 16S, 23S and 5S rRNA together with extra leader, spacer and trailer sequences. In all of seven *E. coli rrn* operons, spacer region between 16S and 23S gene contains one or two tRNA sequences; some operons contain additional one or two tRNA sequences distal to the 5S gene (Figure 1). Ribosomal RNA primary transcript must be further processed by nucleases into individual rRNA molecules which are then post-transcriptionally modified by a number of modification enzymes. The processing of primary transcript into individual mature rRNA molecules involves a number of different endo- and exonucleases and is a fast process as most of the rRNA present in the cells is mature. First rRNA processing events occur before the transcription of *rrn* operon is completed while the final maturation of rRNA takes place in translating ribosomes (Srivastava and Schlessinger, 1990).

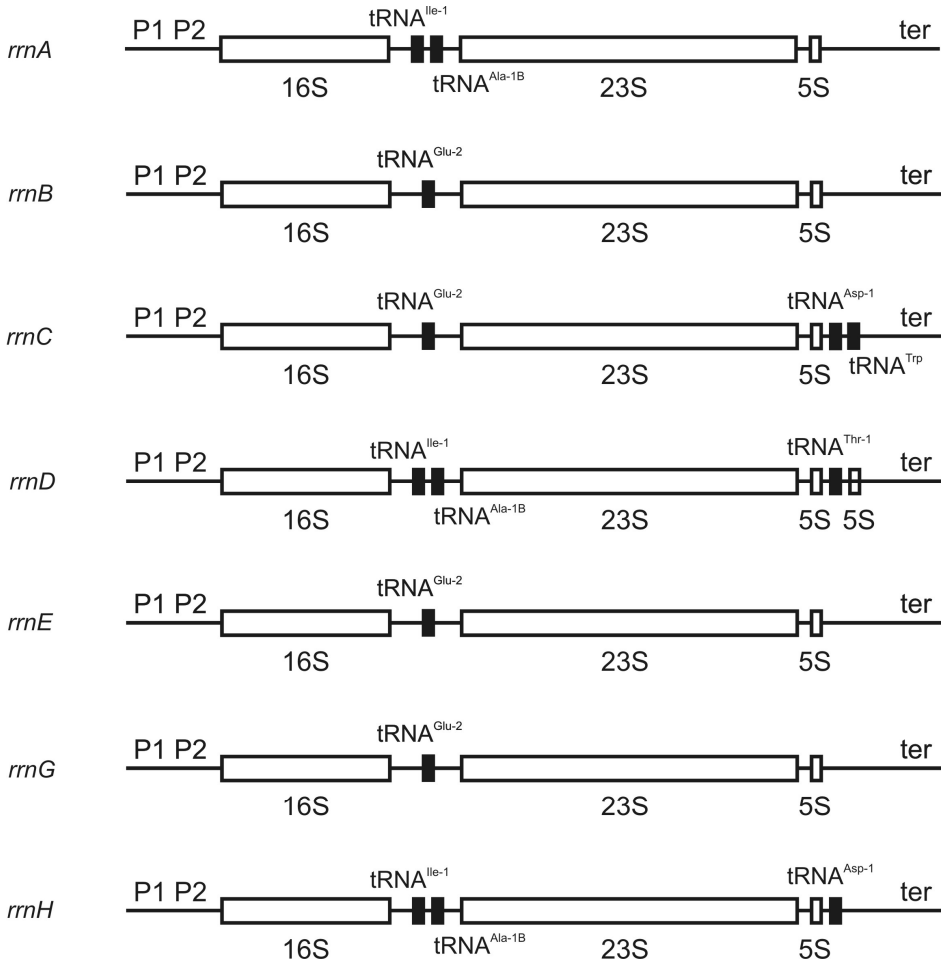

Figure 1. The structure of the rRNA operons in *E. coli*. Open and filled rectangles represent rRNA (16S, 23S, and 5S) and tRNA genes, respectively. The figure also indicates the relative positions of promoters (P1 P2) and terminators (ter). Figure is adapted from (Srivastava and Schlessinger, 1990).

Ribosomal RNA primary transcript is first cleaved by the endoribonuclease RNase III and subsequently processed by other ribonucleases to individual rRNA mature forms (Figure 2, Table 3). RNase III is a double-stranded RNA specific enzyme, which functions as a homodimer and requires divalent metal ion for activity (preferably Mg^{++}) (Robertson et al., 1968). Double-stranded regions in 30S RNA precursor required for RNase III activity are formed by sequences flanking both 16S and 23S rRNA, forming helices known as processing stems. RNase III cleavage takes place already during the transcription of rRNA transcript and results in the release of pre-16S and pre-23S rRNA molecules together with the 3'-terminal part of the primary

transcript, 9S RNA. Pre-16S (17S) rRNA has 115 extra residues in its 5' end and 33 extra residues in its 3' end (Young and Steitz, 1978) whereas pre-23S rRNA has 3 or 7 extra residues in its 5' end (Sirdeshmukh and Schlessinger, 1985) and 8 extra residues in its 3' end (King et al., 1984). 9S RNA contains 5S rRNA sequence and additional sequences that may include one or two distal tRNAs, depending on an exact *rrn* operon sequence (Figure 1).

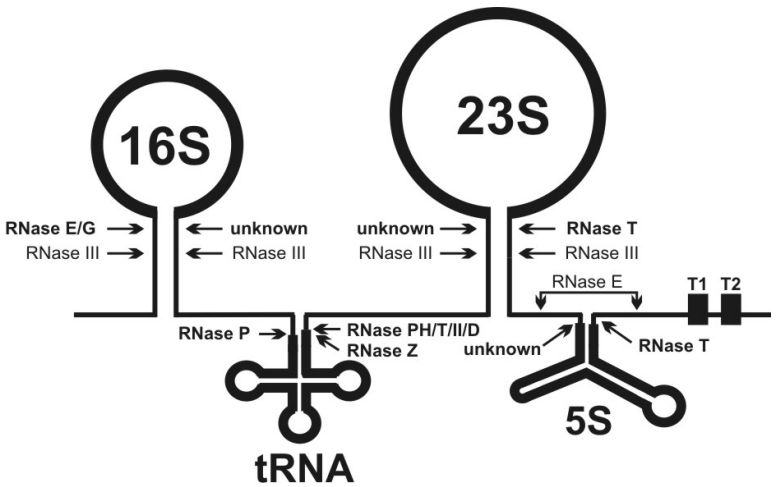

Figure 2. Schematic arrangement of rRNA processing sites. The sites of nucleolytic cleavage and known corresponding enzymes are indicated by arrows. Enzymes responsible for final maturation of individual rRNA termini are shown in bold. tRNA maturation can happen via two pathways – exonucleolytic (RNase PH/TII/D) or endonucleolytic (RNase Z) pathway, as indicated. Figure is adapted from (Liiv, 1998).

It is known that RNase III cleavage sites on 23S rRNA depend whether r-proteins have bound to rRNA or not (Allas et al., 2003). Regarding 5' end of 23S rRNA, RNase III acting on purified 70S or 50S ribosomes results in a 3 nucleotides longer 23S rRNA species, while RNase III acting on de-proteinized RNA (phenol-extracted rRNA from RNase III deficient ribosomes or *in vitro* transcribed rRNA) results in a 7 nucleotides longer 23S rRNA species compared to mature termini (Allas et al., 2003). Interestingly, 3' end processing of 23S rRNA by RNase III is somewhat different. RNase III acting on either purified ribosomes or phenol-extracted RNA from RNase III deficient ribosomes results in 2 or 3 nucleotides longer 3' end than found in mature 23S rRNA (Allas et al., 2003). At the same time, cleavage on *in vitro* transcribed 30S RNA happens at position +8 which has been recognized before as RNase III cleavage site (King et al., 1984). This means that unlike RNase III cleavage at the 5' end where its cleavage sites are determined by the presence of r-proteins, cleavage at the 3' end is not influenced by the presence of r-proteins but is mostly determined by RNA structure.

According to previous data, 16S rRNA can mature in RNase III deficient cells at almost the same rate as in wild-type cells (King and Schlessinger, 1983). In contrast, final maturation of 23S rRNA absolutely requires the initial cleavage by RNase III as in the absence of RNase III only pre-23S rRNA molecules are assembled into ribosomes (King et al., 1984). At the same time, such ribosomes are functional as bacteria lacking RNase III are viable albeit slower growing and defective in translation of some mRNA's (King et al., 1984; Srivastava and Schlessinger, 1990).

1.1. The maturation of 16S rRNA

It was first observed by Murray Deutscher, based on his lab's numerous studies, that none of the presently known exoribonucleases in *E. coli* were required for the maturation of 16S rRNA, leaving the main focus on endoribonucleases. Prime candidate for this was RNase E, as it was known for long time that pre-16S RNA molecules (16.3S) accumulate in an *rne* strain called BUMMER (Dahlberg et al., 1978) and that RNase E is already involved in the processing of 5S rRNA (Apirion and Lassar, 1978; Ghora and Apirion, 1978) and tRNA (Ray and Apirion, 1981). But, it appeared surprisingly that actually two enzymes are required for the final maturation of 16S rRNA 5' end – RNase E and RNase G (CafA) (Li et al., 1999b; Wachi et al., 1999).

RNase E is the main component of RNA degradosome, associating other degradosome components via its scaffold domain (Carpousis, 2007) and it has an extensive role in rRNA and tRNA processing (Apirion and Lassar, 1978; Ghora and Apirion, 1978; Ray and Apirion, 1981). RNase G was first described as a functional homologue of RNase E, sharing an extensive sequence similarity to RNase E N-terminal part and being able to partially suppress temperature-sensitive growth of RNase E mutant strain *ams1* (Wachi et al., 1997; Wachi et al., 1999). The deletion of RNase E or RNase G gene alone does not stop 16S rRNA 5' end from maturation although the maturation rate is greatly reduced (Li et al., 1999b). On the other hand, in the *rne cafA* double mutant no 16S rRNA 5' maturation occurs, and only the initial RNase III cleavage products with 115 extra residues can be detected (Li et al., 1999b). Even though RNase E and RNase G are complementary in their action, their specificity towards 16S rRNA is different. In the absence of RNase E, only two forms of 16S rRNA are detected – mature and +115. In the absence of RNase G, two products appear – +66 and +4/+5 residues in length, the results of RNase E cleavage (Li et al., 1999b; Wachi et al., 1999). Of those, +66 is the primary RNase E cleavage product which is further substrate for RNase G while +4/+5 products are probably the results of less accurate end-maturation process due to the lack of RNase G. The activity of both RNase E and RNase G has been confirmed *in vitro*, where both enzymes were active on different 16S rRNA precursors, in good accordance to *in vivo* data (Li et al., 1999b).

Enzyme(s) responsible for the 3' end maturation of 16S rRNA are still at large although such activity was described and partially purified already in 1976

(Hayes and Vasseur, 1976), unfortunately with no further proof for so far. It was thought to be an endoribonuclease that results in mature 3' termini since no intermediates have been observed. The reaction half-time for the 3' end cleavage is about two-fold slower than is the maturation of 16S rRNA 5' end (King and Schlessinger, 1983), indicating that 16S rRNA 5' and 3' termini are processed independently. This finding is also supported by the fact that 16.3S rRNA accumulated in an RNase E deficient strain does have mature 3' termini (Dahlberg et al., 1978). Furthermore, it was just lately determined that in 21S particles (true precursors to 30S subunits (El Hage and Alix, 2004)) that accumulate in the absence of chaperone DnaK, 5' end of 16S rRNA is matured before 3' end (Al Refaii and Alix, 2009). Taken together, these data support the suggestion that the final maturation of 16S rRNA termini does happen independently while at the same time there is a requirement for the incorporation of at least some r-proteins to the RNA for the final maturation to happen. On the other hand, as 30S subunits containing pre-16S rRNA are not catalytically active, this final maturation has to happen before 30S subunits bind to the 50S subunits to participate in translation.

Table 3. Enzymes involved in the maturation of *Escherichia coli* ribosomal RNA. Listed are enzyme names, corresponding rRNA species and termini, enzyme specificity and resulting termini from nuclease cleavage.

RNA	Terminus	Enzyme	Endo/exo	Result
16S	5'	RNase E	endo	+66; +4/+5
		RNase G	endo	+115; mature
16S	3'	unknown	endo	mature
23S	5'	unknown	endo	mature
23S	3'	RNase T		mature
5S	5'	RNase E	endo	+3
		unknown		mature
5S	3'	RNase E	endo	+3
		RNase T	exo	+1; –1; mature

1.2. The maturation of 23S rRNA

As already noted above, 23S rRNA requires initial RNase III cleavage to be fully matured (King et al., 1984). If there is no RNase III activity present, multiple discrete precursor forms of 23S rRNA accumulate. For example, 5' end can contain from 20 to 97 extra nucleotides, divided between a number of different species (King et al., 1984) while 3' end has only one precursor species with 53 extra nucleotides (King et al., 1984).

It was initially thought that the final maturation of 23S rRNA 3' end is carried out exonucleolytically and that 5' end maturation is performed by an endonuclease, under protein synthesis conditions or conditions favouring protein synthesis, independently of each other (Sirdeshmukh and Schlessinger, 1985; Srivastava and Schlessinger, 1988). While the first assumption was soon proved right, the enzyme responsible for the 5' end maturation of 23S rRNA is unfortunately still at large, despite many efforts to discover it.

Luckily there is much more knowledge present about the 3' end maturation of 23S rRNA and this will be discussed below. It was noted previously that the addition of wt cell extract to ribosomes from RNase III-deficient cells resulted in many different species of 23S rRNA 3' termini, ranging from +8 to –2 nucleotides, indicating an exonuclease activity (Sirdeshmukh and Schlessinger, 1985). This was proven in 1999, when RNase T was shown to be the enzyme mainly responsible for the final maturation of 23S rRNA 3' end (Li et al., 1999a). An exonuclease RNase T had already been described to be required for the end turnover of tRNA (Deutscher et al., 1985) and for the maturation of tRNA (Li and Deutscher, 1994, 1996), 5S rRNA (Li and Deutscher, 1995) and other small stable RNAs (Li et al., 1998). It appeared that only the strains lacking RNase T showed major 23S rRNA 3' end maturation defects (Li et al., 1999a). Somewhat urprisingly, some 3' end trimming took place even in the absence of RNase T, indicating the involvement of other exoribonucleases in the process (Li et al., 1999a). RNase T role in the 3' end maturation process of 23S rRNA was confirmed when purified enzyme was *in vitro* able to efficiently mature both 23S rRNA and ribosomes extracted from RNase T-deficient background (Li et al., 1999a). At the same time, it was also confirmed that 23S rRNA 3' and 5' end maturation happens independently, because 5' end was fully processed in both RNase T-deficient and RNase T-proficient strains (Li et al., 1999a). Thus, it can be concluded that while other exoribonucleases contribute to the initial shortening of 23S rRNA 3' end, only RNase T is required for the final trimming (Li et al., 1999a).

1.3. The maturation of 5S rRNA

The maturation process of 5S rRNA in *E. coli* is poorly understood, although it is known that the complete maturation of 5S rRNA is not essential for cell growth (Li and Deutscher, 1995). A 5S rRNA precursor (9S RNA) accumulates in an RNase E-deficient mutant, consisting of 5S rRNA with 5' extra nucleotides extending to the RNase III cleavage site near the 23S rRNA 3' end and extra nucleotides extending to the operons terminator (Misra and Apirion, 1979). Pre-5S rRNA is released from the 9S RNA by RNase E cleavage, resulting in a product that has extra 3 nucleotides in both 5' and 3' end (Roy et al., 1983). Final maturation of 5S rRNA is inhibited by protein synthesis inhibitor chloramphenicol, suggesting that it happens under protein synthesis conditions (Feunteun et al., 1972; Jordan et al., 1971; Szeberényi et al., 1985).

And, since 5S rRNA precursors have been found in polysomes, this further suggests that final maturation does occur in polysomes (Feunteun et al., 1972).

The maturation of 5S rRNA 3' end is carried out mainly by RNase T, other known exoribonucleases (PNPase, RNase II, PH, D or BN) play little or no role in this process (Li and Deutscher, 1995). It was established that only the absence of RNase T caused incompletely processed 5S rRNA molecules to accumulate (Li and Deutscher, 1995). In the absence of RNase T, predominant precursors contained 2 extra nucleotides in the 3' end as compared to the mature 5S rRNA. Additionally, a series of precursors with up to 10 extra nucleotides were observed, dependent on the exact genetic background (Li and Deutscher, 1995). This finding was in contradiction with previous knowledge that the main 5S precursor has +3 nucleotides in each end, a result of an RNase E cleavage (Roy et al., 1983). Whether those differences are attributable to RNase E specificity differences *in vivo* and *in vitro*, to sequence and structure differences between different *rrn* operons or some yet undescribed nucleolytic activity remains to be studied. RNase T treatment on purified ribosomes from RNase T deficient cells resulted in a rapid conversion of pre-5S rRNA molecules to the mature form, proving its role in the 3' end processing. Isolated pre-5S rRNA molecules with 2 extra nucleotides in the 3' end are rapidly converted to the +1 form by purified RNase T and then, in a slower manner, shortened by two additional nucleotides to generate a -1 product (Li and Deutscher, 1995). Even though such -1 products have not been observed *in vivo*, these were also generated from wild-type 5S rRNA under the same conditions. Taken together, this shows that 5S rRNA 3' end processing is much faster and more accurate when 5S rRNA molecules have been assembled into ribosomes even though their final processing is not a prerequisite for ribosome activity (Li and Deutscher, 1995). In the same study it appeared that the maturation of 3' end of 5S rRNA is independent from the 5' end maturation as 5S rRNA precursors with extended 3' termini had all mature 5' termini (Li and Deutscher, 1995).

Unfortunately, no specific activity responsible for the maturation of 5S rRNA 5' end in *E. coli* has been described to date. It is known that multiple 5' species with either one, two, or three extra nucleotides accumulate in the absence of protein synthesis conditions (Feunteun et al., 1972) and that the same precursor species are observed in RNA pulse-label experiments (Jordan et al., 1970). This suggests that the final maturation of 5S rRNA 5' end is carried out by a yet unidentified exoribonuclease(s) in a similar fashion to 3' end processing although no final conclusion can be drawn.

1.4. The role of other ribonucleases and RNA chaperones in rRNA processing

While there is no direct evidence for PNPase and RNase PH to take part of a specific step in ribosomal RNA processing, deletion of both enzymes together leads to defects in ribosome assembly (Zhou and Deutscher, 1997). PNPase and

RNase PH are the only P_i-dependent 3'-to-5' exoribonucleases known in *E. coli*, with PNPase participating mainly in mRNA decay as one of the components of degradosome (Carpousis, 2007) and RNase PH being involved in tRNA metabolism (Li et al., 1998). Although it was first indicated that both PNPase and RNase PH might play an important role in tRNA metabolism, it appeared later that the absence of PNPase and RNase PH leads to defects in ribosome assembly while tRNA synthesis and processing was left without changes (Zhou and Deutscher, 1997). The absence of PNPase and RNase PH causes cells to acquire a cold-sensitive growth phenotype, a defect that is largely due to the extensive degradation of 23S rRNA and that results in lower than normal level of 50S subunits (Zhou and Deutscher, 1997). Since overexpression of RNase II (3'-to-5' exoribonuclease) can only partially complement the cold-sensitive phenotype of *pnp rph* strain, it shows the specific requirement for at least one P_i-dependent exoribonuclease for the normal cell growth (Zhou and Deutscher, 1997). Whether it is due to the fact that P_i levels influence ribosome metabolism and protein synthesis *in vivo* or some other mechanism, remains to be determined.

Besides ribonucleases that are required for rRNA processing, a group of extra-ribosomal factors are also required. These proteins, alternatively known as RNA chaperones, play an important but yet undetermined role in rRNA processing. At the moment, only 16S rRNA specific RNA chaperones have been characterized, RbfA and RimM being the most studied of them.

RbfA (ribosome binding factor A) was first characterized as a suppressor for cold-sensitive mutation (C23U) in 16S rRNA (Dammel and Noller, 1995). At the same time, deletion of *rbfA* gene led to major growth defects, especially at low temperatures (Dammel and Noller, 1995). Furthermore, ribosome profiles from RbfA-deficient cells showed disrupted balance between the free subunits and ribosomes/polysomes, indicating defects in ribosome biogenesis (Dammel and Noller, 1995). RbfA was found to be associated with the 30S subunits and its deletion caused synthetic lethality with the mutations in 16S rRNA 5' terminal helix (Dammel and Noller, 1995). Later, Jones and Inouye showed that RbfA is a cold-shock protein and that its absence triggers a constitutive cold-shock response in cells (Jones and Inouye, 1996). The exact role of RbfA remained somewhat mystery although it was proposed that RbfA participates in transformation of non-translatable ribosomes to translatable ones at low temperatures (Jones and Inouye, 1996). In 1998 first evidence appeared that RbfA together with RimM participates in the processing of 16S rRNA (Bylund et al., 1998). RimM (ribosome maturation factor M) was identified earlier, when it was shown to be associated specifically with the 30S subunits and that its deficiency effected translational efficiency and led to an accumulation of 17S RNA (Bylund et al., 1997). The slow-growth phenotype of *rimM*-minus cells was rescued by over-expression of RbfA (Bylund et al., 1998), in a similar manner to a cold-sensitive 16S rRNA suppression observed by Dammel and Noller (Dammel and Noller, 1995). Interestingly, it was observed that 16S rRNA processing was defective in both *rimM*-minus and *rbfA*-minus cells and that overexpression of RbfA increased the 16S rRNA processing efficiency in *rimM*-minus background only slightly (Bylund et al., 1998). This finding placed both

RimM and RbfA among proteins needed for the correct and efficient rRNA processing, despite knowing their exact role. The processing defects of 16S rRNA and aberrations in ribosome profiles in *rbfA*-minus cells were later independently confirmed by Xia et al (Xia et al., 2003). Expression of mutated RimM protein in *rimM*-minus background resulted in a similar ribosomal phenotype to *rimM*-minus cells but these defects could be suppressed by mutations in r-proteins S13 and S19 or in helices 31 and 33b of 16S rRNA (Lövgren et al., 2004). Since r-protein S13 interacts with S19 and both S13 and S19 interact with 16S rRNA helices 31 and 33b, this suggests that RimM has role in the correct maturation of ribosome small subunit head region.

As discussed later, the role of RbfA in ribosome biogenesis was also supported by the fact that over-expression of Era could suppress defective ribosome assembly and 16S rRNA processing in RbfA-deficient strain (Inoue et al., 2003). Furthermore, it was soon suggested that RbfA and Era have over-lapping function in ribosome biogenesis, probably via competition for the binding of 30S subunit (Inoue et al., 2006). The binding site for RbfA was shown to be overlapping with binding sites for A and P site tRNAs, it was also in the immediate vicinity of the binding site for Era (Datta et al., 2007). This in turn suggested that in the absence of RbfA, Era could stabilize helix 1 in 16S rRNA, thus overtaking RbfA role and helping the 30S subunits to mature (Datta et al., 2007), a suggestion well supported by the results of Dammel and Noller (Dammel and Noller, 1995). Still, there is one more intriguing point to mention – the binding of RbfA to the 30S subunits shifts helix 44 of 16S rRNA in such a way that 30S:RbfA complex is unable to bind to the 50S subunits (Datta et al., 2007). This on the other hand suggests that RbfA acts as a 'quality sensor' that will not allow the 30S subunits with incompletely matured 16S rRNA 5' end to enter translation initiation cycle. At the same time, RbfA does not have any negative influence on translation *in vitro* or *in vivo*, suggesting that it rather acts selectively on pre-30S subunits, especially under cold-shock conditions, to provide cells with a continuous supply of functional 30S subunits (Datta et al., 2007).

1.5. The maturation of tRNA

RNase P is required for the maturation of most of the *E. coli* tRNA 5' ends (Hartmann et al., 2009) while 3' end maturation can happen via two different pathways. First, it can happen with the help of exonucleases RNase PH/T/II/D (Kelly et al., 1992; Li and Deutscher, 1994, 1995, 1996; Li et al., 1998; Reuven and Deutscher, 1993) or through the endonucleolytic cleavage by RNase Z (for a review see (Redko et al., 2007) and (Hartmann et al., 2009).

2. Modifications in the ribosomal RNA and the role of RNA modification enzymes in ribosome assembly

The exact role of RNA modification enzymes in the ribosome assembly process is not clear, despite all of the efforts done so far. Basically, opinions about their role in ribosome assembly were in past divided into two – (1) only modifications are of importance and enzymes can be left out, and (2) only the (another function of) modification enzyme is of importance and modifications can be left out, since they only act as signals.

As with most things in life, information to support both hypothesis was gathered but in the end only the first hypothesis survived when ribosome assembly is concerned. For example (and this will be covered in more detail below), RluD and RlmE are two of such enzymes in *E. coli* that are required in their active form for the correct assembly of ribosomes (Bügl et al., 2000; Caldas et al., 2000b; Gutgsell et al., 2005; Hager et al., 2002). Intriguingly, even though the lack of their respective modifications will lead to deficiencies in translation, some (known and unknown) second-site mutations can rescue such defects without the re-appearance of corresponding modifications (Ejby et al., 2007; Tan et al., 2002).

TruB, TrmA and Pet56p on the other hand were some of the RNA modification enzymes that were initially shown to be needed for cell growth even when they were catalytically inactive, thus supporting the second hypothesis (Gutgsell et al., 2000; Persson et al., 1992; Sirum-Connolly and Mason, 1993). RluD was in the beginning also implicated to have a second function unrelated to its primary catalytic activity (Gutgsell et al., 2001) but that conclusion was later withdrawn (Gutgsell et al., 2005). Unfortunately, there is a lack of strong evidence to support the suggestion that Pet56p and TrmA have a second function, unlinked to their primary catalytic activity, as originally proposed.

Recently, such evidence have been gathered for KsgA, a highly conserved enzyme responsible for the synthesis of two $m^6{}_2A$ in 16S rRNA in *E. coli* (O'Farrell et al., 2004). KsgA is also involved in ribosome biogenesis (Connolly et al., 2008) and it was proposed to act as a quality control element, segregating those 30S subunits that are still being assembled or processed from those that are fully mature and ready to participate in the translation cycle (Xu et al., 2008). At the same time, KsgA is indicated to have a role in the suppression of cold-sensitive cell growth of a GTPase *era* mutant (Inoue et al., 2007) (described in more detail later) and lately, to have a DNA glycosylase/AP lyase activity (Zhang-Akiyama et al., 2009). This makes KsgA the strongest candidate so far to support the second hypothesis presented above in general although the exact role of its another function and relatedness to the methyltransferase activity/ribosome assembly needs to be confirmed.

What is the exact role of the modifications in ribosomal RNA? It is believed that modified nucleotides confer extra stability and rigidity to RNA structure and in accordance to this, they are mostly found in highly structured RNA species where correct tertiary structure is of utmost importance. On the rRNA

secondary structures, localization of modifications appears to be random. However, when mapped on the ribosome tertiary structures they occur in the functionally most important regions (Ofengand and Del Campo, 2004). This in turn suggests that modified nucleotides may have an important role in protein synthesis, a suggestion well supported by evidence in case of RluD (Gutgsell et al., 2005) and RlmE (Hager et al., 2002) where catalytically inactive enzymes are not able to suppress defects caused by the lack of corresponding modifications in 23S rRNA. Another common role for the modifications in rRNA is to confer either resistance or sensitivity to the ribosome-targeting compounds, especially antibiotics, as is the case with KsgA specific methylations in 16S rRNA (O'Farrell et al., 2004).

Pseudouridine is made post-transcriptionally from uridine residues (Figure 3), in an oligo- or polynucleotide level. Isomerization reaction is catalyzed by pseudouridine synthases in an ATP-independent manner and in bacteria most of the enzymes are responsible for the catalysis of a single modification. Methylation of RNA nucleotides can happen on ribose (2'-hydroxyl) or on nitrogen base (typically on carbon, primary nitrogen or tertiary nitrogen) and similarly to pseudouridylation, most of the methyltransferases are responsible for the catalysis of a single modification. Unlike with pseudouridine synthases that do not require any co-factors, methyltransferases usually require S-AdoMet as a co-factor and methyl group donor.

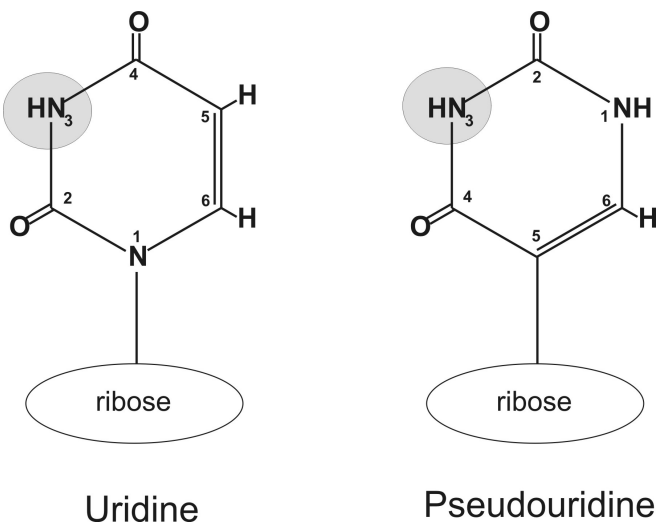

Figure 3. The structure of uridine and pseudouridine. Pseudouridine is made from uridine by the cleavage of N-glycosyl bond, rotation of the uracil ring (C-5 goes to N-1 position), followed by re-formation of glycosyl link as a C-C bond.

2.1. Pseudouridine synthase RluD

RluD (ribosome large subunit pseudouridine synthase D), a pseudouridine synthase responsible for the catalysis of three pseudouridines in helix 69 of 23S rRNA was initially identified and characterized independently by two groups, based on its activity *in vitro* (Huang et al., 1998; Raychaudhuri et al., 1998). Uridines at positions 1911, 1915 and 1917 are conserved among many organisms (with 1915 and 1917 being universally conserved) (Ofengand, 2002) and are specifically pseudouridylated by RluD in *E. coli*. Of those three residues, pseudouridine at position 1915 is further methylated by the methyltransferase RlmH (Ero et al., 2008; Purta et al., 2008).

Even though it was first mentioned in 1994 that a mutation in *rluD* gene causes slow growth in *E. coli*, implicating its role in cell growth (Myler et al., 1994) no further proof of this was provided for few years. At the same time, an allele of *rluD* had been identified previously as a suppressor for the thermosensitive mutation in HflB (FtsH) (Myler et al., 1994), a heat-shock and ATP/Zn^{++}-dependent metalloprotease required for the proteolytic degradation of sigma-32 among other proteins (Schumann, 1999). In this context it is needed to state that the genes for methyltransferase *rlmE* (reviewed in detail below) and *hflB* form a bi-cistronic operon where one of the promoters is controlled by sigma-32 transcription factor and that *rluD* gene is located upstream of *clpB*, a sigma-32 regulated gene encoding a chaperone involved in protein degradation and disaggregation (Doyle and Wickner, 2009). Whether the suppression of $HflB^{ts}$ mutation by RluD was due to its enzymatic activity and role in ribosome biogenesis or due to fact that RluD is located upstream of *clpB*, is unknown to date. There is no information whether such links between *rlmE*, *ftsH*, *rluD* and *clpB* are of any biological importance but even the sole existence of such indirect links between various enzymes that are involved in ribosome biogenesis is highly intriguing.

In 1998, two independent studies were published where *rluD* gene was disrupted and resulting growth phenotype was characterized in more detail. James Ofengand's group used the interruption of *rluD* gene with a miniTn*10* transposon and Daniel Santi's group used the interruption of *rluD* gene with a Kan^{R}-cassette (Huang et al., 1998; Raychaudhuri et al., 1998). Both groups recorded a severe reduction in cellular growth rate in *rluD*-minus cells which was rescued by the introduction of plasmid-borne *rluD* gene (Raychaudhuri et al., 1998), (Huang et al., 1998). Interestingly, a set of faster-growing colonies appeared when *rluD*-minus cells grown in liquid media were plated on LB-plates (Raychaudhuri et al., 1998). Such cells had a growth rate identical to that of wt cells while they were still unable to produce pseudouridines in helix 69 of 23S rRNA. This was probably due to a second-site mutation(s) as the slow-growth phenotype was easily restored by the transduction of interrupted *rluD* gene back to wt background (Raychaudhuri et al., 1998). Few years later a study was published in which it was claimed that catalytically inactive forms of RluD protein are capable of restoring the slow growth phenotype (Gutgsell et

al., 2001). First of all, based on the sequence homology analysis and *in vitro* experiments, aspartate at position 139 in RluD was shown to be the catalytic amino acid responsible for its activity (Gutgsell et al., 2001). Second, it had appeared that the *rluD*-minus strain described by Raychaudhuri et al (Raychaudhuri et al., 1998) already contained a second-site mutation and, based on colony morphology, it was termed *rluD*-minus Tiny whilst the primary disruption strain was termed *rluD*-minus Dust. Tiny strain had ~2-fold longer doubling time whereas Dust had ~5-fold longer doubling time compared to wt cells (Gutgsell et al., 2001). Intriguingly, catalytically inactive RluD mutants RluD(D139N) and RluD(D139T) were still able to form pseudouridines at 23S rRNA positions 1911, 1915 and 1917 *in vivo*, when expressed in *rluD*-minus Dust background but failed to show any activity in *rluD*-minus Tiny background (Gutgsell et al., 2001). Even more, those RluD protein mutants were able to support growth of both Dust and Tiny strains, suggesting that RluD has a second function independent of its catalytic activity.

Unfortunately, these results proved to be wrong when another paper on RluD was published by James Ofengand group few years later (Gutgsell et al., 2005). By that time they had succeeded in constructing a full deletion of *rluD* gene, a strain that was studied in depth and that failed to reproduce any of the intriguing results obtained earlier. Most importantly, this time there was no growth phenotype suppression or RluD-specific pseudouridine synthesis when catalytically inactive forms of RluD proteins were expressed in RluD-deficient cells while active form of RluD resulted in full suppression and re-appearance of pseudouridines (Gutgsell et al., 2005). At the same time, this work revealed many interesting results about RluD role in ribosome assembly, expanding the data known previously (Ofengand et al., 2001).

As shown by Gutgsell and colleagues, the deletion of *rluD* gene led to massive changes in ribosome profile, with the reduction of free 70S ribosomes, increase of free subunits and appearance of new 62S and 39S particles (Gutgsell et al., 2005), similar to the results obtained previously (Ofengand et al., 2001). Interestingly, 62S and 39S particles were observed this time only when lysates were either separated or lysed under low Mg^{++} concentrations (≤10 mM). Lower Mg^{++} concentrations either shifted or converted free 70S ribosomes completely towards the 62S particles, indicating the improperly packed nature of free 70S ribosomes. The 39S particles on the other hand were observable only when 6 mM Mg^{++} concentration was used during lysis. Use of 20 mM Mg^{++} resulted in complete loss of 39S particles, independent of gradient conditions. Although it was claimed that the use of higher Mg^{++} concentration during lysis allows 50S subunits to stay stably associated and therefore no 39S particles are observable, one also cannot rule out differences in lysis efficiency. Nevertheless, closer inspection of 39S particles revealed them to be precursors to the 50S subunits, as they contained incompletely processed 23S rRNA (Gutgsell et al., 2005). Surprisingly, at the same time incompletely processed 16S rRNA was found in the *rluD*-minus 30S subunits (Gutgsell et al., 2005). While there is no clear explanation why the lack of three pseudouridines in 23S rRNA should affect

maturation of 16S rRNA or 30S subunits, similar phenomenon was observed in strains deficient for ribosome large subunit assembly factors SrmB and DeaD (Charollais et al., 2004; Charollais et al., 2003), at that time thought to be an indirect consequence of deficiencies in 50S subunit biogenesis.

It is worth to note that even with the new deletion strain of *rluD* gene, there was still a high frequency of pseudoreversion events (Gutgsell et al., 2005). Such isolated pseudorevertants did still have the disrupted *rluD* gene, lacked RluD-specific pseudouridines but had a growth rate close to that of wt cells and had only modest and slightly variable defects in ribosome profiles (Gutgsell et al., 2005). First light on possible second-site mutations that could suppress defects seen in RluD-deficient cells was shed in 2007, when it was discovered that a point mutation in RF2 (RF2(E172K)) is able to suppress growth defect of *rluD*-minus Dust strain (Ejby et al., 2007). Furthermore, even though *rluD*-minus Dust cells had a 2 to 16- fold higher stop codon read-through rate than that of wt cells, introduction of RF2(E172K) into *rluD*-minus Dust background reduced it back to the wt level with one exception. For some yet unknown reason, cells expressing RF2(E172K) were about 10-fold more accurate on termination on UGA stop codons than wt cells and that was observed both in *rluD*-minus and *rluD*+ background (Ejby et al., 2007). It had been determined previously that free 70S ribosomes from *rluD*-minus strain had ~80% activity of wt ribosomes in *in vitro* poly(Phe) synthesis (Gutgsell et al., 2005), indicating that ribosomes from *rluD*-minus cells are almost fully functional, once they have reached maturation. While 20% difference in the translation rate could not explain big differences seen in growth rates, changed stop codon read-through rates could explain such differences easily. It was also shown previously that while many point mutations in helix 69 of 23S rRNA (U1917C among others) cause strong growth defects and such mutant ribosomes are inactive under *in vitro* conditions (Liiv et al., 2005), ribosomes with deleted helix 69 of 23S rRNA are active and almost as accurate as wt ribosomes *in vitro* although the deletion of helix 69 is dominantly lethal to the cells (Ali et al., 2006). Therefore it was suggested by Ejby et al that at least one pseudouridine in helix 69 of 23S rRNA is involved in translation termination by RF2 and that most if not all defects seen in *rluD*-minus cells are mostly due to errors in translation termination. Especially, disrupted ribosome profile and errors in assembly pathway could be explained as being indirect consequences of global errors in cellular translation, as the synthesis of most of the ribosomal proteins is translationally coupled. Even more, it was determined that *rluD*-minus cells spend about twice the amount of energy compared to wt cells to produce the same amount of biomass (Ejby et al., 2007). At the same time, *rluD*-minus cells have higher expression of heat-shock proteins, further suggesting that translational errors are the cause for later re-folding and/or degradation of mistranslated proteins and therefore also for the extra energy expenditure (Ejby et al., 2007).

Although it was shown that a single point mutation in RF2 is able to rescue *rluD*-minus phenotype, few of the other isolated pseudorevertants still had wt

RF2 sequence, indicating that there are at least two different pathways for pseudoreversion of which only one is known by now (Ejby et al., 2007). Having multiple pathways is also supported by the high frequency of pseudoreversion events observed (Ejby et al., 2007; Gutgsell et al., 2001; Gutgsell et al., 2005).

During the first characterization of RluD, it was suggested that it recognizes helix 69 of 23S rRNA and then isomerizes all uridines to pseudouridines in a position-inspecific manner (Raychaudhuri et al., 1998). Using a set of point-mutations in helix-loop 69, it was shown recently that RluD is highly specific to positions 1911 and 1917 while position 1915 could not be determined due to experimental limits (Leppik et al., 2007). At the same year, it was also determined that 50S subunits are much better and more efficient substrates to RluD than free 23S rRNA (Vaidyanathan et al., 2007). Furthermore, it was implicated that RluD acts on a late step of ribosome assembly, a conclusion which is in very good correlation with results by Leppik et al (Leppik et al., 2007). In conclusion, it can be said that RluD is one of the few RNA modification enzymes that is required for efficient ribosome biogenesis and that acts better on assembled ribosomal subunits than on free RNA.

2.2. RNA methyltransferase RlmE

RlmE (RlmE stands for ribosome large subunit methylase E according to unified nomenclature; previously called RrmJ or FtsJ) was identified and characterized as an RNA methyltransferase involved in ribosome biogenesis simultaneously by two groups in 2000 (Bügl et al., 2000; Caldas et al., 2000a). Caldas et al established first that RlmE is a heat-shock protein which catalyzes the formation of 2'-O-methyluridine in *E. coli* 23S rRNA position 2552 (Um2552) (Caldas et al., 2000a), with a strong preference towards ribosomes rather than free rRNAs as the methyl group acceptor; this finding was independently confirmed by Bügl et al (Bügl et al., 2000). *rlmE* is the upstream gene in a bi-cistronic operon that also encodes for the heat-shock protease HflB (FtsH), this makes RlmE the first modification enzyme linked to the heat-shock response (Bügl et al., 2000). At the same time, U2552 is one of the five universally conserved residues in the A-loop region of 23S rRNA (Moazed and Noller, 1989).

RlmE has homologs in a variety of archaea, eubacteria and eukarya, homologs ranging from 210 to over 840 amino acids in length (Bügl et al., 2000). It has a conserved S-AdoMet binding motif, first found by protein amino acid sequence analysis and later confirmed by crystal structure analysis. Based on 3D structure analysis, one can say for sure that RlmE does indeed have a conserved methyltransferase fold and that it binds S-AdoMet with some of its highly conserved amino acids (Bügl et al., 2000).

On a biological level, the deletion of *rlmE* gene led to a severe reduction in growth rates at all studied temperatures (Bügl et al., 2000; Caldas et al., 2000b), and caused a temperature-sensitive phenotype, with a reduction of maximum

growth temperature by more than 2 °C (Bügl et al., 2000). Not surprisingly, such a decrease in maximal growth temperature has been observed in null mutants for the heat-shock proteins like DnaK, DnaJ and others (reviewed in (Gross, 1996)). Furthermore, *rlmE*-minus cells failed to adjust their growth rate as a response to the temperature shift, indicating the importance of RlmE in adaptation to temperature shifts (Bügl et al., 2000).

In addition to the role RlmE has in rRNA methylation, it was shown to take part of ribosome assembly at the same time. Namely, the lack of RlmE caused an accumulation of free 50S and 30S subunits, at the expense of the 70S ribosomes and polysomes under non-stringent magnesium concentrations (Bügl et al., 2000; Caldas et al., 2000b). Under stringent magnesium concentration that favors dissociation of 70S ribosomes into free subunits, appearance of a new 40S particle was recorded, at the expense of free 50S subunits (Bügl et al., 2000). RNA analysis confirmed 40S particle to be derived from the 50S subunits (Bügl et al., 2000), but as 40S particles were observed only under dissociating conditions, this suggested them to be 'loosely' assembled 50S subunits rather than authentic assembly precursors. Based on a study where *rlmE*-minus ribosomal particles were analysed for their protein content, it appeared that free 50S subunits from wild-type and *rlmE*-minus strain were identical while 40S particles contained at least seven r-proteins in diminished amounts (Hager et al., 2002). These proteins, L5, L16, L18, L25, L27, L28 and L30, belong all to the group of late assembly proteins (Nierhaus, 1991). Based on that finding and on a fact that of all ribosomal particles observed, only 70S ribosomes and free 50S subunits served as a suitable substrate for RlmE while neither free 23S rRNA or other ribosomal particles could not be effectively methylated (Bügl et al., 2000; Caldas et al., 2000a), RlmE was implicated to take part at the very late step of ribosome assembly, well after other known extra-ribosomal factors. What was its exact role in ribosome assembly, remained to be discovered.

In 2002 it was established that it is indeed the methylation activity of RlmE which is needed for normal cell functioning (Hager et al., 2002). This was proven by using a set of different point mutations in proposed active sites of RlmE. It became apparent that all mutated RlmE isoforms incapable of the methylation reaction *in vitro* had growth defects and aberrant ribosome profiles similar to the original *rlmE*-minus strain when expressed in *rlmE*-minus background (Hager et al., 2002). A follow-on study by the same group, using higher number of mutated RlmE isoforms, confirmed previous results (Hager et al., 2004). Furthermore, this time they were able to define the minimal substrate for the RlmE protein to be an unmodified A-loop of 23S rRNA, although the methylation of A-loop took place at a much slower rate compared to 50S subunits (Hager et al., 2004). While it was clear by that time that catalytically active RlmE protein is needed for normal cell functioning, it was not clear which role Um2552 residue had in it.

Previously, it was shown that the *in vitro* protein synthesis rate of *rlmE*-minus cell extract was 1.6 to 2.8-fold lower compared to wt cell extract, when

using *lacZ*-programmed system (Caldas et al., 2000b). Surprisingly, protein synthesis rate of RlmE-deficient cell extract was increased two-fold (compared to unmethylated extracts), when cell extracts were incubated with purified RlmE protein and S-AdoMet beforehand (Caldas et al., 2000b). Based on these results, a suggestion was made that solely the lack of Um2552 in 23S rRNA is responsible for the observed growth, ribosome assembly and translational defects but it took few more years to gather evidence to prove the essentiality of Um2552 for effective translation. Apparently, ribosomes lacking Um2552 have around ten times lower frameshift rates and about 5-fold lower stop codon readthrough rates than wt ribosomes, making them much more accurate (Widerak et al., 2005). Such an increase in accuracy could be due to the enhanced codon-anticodon interaction during aa-tRNA binding, due to the changes in accommodation/proofreading step or due to some other interaction between translation system components. Based on the data that methylation of U2552 affects translation accuracy, an interesting cause was proposed for why RlmE belongs to the heat-shock regulon (Bügl et al., 2000). Namely, even though there are no results showing any influence of heat-shock on U2552 methylation levels, U2552 could be unmethylated under some circumstances when it is advantageous for the cell to gain improved translational accuracy at the expense of speed. This way RlmE could belong to a protein quality control pathway, taking an active part during translation while other heat-shock proteins as chaperones DnaK, GroEL etc act on later steps like protein folding. Furthermore, the essentiality of an Um2552 modification in 23S rRNA for effective translation was one of the first indications that defects seen in ribosome assembly could be due to global errors in translational apparatus and not due to the lack of corresponding modification enzyme.

3. Other extra-ribosomal factors involved in ribosome assembly

RNA helicases, ribosome-dependent GTPases, heat-shock proteins and RNA chaperones are thought to participate in the process of rRNA processing and folding. RNA can and will readily form stable secondary structures, many of which are of wrong conformation, although energetically stable. To overcome such stable structures, there is a specific need for extrinsic factors that could unwind dsRNA helices (RNA helicases), keep RNA in right conformation for the next processing step to take place (RNA chaperones) or change the conformation of already assembled macro-molecular complexes (ribosome-dependent GTPases and heat-shock proteins). Below, an overview of the role of RNA helicases, ribosome-dependent GTPases and heat-shock-proteins in ribosome biogenesis will be given.

3.1. The role of RNA helicases in ribosome assembly

Based on the occurrence and characteristics of conserved motifs in amino acid sequence, helicases are divided into three major superfamilies and two families (named as SF1 to SF5) (for a review see (Cordin et al., 2006)). DExD/H family of putative RNA helicases, consisting of DEAD, DEAH, DExH and DExD-box families, belongs to SF2 and is characterized by eight conserved motifs. The DEAD-box family is by far the largest family among DExD/H helicases, characterized by the presence of nine conserved motifs that are involved in the ATPase and helicase activities. The name of the family was derived from the amino-acid sequence D-E-A-D (Asp-Glu-Ala-Asp) of one of the conserved motifs, Walker B motif.

Usually, DEAD-box proteins contain a core of ~400 amino acids in length that contains conserved motifs and highly variable N- and C-terminal extensions in size and composition. These extensions are thought to give specificity towards corresponding substrates or cofactors or alternatively, to confer additional activities. To date, DEAD-box RNA helicases have been associated with nearly all cellular processes involving RNA, from transcription to mRNA decay.

Escherichia coli has five members of DEAD-box helicases –DeaD/CsdA, SrmB, DbpA, RhlB and RhlE that all have an RNA-dependent ATPase and RNA helicase activities (Iost and Dreyfus, 2006). While their preference towards targets and their activity differ, all of them are able to dissociate short RNA duplexes on an ATP-dependent manner. So far, many different functions have been assigned to them like ribosome biogenesis (DeaD, SrmB and DbpA), mRNA decay (RhlB and DeaD) and translation assistance (DeaD), some of which are reviewed in detail below.

3.1.1. RNA helicase DeaD

DeaD (for DEAD-box helicase, later renamed CsdA for cold-shock DEAD-box protein A (Jones et al., 1996)) was first characterized in 1991 as an hypothetical ATP-dependent RNA helicase which can suppress a temperature-sensitive mutation in ribosomal protein S2 (Toone et al., 1991). S2 is one of the last r-proteins to be assembled into small ribosome subunit and it is required for the binding of S1 to the 30S subunit (Laughrea and Moore, 1978). This indicated that DeaD may have a role in the biogenesis of ribosomes, specifically small ribosomal subunit biogenesis, but it took more than ten years until any of this was proven. In 1996, Jones and colleagues showed that DeaD is a ribosome-associated protein capable of unwinding double-stranded RNA (Jones et al., 1996). Furthermore, it appeared that its expression is heavily up-regulated under cold-shock and that its knockout has a severe cold-sensitive phenotype, causing the appearance of long filamentous cells after prolonged incubation at 15 °C (Jones et al., 1996). Also, they suggested that DeaD is rather a helix-

destabilizing protein than an RNA helicase as it was capable of dissociating dsRNA without the need for ATP (Jones et al., 1996). Few years later, Lu et al also failed to record any ATPase activity of purified DeaD protein, whether in the presence or absence of a variety of polynucleotides (Lu et al., 1999). At the same time, they observed the ability of DeaD to facilitate translation initiation from the highly structured mRNA (Lu et al., 1999). The absence of DeaD ATPase activity was later disproved when it became apparent that it has ATPase activity which is heavily stimulated by the presence of RNA (Bizebard et al., 2004; Prud'homme-Généreux et al., 2004; Turner et al., 2007).

Overall protein expression analysis from *deaD*-minus cells revealed another interesting result where the expression of heat-shock proteins DnaK and GroEL was continuously derepressed in *deaD*-minus cells following a temperature shift from 37 °C to 15 °C. Such a result indicates that DeaD (which itself is a cold-shock induced protein) takes part of the heat-shock response regulation (Jones et al., 1996). This all led to the conclusion that DeaD plays a major role in mRNA translation, especially at low temperature, probably by unwinding stable secondary structures in mRNAs which allows ribosomes to translate successfully. Nevertheless, the question remained – how can DeaD complement the lack of S2 protein? It was proposed that DeaD can suppress the mutation in *rpsB* (which encodes S2 protein) by stimulating S2 translation, for example by destabilizing secondary structures in S2 mRNA. Such enhanced S2 expression could then compensate for the defect in temperature-sensitive S2 protein (Toone et al., 1991). This hypothesis was proven wrong when there was no observable change in neither S2 or S1 expression levels when DeaD was overexpressed from the plasmid in *rpsB*^{ts} cells (Moll et al., 2002). Interestingly, even though DeaD overexpression did not affect S2 expression, it was still capable of restoring both S2 and S1 on the ribosomes via some unknown pathway (Moll et al., 2002). Another hypothesis was that *rpsB* mutation suppression by DeaD overexpression may be due to the functional complementation of S1 by DeaD, as S1 is capable of unwinding dsRNA in an ATP-independent manner similarly to DeaD (Jones et al., 1996; Lu et al., 1999). Somewhat controversially to other results, Moll and colleagues were not able to detect ribosome-associated DeaD, therefore their proposed mechanism for the DeaD-assisted *rpsB*^{ts} suppression was through ribosome assembly pathway where DeaD induces a conformational change in 16S rRNA which in turn results in binding of mutated S2 and consequently, S1 to the ribosome (Moll et al., 2002). While their hypothesis about DeaD being involved in ribosome assembly was proven correct two years later (Charollais et al., 2004), there is so far still no evidence about DeaD being a ribosome small subunit specific assembly factor. Instead, all results published so far indicate that DeaD is involved only in the biogenesis of 50S subunit (Charollais et al., 2004; Peil et al., 2008).

For example, *deaD*-minus cells grown at 20 °C had less polysomes than their wt counterparts and the balance between free ribosomal subunits was also changed, with an increase in the amount of free 30S subunits compared to the 50S subunits together with the appearance of a new, 40S particle (Charollais et

al., 2004). Further analysis with the 40S particle confirmed that it is indeed a precursor for the ribosome large subunit which is incompletely assembled. First of all, it contained mainly incompletely processed pre-23S rRNA derived from the initial RNase III cleavage and second, it contained just a subset of L-proteins (Charollais et al., 2004). According to these results it was claimed that 40S particles are not authentic precursors but are rather mis-assembled ribosomal large subunits whereas the increase in the amount of free 30S subunits is probably mostly due to the lack of functional 50S subunits (Charollais et al., 2004). On the other hand, as shown in the Results part of the thesis, 40S particles are not assembly dead-end products either as they can be matured into at least partially functional large subunits, albeit at much slower rate (Peil et al., 2008).

Though it was previously suggested that DeaD could be a part of small subunit assembly pathway, there was no proof for that as 30S subunits from *deaD*-minus cells were identical to their wt counterparts, both on RNA and protein level (Charollais et al., 2004). Contrary to previous results where DeaD was said to be associated with both ribosome subunits (Jones et al., 1996) or not to be associated with ribosomes at all (Moll et al., 2002), DeaD was shown to be associated mainly with the 50S and pre-50S subunits, with only traces of it extending to the 30S region (Charollais et al., 2004). It was also previously noted that DeaD is present in the 40S fractions from the strain lacking SrmB, another DEAD-box RNA helicase in *E. coli* (Charollais et al., 2003), a finding later confirmed with western blot analysis where DeaD was predominantly found in the 40S particles from *srmB*-minus strain, (Charollais et al., 2004). Even more interestingly, it was found that overexpression of DeaD can rescue ribosome assembly defects in *srmB*-minus strain but not vice versa (Charollais et al., 2004). Somewhat intriguingly, *srmB*-minus *deaD*-minus double mutant cells did not have exaggerated growth phenotype as one might have expected. And finally, since 40S particles from *deaD* and *srmB* differ on their protein content level, it was concluded that DeaD and SrmB act on a different stage of large subunit biogenesis - SrmB was assigned as an early assembly factor and DeaD as a late assembly factor (Charollais et al., 2004).

Shortly after DeaD was shown to be involved in ribosome assembly, it was also shown to be a part of so-called 'cold shock RNA degradosome' (Prud'homme-Généreux et al., 2004). The RNA degradosome is a bacterial multi-protein complex that is responsible for RNA degradation and processing. In *E. coli*, it typically consists of RNase E which acts as a scaffold for other components: RNA helicase RhlB, exoribonuclease PNPase and enolase (Carpousis, 2007). Going through a screen for proteins that could suppress *deaD*-minus cold-sensitive phenotype, Prud'homme-Généreux and colleagues discovered that when RNase E was expressed from a low-copy vector, it was able to partially suppress the *deaD*-minus slow growth phenotype observed at 22 °C (Prud'homme-Généreux et al., 2004). Interestingly, when wt genomic copy of RNase E was replaced with a temperature-sensitive RNase E allele *rne-1*, it amplified the growth defect of *deaD*-minus strain at 22 °C while *rne-1*

mutation alone does not have a growth phenotype (Prud'homme-Généreux et al., 2004). Furthermore, *rne* mRNA life-time was increased about five-fold in *deaD*-minus cells at 22 °C, supporting the role of DeaD in RNA degradosome. As noted before, RNase E is the main component of RNA degradosome, associating with other degradosome components via its scaffold domain. Scaffold domain has two binding sites for RNA helicases (Khemici et al., 2004). To check whether DeaD is able to interact with degradosomes *in vivo*, degradosomes were purified and analyzed for their protein content. While there was almost no DeaD present in degradosomes from cells grown at 37 °C, it was a major component of degradosomes at 15 °C (Prud'homme-Généreux et al., 2004). It also became apparent that RNase E can interact with PNPase and DeaD both *in vitro* and *in vivo*, forming a stable complex that can be immunoprecipitated with RNase E-specific antibody (Prud'homme-Généreux et al., 2004). What's even more interesting, DeaD can replace RhlB on *in vitro* formed minimal degradosomes consisting of RNase E and PNPase. Such minimal degradosomes could degrade a substrate mRNA on an ATP- and phosphate-dependent manner whereby no difference was observed whether degradosomes were formed with RhlB or DeaD (Prud'homme-Généreux et al., 2004). There are possibly two different explanations for that – either that DeaD and RhlB bind simultaneously to RNase E or that there is a heterogeneous population of degradosomes in cold-shocked cells, one population with DeaD and other population with RhlB. Due to the experimental difficulties no proof for either of these hypothesis has been provided so far.

DeaD protein helicase activity *in vivo* was shown by Butland et al (Butland et al., 2007). This was proven by using engineered chloramphenicol acetyl-transferase gene that contained stable secondary structure in the Shine-Dalgarno region, e2-SD. First, over-expression of DeaD did restore e2-SD *cat* mRNA translation to the level of wt e-SD *cat* gene, showing that DeaD can effectively destabilize the secondary structure at the initiation region of e2-SD *cat* mRNA and enhance thereby its translation (Butland et al., 2007). Second, the translation initiation activity of e2-SD *cat* was about 10-fold reduced in *deaD*-minus cells grown at 25 °C but brought back to wt level when growth temperature was raised to 37 °C (Butland et al., 2007). Such an effect is expected as stable RNA secondary structures can be destabilized either by helicases or by raising temperature, thereby lowering their free energies. Taken together, this shows that DeaD indeed does have an active role in translation initiation process, as already proposed by Toone et al (Butland et al., 2007; Toone et al., 1991). The helicase activity of DeaD protein *in vivo* was further proven by mutational analysis in the DEAD motif (DEAD -> DQAD) which abolished both ATPase and helicase activity (Turner et al., 2007). It was also shown in that study that while DeaD C-terminal extension only contributes to optimal functional activity at 25 °C and higher temperatures, it is required for its function at 15 °C (Turner et al., 2007). This result was later questioned when Awano and colleagues failed to observe the requirement for DeaD C-terminal part for its activity at low temperatures (Awano et al., 2007), although it could

be explained by the differences in used expression systems. Later, the need for the helicase activity of DeaD for its cellular functions was confirmed with yet more mutations in the DEAD motif, where DEAD->AEAD and DEAD->DAAD mutants could not complement *deaD*-minus cold-sensitive phenotype (Awano et al., 2007). On the other hand, DEAD->DEAA mutant was able to grow at 15 °C, which either indicates that the last aspartic acid in the DEAD-box is not crucial for helicase activity or such protein retains enough helicase activity to support growth at low temperature (Awano et al., 2007). Relying on previous knowledge, CspA and RNase R were checked for their capability of complementing DeaD deficiency at low temperatures (Awano et al., 2007). While they both were able to support growth, it was at lower level than with wt DeaD protein (Awano et al., 2007). This was probably also a cause why these genes were not revealed on a genetic screen that revealed RhlE as a suppressor (Awano et al., 2007). It also appeared that *deaD*-minus *rnr*-minus double mutant had more pronounced growth defect that could be complemented with over-expression of either RNase R or DeaD while *rnr*-minus alone had no observable growth defect at low temperature (Awano et al., 2007). Furthermore, DeaD and its helicase activity was once again shown to be important for mRNA degradation at low temperature as in the absence of functional DeaD *cspA* mRNA had much longer half-life (Awano et al., 2007). While in very good agreement with results from Prud'homme et al, this also suggests that DeaD might have a primary role in mRNA decay during cold adaptation (Awano et al., 2007; Prud'homme-Généreux et al., 2004).

Two independent genetic screens to find genes that could complement *deaD*-minus cold-sensitive phenotype, revealed *rhlE* (encoding the DEAD-box helicase RhlE) as one of such candidates (Awano et al., 2007; Jain, 2008). Even though RhlE was first described in 1994 (Ohmori, 1994), no distinct cellular function had been assigned to it yet. Until recently, it was only known that RhlE has ATPase and helicase activity *in vitro*, is able to associate with proteins involved in RNA degradation and assists mRNA degradation *in vitro* (Bizebard et al., 2004; Khemici et al., 2004; Raynal and Carpousis, 1999). More light was shed on RhlE function when Chaitanya Jain analyzed its role in *deaD*-minus and *srmB*-minus cells (Jain, 2008). While RhlE over-expression could not rescue the *deaD*-minus slow growth phenotype completely, it was able to reduce doubling time at 20 °C approximately two-fold (Jain, 2008). On the other hand, when RhlE was over-expressed in *srmB*-minus cells, it increased the doubling time over three-fold at 16 °C, at the same time only slight increase in doubling-time was seen in wt cells (Jain, 2008). In contrast, loss of RhlE accentuated growth defects in *deaD*-minus background but diminished the defects in *srmB*-minus background (Jain, 2008). Since these observations were also supported by polysome profile analysis and 23S rRNA 5' end mapping, this led to a proposition of an interesting hypothesis (Jain, 2008). According to this hypothesis, the maturation of 50S subunits proceeds via multiple intermediates with DeaD and SrmB acting on a non-overlapping set of ribosome assembly intermediates that are interconvertible via RhlE. Overexpression of

RhlE shifts the equilibrium towards SrmB pathway, leading to a greater level of ribosome biogenesis defects in *srmB*-minus cells than in *deaD*-minus cells while the lack of RhlE shifts equilibrium towards DeaD pathway, increasing the level of ribosome biogenesis defects in *deaD*-minus background (Jain, 2008). No studies have been performed so far to support this, leaving it to be a speculative, although highly interesting hypothesis.

In 2005, Butland et al conducted a first large-scale proteomics study with *E. coli* to identify protein-protein complexes by using sequential peptide affinity purification (SPA). This revealed many interesting interactions, one of those being between DeaD and many ribosomal proteins (Butland et al., 2005). Follow-on study characterized those interactions in more detail (Butland et al., 2007). As expected for a ribosome-associated protein, most observed interactions were indeed with ribosomal proteins. Somewhat surprisingly, there were more interactions with S-proteins (ten proteins, S1-S7, S13, S15, S20) than with L-proteins (six proteins, L1-L4, L13, L22) or with other proteins (six proteins, YfiF, YgiF, RlmL, SrmB, RNase R, YhiR). Of those interactions, all were determined to be direct protein-protein interactions without the involvement of nucleic acids, except for the interaction with YgiF which was lost after nuclease treatment. All identified proteins are either RNA- or DNA-binding proteins involved in many different areas as translation (r-proteins), ribosome assembly (SrmB), rRNA modification (RlmL) or RNA processing (RNase R). Such functional diversity suggests that DeaD can play more of a general role in the cells, being involved in processes like translation initiation, RNA processing, ribosome assembly etc as an RNA helicase (Butland et al., 2007).

While there is now enough evidence that DeaD is needed for normal cell growth on many levels, being involved in translation initiation, RNA decay and cold-shock adaptation, its exact role in ribosome assembly needs to be clarified. Even though the lack of DeaD protein leads to defined changes in ribosome profile and RNA processing defects, this could be an indirect effect caused by erroneous translation. Similar phenomenon has been observed for the RNA modification enzymes RlmE and RluD, where there is now direct evidence that the observed defects in ribosome assembly are most likely caused by errors in translation which in turn are caused by the lack of corresponding rRNA modifications.

3.1.2. RNA helicase SrmB

SrmB was the first of five DEAD-box RNA helicases identified in *E. coli* (Nishi et al., 1988). It was discovered in a search for second-site mutations that could suppress ribosome assembly defects in r-protein L24-deficient cells as L24 is essential for the assembly of ribosomes in *E. coli* (Cabezón et al., 1977). Previously it had been shown that a temperature-sensitive mutation in L24 causes protein instability and disrupts interaction between 23S rRNA and L24, thereby causing lethality through errors in ribosome assembly (Nishi and

Schnier, 1986). Over-expression of SrmB, on the other hand, was enough to allow L24-deficient cells revert the defects in ribosome assembly (Nishi et al., 1988). Sequence alignment analysis revealed high homology of SrmB to eIF-4A and p68, two well-known human proteins. Like eIF-4A, SrmB had nucleic acid dependent ATPase activity, although more than an order of magnitude greater than eIF-4A did (Nishi et al., 1988). In contrast to eIF-4A, SrmB was capable of binding to RNA alone, without the help of other proteins whereas eIF-4B is required for the binding of eIF-4A to the RNA substrate (Nishi et al., 1988).

The mechanism by which SrmB could rescue ribosome assembly defects caused by the temperature-sensitive mutation in L24 remained unclear for quite some time although a following hypothesis was originally proposed. According to this hypothesis, SrmB could bind to 23S rRNA regions other than those occupied by L24 during ribosome assembly process and thereby protect unstable assembly precursors from the degradation or, alternatively, to help rRNA to acquire its correct tertiary structure (Nishi et al., 1988). Unfortunately, there was no further evidence to support this or any other hypothesis for more than fifteen years until in 2003, Charollais and colleagues provided evidence about the role of SrmB in ribosome assembly (Charollais et al., 2003). They constructed a *srmB* deletion strain which displayed a slow-growth phenotype at 30 °C, a defect that became even more pronounced at lower temperatures. The link between SrmB and L24 (established by Nishi and colleagues (Nishi et al., 1988)) was confirmed by analyzing the growth properties of both single and double mutant strains. It appeared that while *srmB*-minus and *rplX19* single mutants showed no difference compared to wt cells when grown at 37 °C, *srmB rplX19* double mutant was severely impaired in growth (Charollais et al., 2003). Since such results suggested possible defects in ribosome assembly, ribosome profiles were analyzed next. Compared to the wt cells, ribosome profile of *srmB*-minus cells was considerably changed. Namely, there was a large increase in the amount of free 30S subunits and an appearance of a new 40S particle (Charollais et al., 2003). Furthermore, the normal 1:2 ratio between free 30S and 50S subunits was changed to 2:1 ratio, suggesting that there is a deficiency in the ribosome large subunit biogenesis. Closer examination revealed the presence of incompletely processed 23S rRNA in the 40S particles and 17S rRNA in the 30S subunits from *srmB*-minus cells. First result was attributed directly to the lack of SrmB that caused defects in 50S subunit assembly while the latter was attributed to be an indirect consequence of the deficiency of 50S subunits rather than the direct involvement of SrmB in 30S subunit biogenesis. Proposal that 40S particles are precursors to mature 50S subunits was fully supported by the protein content analysis. Namely, five r-proteins (L13, L28, L34, L35, L36) were missing and nine r-proteins (L6, L7/L12, L14, L16, L25, L27, L31, L32 and L33) were present in reduced amounts in the 40S particles from *srmB*-minus cells; 50S and 30S subunits on the other hand were identical to their wt counterparts (Charollais et al., 2003). With an exception of L13, all other r-proteins belong to the so-called 'late' assembly step proteins (Nierhaus, 1991) showing the immature and heterogeneous nature of the 40S particles from

srmB-minus cells. The role of SrmB in the 50S subunit assembly was further supported by the fact that it co-sedimented with the 50S subunit precursors and was able to specifically bind to the 40S particles instead of mature 50S subunits (Charollais et al., 2003). Based on the protein content analysis, Charollais et al proposed that SrmB is involved in an early step of ribosome large subunit assembly. The most intriguing result was the absence of r-protein L13 from the 40S particles. Based on this, it was proposed that SrmB is needed for the recruitment of L13 to the subunit precursors and that the absence of SrmB leads ribosome assembly to an alternative and slower assembly pathway, resulting in the appearance of the 40S particles (Charollais et al., 2003). While the binding of L13 to 23S rRNA *in vitro* depends on the presence of L20 (and L20 was present in the 40S particles), it could be that L20 is not correctly positioned for the binding of L13 to the 40S particles in *srmB*-minus cells. This in turn is in good agreement with the previously established link between SrmB and L24 when it was shown *in vitro* that in the absence of L24, L20 can take over the initiator function (Franceschi and Nierhaus, 1988). Therefore, if in the absence of SrmB L20 might not be able to replace L24, this might lead to the observed synthetic lethality.

As already described before, there is also some evidence for the interactions between SrmB and other DEAD-box helicases DeaD and RhlE (Charollais et al., 2004; Jain, 2008). For example, over-expression of DeaD can rescue ribosome assembly defects in *srmB*-minus strain but not vice versa, supporting the assignment of SrmB as an early assembly factor (Charollais et al., 2004). On the other hand, over-expression of RhlE in *srmB*-minus cells increased the observed growth defect at 16 °C while the loss of RhlE in *srmB*-minus background diminished growth defects almost completely (Jain, 2008). Therefore, it remains to be confirmed whether SrmB acts in ribosome assembly as an RNA helicase or as an RNA chaperone and what its real mission is. For now one can only say that SrmB is indeed involved in ribosome biogenesis, together with other RNA helicases like DeaD, RhlE and DbpA.

3.1.3. RNA helicase DbpA

DbpA (DEAD-box protein A) was the second of identified DEAD-box proteins in *E. coli*, found shortly after the discovery of SrmB in 1988 (Iggo et al., 1990; Nishi et al., 1988). It was identified and cloned during a search for the relative of eukaryotic protein p68 in *Escherichia coli*, without any indications about its potential substrate or role in cell (Iggo et al., 1990). DbpA consists of two domains – C-terminal domain that confers specificity for 23S rRNA (Kossen et al., 2002) and N-terminal domain that bears ATPase and helicase activity (Elles and Uhlenbeck, 2008).

After the initial discovery of DbpA protein, it took few years to establish whether it is capable of hydrolyzing ATP in an RNA-dependent manner, but that discovery contained a big surprise – ATPase activity of DbpA was

triggered only in the presence of 23S rRNA (Fuller-Pace et al., 1993). Furthermore, DbpA was highly specific to bacterial 23S rRNA. Total RNA from yeast or HeLa cells, rRNA from rabbit or total tRNA from *E. coli* failed to trigger any ATPase activity; neither did the same single- or double-stranded DNA (Fuller-Pace et al., 1993). Based on the ATPase activity tests, it was proposed that DbpA recognizes rather a specific sequence than a secondary/tertiary structure, unless such a structure is stable enough to withstand RNA denaturation. Two years later it became apparent that only 93 nucleotides long 23S rRNA sequence is both necessary and sufficient for the activation of ATPase activity of DbpA (Nicol and Fuller-Pace, 1995). This sequence is a part of domain V in 23S rRNA (nucleotides 2496–2588) that forms a part of the peptidyltransferase center and interacts with A- and P-site tRNAs (Moazed and Noller, 1989). Furthermore, Nicol and Fuller-Pace confirmed that together with primary sequence, 23S rRNA secondary structure is of central importance for its recognition by DbpA, especially the three stem-loops located within that fragment (Nicol and Fuller-Pace, 1995). While DbpA was able to bind minimal substrate RNAs with disrupted stem-loops as efficiently as wt RNA, its ATPase activity was many times lower on such substrates (Nicol and Fuller-Pace, 1995). In a search for other 23S rRNA regions capable of stimulation of ATPase activity of DbpA, four such regions were found by Böddeker et al (Böddeker et al., 1997). Of these four regions, only one (spanning nucleotides 2500–2600) was capable of achieving 100% stimulation of ATPase activity and that region was similar to that originally reported by Nicol and Fuller-Pace (Nicol and Fuller-Pace, 1995). Other three 23S rRNA regions triggered ATPase activity of up to 60% and while spread around all over 23S rRNA, they all were rich in stem-loop structures, indicating again the importance of secondary structure elements for the substrate recognition by DbpA. Only some time later was it discovered that the hairpin 92 together with adjacent 5' and 3' single-stranded sequences within 23S rRNA is the main structural element that is needed to trigger ATPase activity of the DbpA protein (Tsu et al., 2001). Furthermore, kinetic studies revealed that neither free 50S subunits or 70S ribosomes are capable of ATPase activity stimulation of DbpA (Tsu and Uhlenbeck, 1998), suggesting that it has a role in ribosome biogenesis rather than in a generic translation machinery as already proposed before.

Intriguingly, tests to determine whether DbpA is an RNA helicase concluded with a finding that DbpA can destabilize RNA:RNA and RNA:DNA complexes on an ATP-independent manner (Böddeker et al., 1997). Just a short time later, Pugh et al demonstrated that under their experimental conditions, DbpA bears no detectable helicase activity *in vitro*, whether ATP-dependent or -independent (Pugh et al., 1999). The question whether DbpA acts as an RNA helicase and under what conditions was addressed again in 2001 by two groups. Henn and colleagues used atomic force microscopy while Diges and Uhlenbeck used traditional helicase assays (Diges and Uhlenbeck, 2001; Henn et al., 2001). Atomic force microscopy study demonstrated that DbpA can unwind long

dsRNA sequences with 5' overhangs processively in an ATP-dependent manner (Henn et al., 2001). In contrast, Diges and Uhlenbeck determined that DbpA is a non-processive ATP-dependent RNA helicase and that its activity is highly stimulated by the hairpin 92 of 23S rRNA (Diges and Uhlenbeck, 2001). Discrepancies between the three helicase assay results (Diges and Uhlenbeck, 2001; Henn et al., 2001; Pugh et al., 1999) could be explained in many ways, as proposed by Diges and Uhlenbeck (Diges and Uhlenbeck, 2001). First, the helicase substrate used by Pugh et al was probably too stable and it did not contain single-stranded region between the substrate helix and hairpin 92, accounting for the not detected helicase activity. Second, it had been shown that the C-terminal part of DbpA is needed for the recognition and binding to the hairpin 92 of 23S rRNA, thereby 'locking' DbpA to its substrate in the presence of 23S rRNA. In the absence of hairpin 92 (as was in the atomic force microscopy experiments), the N-terminal part of DbpA could still maintain its non-specific affinity towards RNA substrate and thereby disrupt long helices in a non-specific manner. While all these explanations seemed plausible, the question still remained – does DbpA possess any helicase activity?

To address this issue once and for all, Diges and Uhlenbeck conducted a follow-on study using a more exhaustive set of different dsRNA substrates (Diges and Uhlenbeck, 2005). This resulted in a finding that DbpA is a 3' -> 5' RNA helicase that requires a single-stranded loading site 3' of the substrate helix. Furthermore, it appeared that DbpA is a weakly processive RNA helicase and that its helicase activity depends on the presence of hairpin 92 of 23S rRNA (Diges and Uhlenbeck, 2005).

Unfortunately, there is still no information about the role of DbpA *in vivo* although based on its high specificity towards 23S rRNA a role in translation or ribosome assembly was proposed already in 1993 and thereafter (Fuller-Pace et al., 1993; Tsu and Uhlenbeck, 1998). DbpA knock-out cells display normal growth and ribosome assembly phenotype, indicating that whatever its role in cells is, it is not essential for cell viability (Iost and Dreyfus, 2006; Peil et al., 2008). Interestingly, just recently Elles and Uhlenbeck succeeded in the isolation of DbpA point mutation (DbpA(R331A)) that abolishes its ATPase and helicase activity and results in a dominant slow growth phenotype (Elles and Uhlenbeck, 2008). While no clear explanation can be given for such a phenomenon, one could hypothesize that in the absence of DbpA protein (whether wt or mutated) other DEAD-box helicases take over its function without any visible loss of viability. On the other hand, when catalytically inactive but otherwise 23S rRNA hairpin 92 specific DbpA with its intact C-terminal region is present in the cells, it out-competes wt DbpA and causes slow-growth phenotype via yet unknown mechanism. Thus far this is the only clear growth defect associated with the RNA helicase DbpA and whilst too early to draw any final conclusions about the role of DbpA in ribosome assembly or cell growth, the picture is slowly clearing up.

3.2. The role of ribosome-dependent GTPases in ribosome assembly

As of today, there is direct evidence for the involvement of four small GTPases in ribosome biogenesis in *E. coli*. First two of the proteins, Era and RsgA, are involved in the assembly of 30S subunit while other two, ObgE and Der, are involved in the assembly of 50S subunit. Three out of four GTPases mentioned (Era, ObgE and Der) are essential proteins for cell viability and for most of them many different cellular functions have been assigned previously. One common link connecting above-mentioned GTPases is that they all are conserved among many phyla (Caldon and March, 2003) and that they have a role in ribosome biogenesis (Karbstein, 2007). This is also one of the reasons why those GTPases together with other extra-ribosomal factors are such interesting targets for antimicrobial compound development (Comartin and Brown, 2006; Maguire, 2009) and why their role in ribosome biogenesis needs to be studied further. Another interesting property of some small GTPases is that besides their direct involvement in ribosome biogenesis, their over-expression can rescue ribosome assembly defects seen in the RNA methyl-transferase RlmE, RNA chaperone RbfA and GTPase RsgA deficient strains (Campbell and Brown, 2008; Inoue et al., 2003; Tan et al., 2002), thereby linking them to ribosome biogenesis both directly and indirectly via multi-protein network.

3.2.1. GTPase Era

Era (*E. coli* Ras-like protein) is an essential *Escherichia coli* small G-protein, characterized to take part in various cellular processes like cell division and carbon assimilation among others (for the review see (Caldon and March, 2003)). It is composed of two domains, an N-terminal Ras-like domain and C-terminal domain responsible for RNA binding (Johnstone et al., 1999). Era is capable of binding both free 16S rRNA and ribosome 30S subunits in a nucleotide dependent manner (GDP- and GTP-bound forms of Era have reduced binding ability compared to Era alone) and its depletion leads to major translational defects in S100 fraction/*ompF-lpp* mRNA based cell-free translation system (Sayed et al., 1999). Depletion of Era protein also led to major growth defects and accumulation of 17S RNA, a precursor to 16S rRNA, together with visible defects in ribosome profiles (Inoue et al., 2003), suggesting that it acts as a ribosome assembly factor among its other functions. Its role in ribosome biogenesis was further supported by the fact that over-expression of Era can suppress defective ribosome assembly and 16S rRNA processing in an RNA chaperone RbfA-deficient strain while at the same time over-expression of RbfA was not capable of suppressing ribosome assembly defects in Era-depleted cells (Inoue et al., 2003). On the other hand, over-expression of Era could not suppress the C23U mutation in 16S rRNA or the

deletion of *rimM*, both of which were suppressed by the over-expression of RbfA, indicating complementary but still different role for both RbfA and Era (Inoue et al., 2003). Even though the over-expression of wt Era was able to suppress growth and ribosome assembly defects in *rbfA*-minus strain, over-expression of Era(E200K) protein (the mutation that causes cold-sensitive phenotype in *era*-minus background) caused more severe growth defects and two-fold higher accumulation of 17S RNA instead (Inoue et al., 2006). This was thought to happen via competition for the binding of 30S subunit by chromosome-encoded wt Era and plasmid-encoded Era(E200K) protein, resulting in defective 16S rRNA processing. Interestingly, it was found previously that cold-sensitive phenotype of Era(E200K) could be suppressed by the over-expression of *ksgA* gene (Lu and Inouye, 1998). KsgA is a 16S rRNA adenosine dimethyltransferase (O'Farrell et al., 2004) that was just recently shown to participate in ribosome assembly (Connolly et al., 2008) and that has a secondary DNA glycosylase/AP lyase activity (Zhang-Akiyama et al., 2009). The mechanism by which KsgA over-expression could suppress the cold-sensitivity of the Era(E200K) mutation was unknown at that time, but recently there was an interesting breakthrough when mutational analysis revealed that KsgA has another function, unlinked to its methyltransferase activity (Inoue et al., 2007). Mutated forms of KsgA protein that had a severely reduced methyltransferase activity were still able to suppress the defects in *era*/Era(E200K) strain while the mutation in KsgA C-terminal domain that did not affect its methyltransferase activity (KsgA(R248A)) failed to suppress the growth defects (Inoue et al., 2007). It also appeared that over-expression of KsgA does not repair defective 16S rRNA processing in Era(E200K) strain, indicating that Era(E200K) cold-sensitivity is not due to the accumulation of 17S RNA (Inoue et al., 2007). On contrary, over-expression of wt KsgA but not KsgA(R248A) protein made cells highly sensitive to organic acids, showing that KsgA has indeed a second activity in the cells, responsible for the suppression of Era(E200K)^{cs} phenotype and regulation of acid shock response whilst unlinked to its methyltransferase activity. The latest report that KsgA has DNA glycosylase/AP lyase activity (Zhang-Akiyama et al., 2009) fits well with the discovery of Inoue et al (Inoue et al., 2007) although it is not known to date whether the reported secondary activities are the same or not.

3.2.2. GTPase RsgA

RsgA (ribosome small subunit dependent GTPase A, previously called YjeQ) was first characterized as a novel GTPase in *E. coli*, essential for cell viability (Arigoni et al., 1998), but its essentiality was later disproved (Himeno et al., 2004). RsgA was found to co-purify exclusively with ribosomes in the cell and *in vitro* binding assays revealed its greater affinity towards ribosome 30S subunit than towards 50S subunits or 70S ribosomes (Daigle and Brown, 2004). The binding affinity of RsgA was determined by the presence of guanosine

nucleotides - RsgA was almost exclusively bound to the 30S subunits in the presence of GDPNP while in the presence of GDP or GTP some amount of RsgA was still detectable in the supernatant (Daigle and Brown, 2004; Himeno et al., 2004). Furthermore, GTPase activity of RsgA was strongly stimulated by the presence of ribosomes, especially by the 30S subunits (Daigle and Brown, 2004), (Himeno et al., 2004) and its binding specificity towards ribosomes was determined by its N-terminal region (Daigle and Brown, 2004). GTPase activity of RsgA was greatly reduced by A-site specific antibiotics while antibiotics binding to P-site or peptidyltransferase center failed to affect the GTPase activity of RsgA, suggesting that the binding site for RsgA on ribosome at least partially overlaps with the A-site region of 30S subunit (Himeno et al., 2004).

Although *rsgA* was previously reported to be an essential gene for cell viability, Himeno et al succeeded in the construction of *rsgA* deletion strain. *rsgA*-minus strain had about 2.5-fold longer doubling time and disrupted ribosome profile compared to wt strain, where 70S ribosome fraction was greatly reduced with a concomitant increase in the amount of free 50S and 30S subunits (Himeno et al., 2004). Unfortunately, the poor resolution of ribosome profiles could not allow one to estimate whether there were any intermediate particles present or not. Nevertheless, the fact that free 30S subunits from RsgA-deficient strain were less active in the stimulation of GTPase activity of RsgA than 30S subunits obtained from 70S ribosomes via dissociation, indicated that they were not fully matured (Himeno et al., 2004). This finding was also supported by the fact that free 30S subunits from RsgA-deficient strain contained a large amount of 17S RNA, a precursor to 16S rRNA (Himeno et al., 2004). Furthermore, it was later determined that the binding of RsgA to the 30S subunit induces conformational changes around A- and P-site and helix 44 of 16S rRNA that are partially restored after GTP hydrolysis by RsgA and its dissociation from 30S subunit (Kimura et al., 2008), confirming its participation in ribosome biogenesis.

Recently a number of genes were identified that were able to suppress the growth and/or ribosome biogenesis defects seen in *rsgA*-minus strain when over-expressed. Interestingly, two of those genes, GTPases *infB* and *era*, were able to suppress both growth and ribosome assembly defects on an expression level dependent manner (Campbell and Brown, 2008). A complementary screen identified additional seven genes whose deletion led to more severe growth defects than seen in the single mutants for those genes or *rsgA*-minus alone. Most interesting of those genes were *rimM*, *rluD* and *ksgA* (Campbell and Brown, 2008). RimM and RluD have been previously shown to participate in ribosome assembly (discussed in detail above) and KsgA is an universally conserved 16S rRNA specific methyltransferase also involved in ribosome biogenesis (Connolly et al., 2008). Although Campbell and Brown suggested that the enhanced slow growth seen in *rluD rsgA* and *rimM rsgA* double mutant strains is because they function in the same pathway as RsgA, it is highly unlikely, at least with RluD. Namely, RluD is a 50S subunit specific factor (even though the lack of RluD does disrupt 30S assembly) while RsgA is a 30S

subunit specific factor, meaning that they are acting on different substrates during ribosome assembly. On the other hand, since the lack of either RluD or RsgA causes defects in their respective subunit assembly, it is logical to expect even bigger defects in double mutant strain, exactly as was seen by Campbell and Brown (Campbell and Brown, 2008).

3.2.3. GTPase ObgE

Escherichia coli protein ObgE (also called $CgtA_E$) belongs to an evolutionarily conserved group of essential GTP-binding proteins (Czyz and Wegrzyn, 2005). While many different functions have been assigned to Obg proteins, ranging from chromosome segregation to stress response (Czyz and Wegrzyn, 2005), only ribosome-associated activities will be covered here. First implications that ObgE (and other so-called small GTPases in that sense) might have a role in ribosome assembly in *E. coli* were established in 2002, when it appeared that over-expression of ObgE or Der, another GTPase in *E. coli* (reviewed below), can rescue the deletion of *rlmE* gene (Tan et al., 2002), encoding for the enzyme that methylates U2552 in *E. coli* 23S rRNA (Bügl et al., 2000; Caldas et al., 2000a). The deletion of *rlmE* gene causes major defects in growth and ribosome assembly, as described in detail above. To much of a surprise, growth and assembly defects in *rlmE*-minus strain were rescued by over-expressing either Der or ObgE (Tan et al., 2002). Although it is still unknown how the over-expression of two GTPases can suppress RlmE deficiency, it was proposed to happen on a level of ribosome structure stabilization and not by a stimulation of complementary methyltransferases (Tan et al., 2002). Next suggestion that *E. coli* ObgE might have a role in ribosome biogenesis was published by Wout and colleagues when they discovered that ObgE co-sediments with the 50S subunit and co-purifies with the RNA helicase DeaD and (p)ppGpp synthetase/ hydrolase SpoT (Wout et al., 2004). These results were followed by a paper where the involvement of ObgE in ribosome biogenesis in *E. coli* was directly shown (Sato et al., 2005), a result later confirmed by an independent study (Jiang et al., 2006). Both groups discovered that the depletion of ObgE leads to changes in ribosome profiles, with the accumulation of free subunits and reduction of free 70S ribosomes (Jiang et al., 2006; Sato et al., 2005). The involvement of ObgE in ribosome biogenesis was further confirmed by a fact that ObgE co-sedimented with both free 30S and 50S subunits but not with the 70S ribosomes (Jiang et al., 2006; Sato et al., 2005; Wout et al., 2004) and that it was able to bind to 16S and 23S rRNA in the presence of GTP and magnesium ions (Sato et al., 2005). Even more, ObgE-depleted cells accumulated pre-16S rRNA (Sato et al., 2005) and pre-23S rRNA (Jiang et al., 2006), defective in the maturation of both 5' and 3' ends. At the same time, ObgE was shown to be associated with a number of r-proteins (S3-S5, S13, S16, L2, L4, L16–17) and extra-ribosomal factors involved in ribosome biogenesis (RNA helicase DeaD being the most interesting of those) (Sato et al., 2005) while the 40S

particles from ObgE-depleted cells had decreased amount of r-proteins L16, L33 and L34, similar to that observed in *deaD*-minus (Charollais et al., 2004), *srmB*-minus (Charollais et al., 2003) and *rlmE*-minus (Hager et al., 2002) strains. Taken together, this data strongly points ObgE to take part in the late step of ribosome 50S subunit assembly, especially as ObgE was found to be associated only with the 'near-50S' or 50S particles but not with the 40S particles of various origin (Jiang et al., 2006). There is also some intriguing evidence that ObgE regulates the level of cellular (p)ppGpp during exponential growth, by controlling the hydrolase activity of SpoT via direct interaction between two proteins on pre-50S subunits that are being assembled (Jiang et al., 2007). Whether there exists such a link between ObgE, SpoT and ribosome assembly, remains to be seen.

3.2.4. GTPase Der

Der (double-Era-like domains, previously called EngA) is an essential GTPase for cell viability and unique as it has two tandemly repeated GTP-binding domains at the N-terminal region followed by a C-terminal domain (Hwang and Inouye, 2001). As already mentioned above, Der and ObgE were shown to be the suppressors for the deletion of RlmE, a heat-shock dependent enzyme that methylates U2552 in *E. coli* 23S rRNA (Tan et al., 2002); that was also the first implication about the role of Der in ribosome biogenesis. Few years later it was confirmed that Der itself is essential for the normal biogenesis of the 50S subunits in *E. coli* (Bharat et al., 2006; Hwang and Inouye, 2006). Namely, when cells were depleted for Der protein, their ribosome profiles showed major changes - the amount of free subunits was significantly increased at the expense of the amount of polysomes and free 70S ribosomes (Bharat et al., 2006; Hwang and Inouye, 2006), similar to that seen in other ribosome assembly deficient strains. When Der-depleted ribosomes were dissociated at low Mg^{++} concentrations, new particles appeared in the 40S region, their exact sedimentation coefficient being dependent on the Mg^{++} concentration used (Hwang and Inouye, 2006). Those 40S particles were found to lack r-proteins L9 and L18 and to contain L2 and L6 in slightly diminished amounts (Hwang and Inouye, 2006). Furthermore, Der-depleted cell extracts contained precursors to 16S and 23S rRNA, incompletely processed at both 5' and 3' ends (Hwang and Inouye, 2006). The role of Der in large subunit assembly was also supported by the fact that Der co-fractionated only with the ribosome 50S subunits but not with the 70S ribosomes or 30S subunits (Hwang and Inouye, 2006), (Bharat et al., 2006).

It is worth to note that although Der is essential for cell growth, it was initially shown that its absence can be compensated on normal growth temperature with Der protein in which either one of the two but not both GTP-binding domains have a point mutation (N118D and N321D) (Hwang and Inouye, 2006). At the same time, both GTP-binding domains were shown to be

required for cell growth at low temperatures (Hwang and Inouye, 2006). Using a different set of point mutations in GTP-binding domains in Der (K15A, S16A, K216A, S217A), Bharat and colleagues failed to record the ability of those mutants to support growth and suppress ribosome assembly defects except for only one mutation (K15A) (Bharat et al., 2006). This in turn indicates that both GTP-binding domains in Der are indispensable for its cellular function (Bharat et al., 2006) and that DerN118D and DerN321D used by Hwang and Inouye (Hwang and Inouye, 2006) still retained some of its functionality in the cell at 37 °C and 42 °C, their lower activity being compensated for with over-expression (Hwang and Inouye, 2008).

Interestingly, growth and ribosome assembly defects caused by the lack of functional Der protein could be rescued by the over-expression of RelA protein (Hwang and Inouye, 2008), a (p)ppGpp synthetase (Justesen et al., 1986). Furthermore, it was confirmed that it is indeed the (p)ppGpp synthetic activity of RelA what is needed for the suppression of both growth and ribosome profile defects in *der*/Der(N118D) and *der*/Der(N321D) strains at 30 °C while over-expression of RelA cannot support growth of *der*-minus cells (Hwang and Inouye, 2008). How does RelA exactly suppress the deficiency of Der in cells, remains to be confirmed but it seems to depend mainly on the accumulation of (p)ppGpp. One possibility is that the accumulation of (p)ppGpp helps to associate mutant Der protein with the 50S subunits, or that it slows down rRNA synthesis and allows ribosome subunits to mature in a correct but somewhat slower manner. It is worth to remind here that GTPase ObgE (described in detail above) has interactions and co-sediments with SpoT, a partner of RelA enzyme. Whether it is just a coincidence that two GTPases capable of suppressing defects caused by the methyltransferase RlmE deficiency have interactions with (p)ppGpp pathway enzymes (ObgE with SpoT and Der with RelA) is an interesting point that needs to be studied further.

3.3. The role of heat-shock proteins in ribosome assembly

The role of heat-shock proteins in ribosome assembly in *E. coli* has been studied in depth, mostly by J.H. Alix and his group. So far, most of the work involving the role of heat-shock proteins in ribosome assembly has been done in cells lacking either DnaK or DnaJ or both (Al Refaii and Alix, 2009; Alix and Guérin, 1993; El Hage et al., 2001) and there is evidence for the involvement of GroEL and other chaperones in ribosome assembly (El Hage et al., 2001).

First indication that DnaK is involved in ribosome biogenesis came in 1993, with the discovery that precursors to ribosomal subunits accumulate in *dnaK*^{ts} strains grown at elevated temperatures (Alix and Guérin, 1993). This was determined to be a direct consequence related to the lack of functional DnaK protein, not by either stringent response or uncontrolled heat-shock response.

Few years later came the first evidence that together with DnaK and DnaJ, GroEL and possibly other heat-shock proteins participate in ribosome biogenesis in *E. coli* (El Hage et al., 2001). Unlike with the *dnaK*^{ts} strain used before, the full deletion of DnaK caused defects only in the assembly of 50S subunits, leaving the assembly of 30S subunit untouched. Furthermore, DnaK and DnaJ were shown not to be required for ribosome assembly at up to 42 °C, in case other sigma-32 dependent heat-shock proteins are over-expressed at the same time. In the same work it was established that *groEL*^{ts} strains exhibit abnormal ribosome patterns at high temperatures where the deficiency of functional GroEL protein seemed to affect only the assembly of 50S subunits, leaving 30S maturation unaffected. On the other hand, it was shown that the over-expression of GroEL/GroES chaperonins was enough to at least partially compensate for the defects in ribosome assembly due to the lack of functional DnaK protein (El Hage et al., 2001).

Precursor ribosomal particles from DnaK-deficient cells were at first indicated to be capable of final maturation since they were shifted towards the 70S ribosomes in time (Alix and Guérin, 1993). This finding was confirmed later, by showing that abnormal ribosomal particles found in *dnaK*^{ts} cells are indeed true precursors to mature subunits (El Hage and Alix, 2004) which are capable of final maturation in the lack of DnaK chaperone, although at a much slower rate than in wt cells. The 21S particles from *dnaK*^{ts} cells contained pre-16S rRNA and lacked a number of late assembly step S-proteins, whereas 45S particles contained pre-23S rRNA and lacked a number of late assembly step L-proteins (El Hage and Alix, 2004). By following the kinetics of rRNA processing, it was clear that precursor forms of rRNA contained in the 21S and 45S precursor particles were processed into their mature forms, together with the shift of precursor particles towards completely assembled 50S and 30S subunits (Alix and Guérin, 1993; El Hage and Alix, 2004). An interesting common feature of both 21S and 45S precursor particles was that they lacked some late assembly step proteins, suggesting that DnaK has a role in a late step of ribosome assembly. Furthermore, the incorporation of r-proteins missing from the 21S subunits in *in vitro* reconstitution requires an energy-dependent conformational change and it was previously suggested that DnaK participates in this change (Maki et al., 2002; Maki et al., 2003).

The suggestion of DnaK might be involved in the conformational change during the assembly of 30S subunit both *in vitro* and *in vivo* resulted in a very heated debate, with Gloria Culver and colleagues on one side and Jean-Harve Alix and Knud Nierhaus on the other side. First, it was shown by Gloria Culver and colleagues that purified DnaK chaperone system components facilitate 30S subunit reconstitution at low temperatures (Maki et al., 2002). Without the heating step that is required for a conformational change during the 30S assembly process, RI precursors accumulate and reconstitution stalls. Surprisingly, the addition of purified DnaK, DnaJ, GrpE proteins and ATP could activate the conversion of RI precursors to the 30S particles without any heating step, resulting in the 30S subunits active in tRNA binding, although

30% less active than normally reconstituted 30S subunits (Maki et al., 2002). These findings were heavily criticized by J-H. Alix and K. Nierhaus who failed to record any of the DnaK-facilitated effects on the 30S subunit reconstitution (Alix and Nierhaus, 2003). Instead of tRNA binding assay, they used poly(Phe) synthesis assay to follow the kinetics of 30S subunit reconstitution, with or without chaperones. This resulted in a finding that while the DnaK protein used throughout the reconstitution was active, it had no effect of whatsoever on the measured poly(Phe) activity (Alix and Nierhaus, 2003), a result in striking contrast with that of Culver group (Maki et al., 2002). Just a short while later, Maki et al published a follow-on note to explain for the accounted controversies between the results of two groups (Maki et al., 2003). The main reasons that resulted in dissimilar results in the end were most probably due to different experimental setup used. For example, the purification of reconstituted subunits to eliminate bound DnaK before functional assays was shown to be crucial, as were some other specific details. Furthermore, using now a completely defined poly(Phe) synthesis assay instead of the previously used tRNA binding assay, Maki et al were able to show again that DnaK facilitated 30S subunits are catalytically active, albeit at a lower level than normally reconstituted 30S subunits (Maki et al., 2003).

The fact that DnaK is absolutely needed for the late assembly step(s) at high temperatures suggests it to act as a quality control mechanism under heat-shock conditions, to stop cells from wasting crucial energy in defective translation by keeping the number of available translationally active ribosomes low. At the same time, chaperones seem to participate in the ribosome assembly also in other ways, most likely by inducing conformational changes needed for final maturation of subunits.

RESULTS AND DISCUSSION

Objectives of the present study:
To date, the involvement of many extrinsic factors in ribosome assembly in *E. coli* has been shown. Among those factors, RNA modification enzymes and RNA helicases play an important part. Until only recently, ribosome assembly was thought to happen via certain pre-determined pathways that resulted in mature ribosomes. The identification of many similar but still different precursor particles from ribosome assembly deficient strains raised the question whether such *in vivo* precursors can indeed be matured or are they assembly 'dead-end' products, incapable of final maturation. Furthermore, the role of RNA modifications and modification enzymes in ribosome biogenesis and translational apparatus has been a constant cause for discussions. To try to answer some of those questions, following aims were put up:

1. To characterize the role of pseudouridine synthase RluD in ribosome assembly and to describe assembly defects in RluD-deficient cells.
2. To characterize the specificity of pseudouridine synthase RluD in detail.
3. To identify and characterize pseudouridine methyltransferase responsible for the synthesis of methylated pseudouridine at position 1915 in 23S rRNA.
4. To characterize the role of RNA helicases DeaD and DbpA in ribosome assembly, to describe assembly defects in DeaD-deficient cells and to analyze the maturation and functional properties of ribosomal precursors in DeaD-deficient cells.

Ref I. Pseudouridine synthase RluD has an important role in ribosome assembly

Pseudouridine is the most abundant single ribosomal RNA modification found in all organisms that is made post-transcriptionally from uridine residues, in an oligo- or polynucleotide level by pseudouridine synthases in an ATP-independent manner. There are two interesting points in pseudouridine formation – first, on what basis is the nucleotide selection made and, second, what purpose does the pseudouridine itself serve. So far, pseudouridines have been found only in RNA molecules whose tertiary structure is crucial for their function, i.e. rRNA, tRNA, snRNA, snoRNA, and tmRNA.

There are altogether ten pseudouridine synthases that have been divided into four families, based on their amino acid sequence homology (I, Table 3). Of those ten proteins, three are responsible for the synthesis of pseudouridines in tRNAs while rest of the enzymes catalyze pseudouridines in rRNA molecules. Thus, all predicted pseudouridine synthases in *E. coli* have been connected to

their respective pseudouridines (I, Table 3). One common feature amongst all known pseudouridine synthases is the presence of catalytic aspartic acid residue, located in a conserved motif II (Ofengand Rudd 2000). Mutations of this aspartic acid render enzymes catalytically inactive, as determined by both *in vitro* and *in vivo* assays (Ofengand et al., 2001).

Pseudouridines corresponding to each of the enzymes have been determined by individually knocking out single synthase genes and screening for the disappearance of pseudouridine at the same time. Of all pseudouridine synthase genes, only the deletion of *rluD* caused defects in cell growth-rate, indicating its importance for cell viability (Huang et al., 1998; Raychaudhuri et al., 1998). Since pseudouridines made by RluD are located in the highly conserved region of 23S rRNA (Ofengand, 2002), we tested the possibility that the absence of these three pseudouridines might affect ribosome assembly.

Exponentially growing cells were lysed and their ribosome content was analyzed by sucrose gradient centrifugation at different Mg^{++} concentrations (I, Figure 6). In panel A, ribosome profiles corresponding to wt, *rluD*-minus/ pRluD and *rluD*-minus strains are shown. Under used conditions, wt ribosome profile consists of a large peak corresponding to the 70S ribosomes and small peaks corresponding to free 50S and 30S subunits, represented by wt MG1655 strain ribosome profile. In contrast, deletion of *rluD* gene (designated as *rluD*-minus Dust strain by Gutgsell et al (Gutgsell et al., 2001)) on the other hand caused major changes to ribosome profile. First, the balance between the 70S ribosomes and free subunits was heavily disrupted as the amount of free subunits was much higher than in wt cells, at the expense of 70S ribosomes (I, Figure 6A). Second, an additional particle designated as 39S was accumulated in the cells. Interestingly, the mobility of the ribosomal small subunits in the sucrose gradients was shifted from 30S to 27S in spite of the fact that RluD makes pseudouridine residues only in 23S rRNA. Defects in ribosome profiles were restored to wt appearance when wt RluD protein was expressed from a plasmid in *rluD*-minus background. The presence of the abnormal ribosomal particles means either that there is an assembly defect due to the disruption of the *rluD* gene, or that ribosomal particles lacking these specific pseudouridines are unstable. To analyze the stability of the ribosomal particles during sample processing, we prepared the lysate at 6 mM Mg^{++} and then adjusted it to 15 or 20 mM Mg^{++} concentration before centrifugation at the chosen Mg^{++} concentration since it is known that Mg^{++} ions stabilize ribosomes. If the 39S particles were derived from the native 50S subunits because of their instability, one would expect the 39S peak to disappear when Mg^{++} concentration is increased. As seen in (I, Figure 6A), this was not the case, meaning that 39S ribosomal particles are stable during sample processing. Furthermore, 39S particles contained L-proteins (L. Peil & J. Remme, unpublished data) and precursor 23S rRNA (Figure 4), (Gutgsell et al., 2005), indicating that those particles are likely to be precursors to 50S subunits. Thus, from these results one can conclude that the absence of RluD protein leads to the assembly defect of both ribosome subunits.

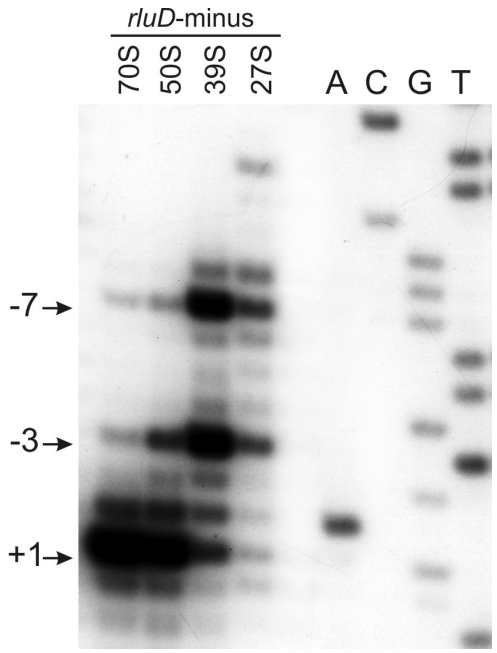

Figure 4. Analysis of 5' termini of *rluD*-minus strain 23S rRNA.
Ribosomal particles were separated by sucrose gradient centrifugation, RNA was precipitated and purified and 5' termini were determined by primer extension analysis. Positions of mature ('+1') and precursor ('-3' and '-7') 23S rRNA 5' termini are indicated, together with corresponding DNA sequence.

Alternatively to the hypothesis described above, ribosome assembly defects could have been caused independently of RluD catalytic activity or pseudouridines, for example when RluD has a second function in the cells, unlinked to its primary catalytic activity. This was checked by transforming *rluD*-minus Tiny cells with plasmids containing *rluD* sequences coding for catalytically inactive forms of RluD and recording corresponding ribosome profiles (I, Figure 6B). *rluD*-minus Tiny cells have an unidentified second-site mutation that is able to partially suppress slow-growth phenotype of *rluD*-minus Dust cells (Gutgsell et al., 2001). That second-site mutation is also capable of partially suppressing ribosome assembly defect (I, compare Figure 6A and 6B). In general, there are no 39S particles in *rluD*-minus Tiny cells although there still are abnormal 42S particles present in the pattern. Furthermore, 27S particles have shifted back to the 30S region in *rluD*-minus Tiny cells when compared to *rluD*-minus Dust cells (I, Figure 6). Taken together, this all indicates a partial suppression of ribosome assembly defects in *rluD*-minus Tiny cells when compared to *rluD*-minus Dust cells.

The essentiality of RluD catalytic activity was analyzed in *rluD*-minus Tiny cells instead of the original *rluD*-minus Dust cells because of previously

published intriguing results. Namely, Gutgsell and colleagues had discovered the re-appearance of RluD-specific pseudouridines in *rluD*-minus Dust cells expressing mutated RluD protein from the plasmid (Gutgsell et al., 2001), (I, Figure 5B). Interestingly, they failed to observe similar results for *rluD*-minus Tiny cells (Gutgsell et al., 2001), (I, Figure 5A). Based on the fact that *rluD*-minus Dust cells had restored pseudouridines in helix 69 of 23S rRNA in the presence of mutated RluD protein, they were not a suitable platform for further studies on a possible second function of RluD. Therefore, *rluD*-minus Tiny strain was selected instead for following studies. To much of a surprise, mutated forms of RluD protein were able to rescue aberrant ribosome profiles of *rluD*-Tiny cells back to wt appearance (I, Figure 6B) whereas no pseudouridines were made in helix 69 of 23S rRNA (I, Figure 5A). This result suggested intriguingly that RluD has a second function in cells which is independent of its pseudouridine catalytic activity and which is responsible for the correct ribosome assembly. While being the first such indication for pseudouridine synthases, there was evidence for similar phenomenon among other RNA modifying enzymes. For example, lack of Pet56p (2'-O-methyltransferase specific for G2270 in 21S rRNA in yeast mitochondria) caused massive defects in ribosome large subunit assembly (Sirum-Connolly and Mason, 1993) that were implicated to be rescued with the introduction of catalytically inactive Pet56p protein. Later it was mentioned that while Pet56p is normally essential for the formation of functional mitochondrial ribosomes, extragenic mutations have been obtained that suppress, albeit weakly, Pet56p loss-of-function mutations (Sirum-Connolly and Mason, 1995). Thus, it can be concluded that neither the Pet56p-catalyzed ribose methylation nor the Pet56p protein itself is absolutely required for the synthesis of a functional ribosome.

Unfortunately, future studies proved the hypothesis about the potential second function of RluD wrong due to simple errors in the experimental system. Most importantly, original *rluD*-minus Dust and Tiny cells appeared to contain two copies of *rluD* gene, of which only one was disrupted by a miniTn*10* cassette, a result established by colony PCR and Southern hybridization analysis (Figure 5). Initial screening of *rluD*-minus strains by PCR with primers complementary to *rluD* gene resulted in a finding that all *rluD*-minus strains produced an extra band similar in length to wt *rluD* gene, together with the longer band corresponding to disrupted *rluD* gene (Figure 5A). To determine the origin of both products seen in PCR analysis, we used Southern hybridization analysis. For that, partial digestion of corresponding genomic DNA and hybridization with *rluD* gene specific labeled probes was performed (Figure 5B). There were two options as in what configuration wt and disrupted *rluD* gene could have been in the genome, presented in Figure 6. Based on the experimental data presented in Figure 5, we concluded that all *rluD*-minus strains contained an extra copy of wt *rluD* gene adjacent to the miniTn*10*-interrupted *rluD* gene instead of it being in front of the miniTn*10*-interrupted *rluD* gene, as indicated in Figure 6 (compare hypothetical fragments shown in Figure 6 to experimental data shown in Figure 5).

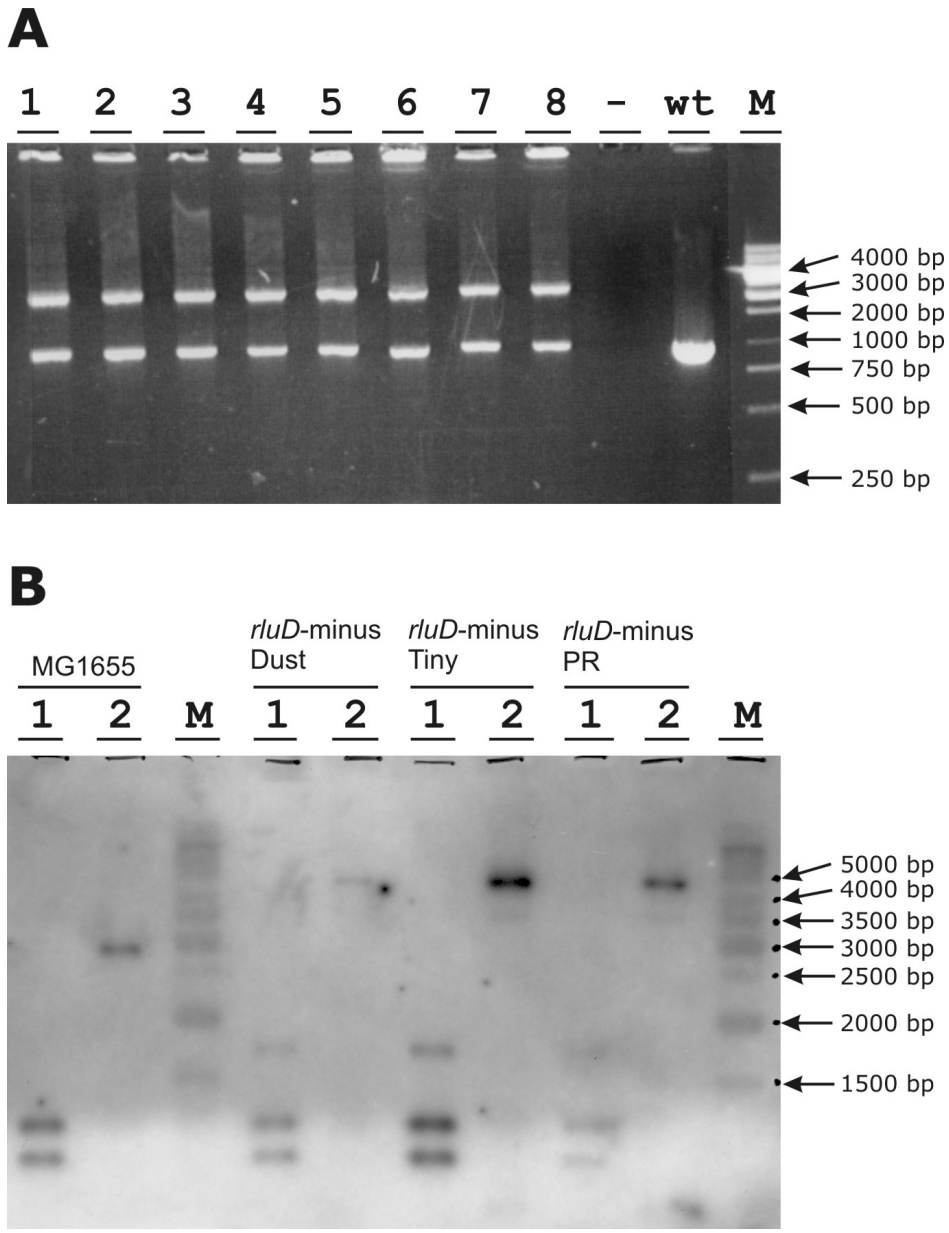

Figure 5. Analysis of wt and *rluD*-minus strains by colony PCR (A) and Southern hybridization (B).
(A). Lanes 1–3: *rluD*-minus Dust; lanes 4–6: *rluD*-minus Tiny; lanes 7–8: *rluD*-minus PR; lane '–': empty reaction; lane 'wt': MG1655; lane 'M': molecular weight marker. Products were separated in 1% agarose gel and visualized by UV.
(B). Lanes marked as '1': *Mlu*I digestion of genomic DNA, lanes marked as '2': *BssH*II digested genomic DNA, lane 'M': molecular weight marker. Filter was hybridized with radioactively labeled *rluD*-specific probe and autoradiographed. Positions of molecular weight markers are indicated.

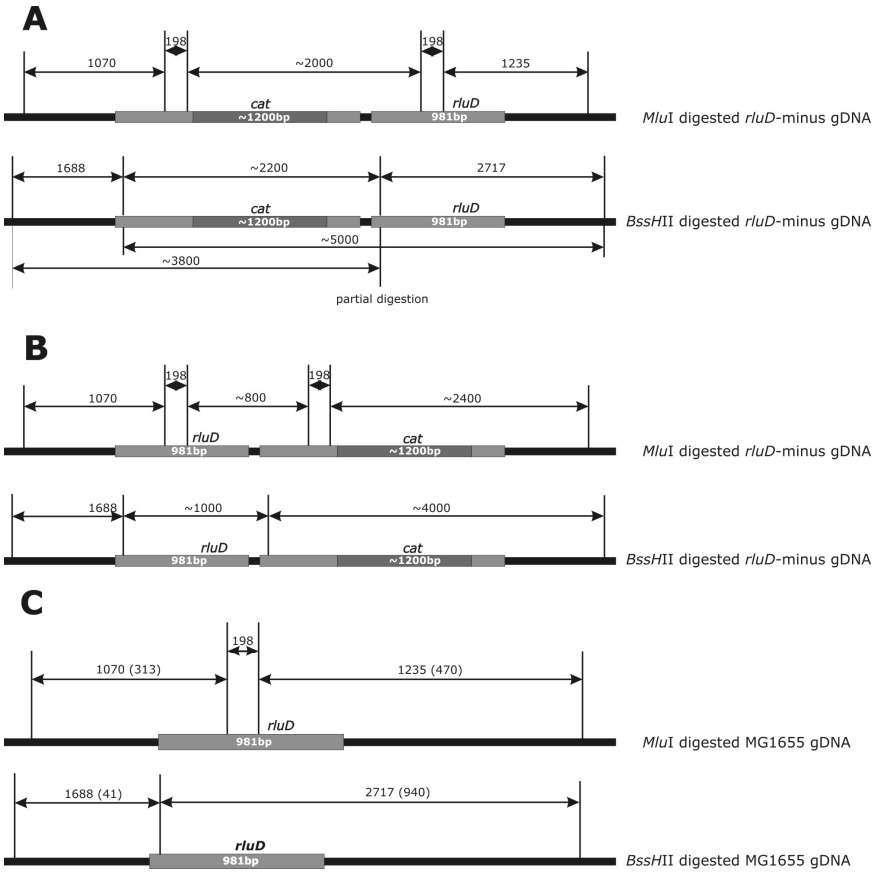

Figure 6. Schematic representation of hypothetical DNA fragments produced by genomic DNA digestion with two different restriction enzymes, figure is not to scale.
(A) Hypothetical genomic DNA fragments produced in *rluD*-minus strains when extra copy of *rluD* gene is placed after disrupted gene.
(B) Hypothetical genomic DNA fragments produced in *rluD*-minus strains when extra copy of *rluD* gene is placed in front of disrupted gene.
(C) Hypothetical genomic DNA fragments produced in wt MG1655 strain with single rluD gene. Fragment lengths in brackets correspond to regions complementary to *rluD* gene.

This in turn effectively meant that previous intriguing results about catalytically inactive form of RluD being able to rescue ribosome assembly and growth defects were most likely due to the second copy of *rluD* gene present in the genome. This could have happened via many ways. First, recombination between genomic copy of wt *rluD* gene and mutated copy of *rluD* gene in the plasmid could have happened, resulting in the re-appearance of RluD-specific pseudouridines and rescue of previously observed defects. Second, since all original *rluD*-minus strains initially did not contain RluD-specific pseudouridines (I, Figure 5), this means that the expression of an extra wt copy of *rluD*

gene was negligible. On the other hand, plasmid transformation of *rluD*-minus Dust cells and numerous follow-on passages might have been enough for the accumulation of mutations to allow for enough of the wt *rluD* gene expression responsible for the re-appearance of RluD-specific pseudouridines. Nevertheless, this second copy of wt *rluD* gene was not the reason for the high frequency of pseudoreversion events observed, since similar events took place in a number of other full deletion strains of *rluD* gene (Ejby et al., 2007; Gutgsell et al., 2005).

Results presented here were confirmed indirectly when a study was published later with a newly constructed *rluD* deletion strain. In that work no rescue of slow-growth phenotype or ribosome assembly defects by mutated forms of RluD protein were observed (Gutgsell et al., 2005). Therefore, as intriguing as the hypothesis of RluD having a second function in the cells was, it is confirmed by now that all observed defects are either due to the lack of pseudouridines or absence of catalytically active RluD protein. At the moment, more evidence is pointing towards the hypothesis that the lack of pseudouridines is responsible for the initial assembly defects, but that these defects can be overcome by a number of second-site suppressor mutations. So-called pseudorevertants lack RluD-specific pseudouridines but have a normal growth phenotype (Ejby et al., 2007; Gutgsell et al., 2001; Gutgsell et al., 2005) and ribosomal profiles (Gutgsell et al., 2005). As of today, only one of many suppressor mutations has been identified and characterized in detail, a point mutation in RF2 (Ejby et al., 2007). The discovery that the introduction of RF2(E172K) into *rluD*-minus Dust background reduced otherwise 2 to 16- fold higher stop codon read-through rate back to the wt level (Ejby et al., 2007) was a clear indication that at least one of RluD-specific pseudouridines in helix-loop 69 of 23S rRNA is involved in translation termination by RF2. This suggestion was well supported by both overlaid 3D structures of ribosome and RF2 that showed the close proximity of RF2(E172K) mutation to helix-loop 69 (Ejby et al., 2007) and results by Ali et al where only subunit association, translation initiation, termination, or ribosome recycling were shown to be affected by the absence of helix-loop 69 (Ali et al., 2006).

Therefore, one can conclude that the defects seen in *rluD*-minus cells are mostly due the translational deficiencies of ribosomes (at termination step), likely caused by the lack of three RluD-specific pseudouridines in helix-loop 69 of 23S rRNA. In addition, it has been shown for methyltransferase RlmE (RrmJ/FtsJ) that not the catalytically inactive enzyme but its product, a 2'-O-methylated U2552 in 23S rRNA, is needed for correct ribosome assembly (Hager et al., 2002; Hager et al., 2004). Lack of Um2552 affects translation *in vitro* (Caldas et al., 2000b) and *in vivo*, causing ribosomes to become much more accurate (Widerak et al., 2005) which in turn likely affects ribosome assembly, similarly to that seen in *rluD*-minus cells.

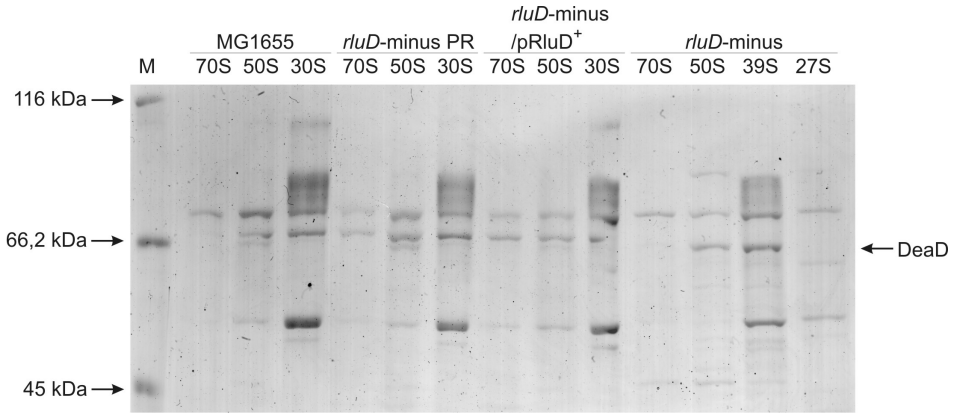

Figure 7. Analysis of non-ribosomal proteins in the ribosomal fractions by SDS-PAGE and Coomassie blue staining.
Ribosomal particles were separated by sucrose gradient centrifugation, proteins were extracted by TCA precipitation, and separated on 7% SDS-PAGE. Positions of protein marker bands and DeaD protein are indicated, as are ribosomal particles.

One more interesting result was revealed by protein analysis that needs to be discussed. A protein with an apparent molecular weight of ~65 kDa was found mainly in the the 39S but also to a lesser extent in the 50S particles although not in the 70S or 27S particles of the *rluD*-minus Dust strain (Figure 7). This protein was identified as an RNA helicase DeaD (N-terminal sequence was determined by Edman degradation to be E/S/G/H/A-E-F-E-T/Q/A-X-F-A-D-L/K), a protein which is involved in many processes in the cell (reviewed in detail above) and which was later shown to be required for the normal ribosome assembly in *E. coli* (Charollais et al., 2004). As shown in Figure 7, DeaD protein was also present in the 50S subunits of wt strain, *rluD*-minus Dust carrying the rescue plasmid pRluD+, and in a pseudorevertant of the *rluD*-minus Dust strain (*rluD*-minus/PR) but in a lesser amount than was found in the 50S subunits of *rluD*-minus Dust cells. The presence of DeaD protein at increased level in the ribosomal fractions of *rluD*-minus Dust cells indicate that the expression of DeaD protein is either induced upon disruption of *rluD* gene or that DeaD accumulates in incompletely assembled ribosomal particles. While there is no direct evidence that the lack of RluD specifically causes DeaD to accumulate, second hypothesis is well supported. Namely, DeaD has been shown to suppress a mutation in S2 (Toone et al., 1991), thereby linking it to ribosome biogenesis. At the same time, DeaD has been shown to be bound to the assembly precursor particles in RNA helicase SrmB-deficient cells and over-expression of DeaD is enough to rescue ribosome assembly defects in *srmB*-minus strain (Charollais et al., 2004).

Based on the data presented here, we can conclude that the deletion of pseudouridine synthase *rluD* gene leads to major defects in ribosome assembly.

Although initial results suggested that such defects are caused by the lack of RluD protein, it can now be concluded that the defects seen are mostly due to the absence of corresponding pseudouridines that cause deficiency in translation, probably in the termination step. In addition, we have linked together pseudouridine synthase RluD and RNA helicase DeaD in ribosome biogenesis, a point further discussed below.

Ref II. Pseudouridine synthase RluD is highly specific towards uridines at positions 1911 and 1917 and it is involved in the late step of ribosome assembly

It had been proposed previously by Raychaudhuri and colleagues that RluD recognizes helix 69 in 23S rRNA and then isomerizes all uridines in the loop region to pseudouridines, in a position-independent manner (Raychaudhuri et al., 1998). This suggestion was supported by the finding that RluD protein acting on a naked 23S rRNA was quite unspecific, since it isomerized more than three uridines per 23S rRNA molecule (Wrzesinski et al., 2000). We succeeded to show that in contrast to previous suggestions, RluD is highly specific towards uridines at positions 1911, 1915 and 1917 in 23S rRNA (II, Figure 2). In this work, we tested whether RluD is able to modify uridines at other positions of helix-loop 69 in 23S rRNA, by using single nucleotide substitutions A1912U, C1914U, A1916U and A1919U. Mutant genes for 23S rRNA were expressed *in vivo* and pseudouridines were determined by chemical modification followed by primer extension analysis. Since uridine at position 1915 is further methylated at N3 position, it was not possible to determine the presence of pseudouridine at this position as $m^3\Psi$ itself causes primer extension stop independent of any further chemical treatment. Three of four analyzed mutant RNAs (A1912U, C1914U and A1919U) all contained pseudouridines only in positions 1911 and 1917, similarly to wt 23S rRNA (II, Figure 2). Interestingly, in A1916U mutant no pseudouridines could be detected in 50S subunits and pseudouridines were barely detectable in 70S ribosomes. This suggested that A1916 serves as an important specificity determinant for RluD, a conclusion supported by additional mutagenesis. Namely, of all used mutations, only A1916U and A1916G had effect on pseudouridines synthesis by RluD (II, Figure 4). As already mentioned, in case of A1916U no pseudouridines could be detected either in the 70S ribosomes or in free 50S subunits. In case of A1916G, pseudouridines were present in the 70S ribosomes but not in free 50S subunits.

Next we wanted to identify whether RluD acts during early assembly on naked 23S rRNA or during late assembly. In latter case RluD can require the presence of r-proteins or a conformational change of rRNA. For that purpose we used *deaD*-minus strain which was previously shown to be deficient in ribosomal large subunit assembly (Charollais et al., 2004). Furthermore, the 40S particles accumulating in this strain are assembly precursors to 50S subunits

(IV, Figure 3) and since DeaD was implicated to be a late assembly factor (Charollais et al., 2004), this provided perfect test-bed. The presence of RluD-specific pseudouridines was determined from wt and *deaD*-minus ribosomal particles. Expectedly, wt 70S and 50S particles contained RluD-specific pseudouridines, as did the 70S ribosomes from *deaD*-minus cells (II, Figure 5). The 50S and 40S particles on the other hand contained only traces of RluD-specific pseudouridines, indicating that RluD is active in the absence of DeaD and that it acts on a late assembly step. In addition, we have determined that free 50S particles from the *deaD*-minus strain have low functional activity, probably due to incomplete assembly (IV, Table 2). The finding that RluD acts on a late assembly step was fully supported by the finding by Vaidyanathan et al (Vaidyanathan et al., 2007). They determined that free 50S subunits are much better and more efficient substrates to RluD than free 23S rRNA (Vaidyanathan et al., 2007), once again implicating that RluD acts on a late step of ribosome assembly, a conclusion in very good correlation with our results described above.

In conclusion, these results make RluD one of the few RNA modification enzymes (together with RlmE (Bügl et al., 2000) and RsmE (Basturea and Deutscher, 2007)) that is required for the efficient ribosome biogenesis and that acts better on assembled ribosomal subunits than on free RNA. Based on the data known previously and presented above, one can conclude that products of pseudouridine synthase RluD are needed for the correct assembly of ribosomes.

The question that still remains unanswered is - what is needed for and how does RluD recognize its substrate? At the moment there are two alternatives as how this can happen. First, r-proteins may be needed for the RluD directed isomerization of uridines in helix-loop 69 or, alternatively, proper folding of helix-loop 69 needed for the RluD directed isomerization of uridines in helix-loop 69 occurs only during the late step of 50S subunit assembly. Unfortunately, no experimental data has been collected yet to support either of those hypothesis.

Ref III. Identification of a pseudouridine methyltransferase RlmH

As described above, helix-loop 69 of *E. coli* 23S rRNA contains three modified nucleosides: pseudouridines at positions 1911 and 1917 and $m^3\Psi$ at position 1915. Pseudouridines in helix-loop 69 are synthesized by a pseudouridine synthase RluD while the enzyme responsible for the methylation of pseudouridine at position 1915 remained unknown for long time. In this paper, we identified RlmH as the first pseudouridine specific methyltransferase. The identity of modified residue at position 1915 in *E. coli* 23S rRNA was determined to be 3-methylpseudouridine, as proposed previously (Kowalak et al., 1996). This is the first and only modified pseudouridine residue found in bacterial rRNA to date while there are three different species of modified pseudouridines found in Eukarya: m^1-pseudouridine, 2'-O-methylpseudouridine

and m^1acp^3-pseudouridine (Kowalak et al., 1996). There is also some evidence that $m^3\Psi$ is present in *Zea mays* chloroplasts (Ofengand et al.), a point further discussed below.

RlmH (previously called YbeA) was identified as a pseudouridine methyltransferase from a set of 11 putative methyltransferase knock-out strains (part of KEIO collection). Candidate genes for the initial screen were selected based on their functional annotation and availability of the knock-out strains. From each of the strains, total rRNA was extracted and screened for the presence of $m^3\Psi$ at position 1915 in 23S rRNA by primer extension analysis (III, Figure 2). Ten out of eleven knock-out strains together with wt strain had a strong stop signal, corresponding to a methylated pseudouridine at position 1915 of 23S rRNA. *rlmH*-minus strain on the other hand had no stop signal, indicating the absence of corresponding methylated pseudouridine. The ability of RlmH to actually methylate pseudouridine at position 1915 was further confirmed by HPLC analysis of nucleoside composition of corresponding 23S rRNA fragment from different strains – wt, *rlmH*, *rlmH*/pRlmH and 23S rRNA treated with RlmH *in vitro* (III, Figure 3). After the discovery of RluD, there had been no direct confirmation whether uridine or pseudouridine serves as a substrate for a subsequent methylation reaction although it was proposed that pseudouridine is a better substrate (Raychaudhuri et al., 1998). In good correlation with that proposal, we discovered that RlmH heavily prefers pseudouridine to uridine as a substrate for methylation reaction. (III, Figure 4). Furthermore, it appeared that RlmH requires pseudouridine at position 1915 to be presented in the 70S ribosomes and not in free 50S subunits for the methylation. Based on that data, one can conclude that RluD is also specific to 70S ribosomes as it is required to act before RlmH. In accordance, Purta et al have modeled RluD into 70S ribosome according to our finding (Purta et al., 2008). These results indicate that RlmH to takes part in the late step of ribosome assembly, as RlmH acts well after pseudouridine synthase RluD (RluD had already been shown to be a late step modifying enzyme (II)) and RlmH requires 50S subunit to be associated with 30S subunit.

An interesting feature of *rlmH* gene that needs to be discussed is that it is a widely conserved gene. Based on its amino acid sequence, RlmH belongs to the COG1576 cluster of SPOUT superfamily methyltransferases (Tkaczuk et al., 2007). Clusters of Orthologous Groups (COGs) is a systematic grouping of gene families from completely sequenced genomes (Tatusov et al., 1997), (Tatusov et al., 2003). Since the number of sequenced genomes is big and increasing fast, only one representative was taken from each class or family of organisms for sequence alignment analysis (III, Figure 5 and III, Supplementary Figure 1, correspondingly). Sequence alignment analysis revealed two main results, both of which are discussed in detail below. First, based on our sequence analysis, members of COG1576 were found in all three domains of life and second, RlmH homologs are well conserved, especially in their C-terminal part.

In Eukarya, we found COG1576 representatives only in green plants (*Viridiplantae*), with NP_196624 from *Arabidopsis thaliana* being annotated as a chloroplast protein (III, Figure 5 and Supplementary Table 1). This finding supports the discovery of $m^3\Psi$ from *Zea mays* chloroplasts (Ofengand et al.) but since *Zea mays* genome was not available in either COG or RefSeq databases at the time of our analysis, there was no way to confirm the presence of an RlmH ortholog in maize genome. Similarly, in a back-to-back publication by Purta et al (Purta et al., 2008), COG1576 members in plants were only found in chloroplasts and so far there is no evidence to support the presence of $m^3\Psi$ in cytoplasmic ribosomes. In Archaea, we found COG1576 members to be present only in the phylum *Euryarchaeota*, a finding supported by Purta et al (Purta et al., 2008). In contrast to Eukarya and Archaea, members of COG1576 were present in most major lineages in Bacteria. According to our data, the corresponding gene was present in phyla *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Deinococcus-Thermus*, *Firmicutes*, *Fuso-bacteria*, *Lentisphaerae*, *Proteobacteria*, *Synergistetes*, *Thermotogae*, and *Verrucomicrobia* (III, Figure 5, Supplementary Figure 1 and Supplementary Table 1). Interestingly, Purta et al failed to find any *rlmH* ortholog in *Actinobacteria* and *Cyanobacteria*, probably due to different sequence selection criteria. At the same time, they made an interesting observation that in each organism that had an RlmH ortholog also an RluD ortholog was present. This in turn is in very good correlation with our data that shows the requirement for prior pseudouridine formation before the methylation by RlmH can happen (III). One more interesting correlation was revealed by the sequence alignment analysis. Namely, while RlmH was present in *Mycoplasma pulmonis* and *Ureaplasma urealyticum*, it was absent in *Mycoplasma pneumoniae* and *Mycoplasma genitalium* among *Firmicutes*, it was also missing in Rickettsiales among alpha-proteobacteria, all of who are parasites with small genomes. The fact that such organisms have lost the *rlmH* gene is interesting, though no clue about its biological relevance can yet be drawn.

Coming back to the second conclusion drawn based on sequence alignment, we found that six amino acids were conserved among a smaller set of analyzed sequences encompassing a member from each class of organisms (III, Figure 5). Of those six amino acids, two glycines (G103 and G107, *E. coli* numbering) are in the predicted SAM-binding region and one arginine (R142) is a predicted catalytic amino acid. An arginine in a similar context is conserved in the methyltransferase TrmD and has shown to be part of its catalytic center (Elkins et al., 2003), similarly to another SPOUT methyltransferase TrmH (Watanabe et al., 2005). Similar results were obtained with a larger set of sequences, encompassing a member from each family of organisms (III, Supplementary Figure 1). Even though the total number of universally conserved amino acids was reduced from six to four, one of them was still R142, strengthening the hypothesis about its importance as a catalytic amino acid.

In conclusion, we have identified a widely conserved RlmH protein as a pseudouridine methyltransferase that requires pseudouridine presented in the

70S ribosome as a substrate for efficient methylation. These results together with data from Purta and colleagues (Purta et al., 2008) implicates that RlmH is one of the latest known rRNA modification enzymes acting in ribosome biogenesis, most probably during the first steps of translation. Since it has been known for a long time that final maturation of rRNA termini takes place under conditions favouring protein synthesis (Srivastava and Schlessinger, 1990), it is not too surprising that some RNA modifications are made so late in the assembly process. Furthermore, as the absence of RlmH protein leads to slightly reduced amounts of modified nucleosides in the 23S rRNA fragment 1778-1921 (III, Table 1), one might postulate the role of RlmH to be in ribosome biogenesis quality control. On the other hand, since cells lacking functional RlmH protein had a clear competitional growth disadvantage against wt cells (Purta et al., 2008), this might indicate the functional importance of $m^3\Psi$ for efficient translation, similar to results observed in the absence of RlmE (Caldas et al., 2000b; Widerak et al., 2005) or RluD (Ejby et al., 2007).

Ref IV. RNA helicase DeaD is involved in the ribosome assembly

Having established previously that RNA helicase DeaD is involved in ribosome biogenesis, being over-represented in ribosomal particles from pseudouridine synthase RluD deficient strain (Ref I), we decided to analyze ribosome assembly in *E. coli* cells lacking RNA helicases DeaD and DbpA. For that, we used a previously available *deaD*-minus strain and self-constructed *dbpA*-minus strain. DbpA was chosen for the analysis because of its high specificity towards *E. coli* 23S rRNA, suggesting its involvement in ribosome biogenesis.

First analysis focused on the ribosomal particle content of wt and both deletion strains (IV, Figure 1). In wt cells, major peak was formed by the 70S ribosomes with low levels of free 50S and 30S subunits, both at 25 and 37 °C (IV, Figure 1A). To much of our surprise, *dbpA*-minus cells exhibited similar ribosome pattern to wt cells (IV, Figure 1C), even though DbpA is highly specific to 23S rRNA (Fuller-Pace et al., 1993) and suggested to be involved in ribosome biogenesis. This result, together with the results from the growth rate experiment allowed us to conclude that the deletion of *dbpA* has no detrimental effect to ribosome biogenesis under the used experimental conditions, therefore no further experiments were carried out with this strain.

In contrast, deletion of *deaD* gene caused major defects in ribosome pattern, more so at lower growth temperature. The balance between 70S ribosomes and free subunits was disrupted, with much more 30S subunits being accumulated in *deaD*-minus cells than 50S subunits, together with the appearance of a new 40S particle (IV, Figure 1B). This observed pattern was similar to the one originally published by Charollais et al (Charollais et al., 2004), suggesting that the disruption of *deaD* gene leads to defects in ribosome assembly. RNA content analysis revealed that 40S particles contained exclusively 23S rRNA, a feature common to ribosome large subunit (IV, Figure 2). Furthermore, by analyzing

the 5' end of 23S rRNA extracted from both 50S and 40S particles of *deaD*-minus cells, we came to a conclusion that they contained a large amount of immature 23S rRNA (IV, Table 1). This conclusion was based on the fact that final maturation of 23S rRNA requires initial cleavage by RNase III (King et al., 1984) and that RNase III cleavage site specificity is determined by the presence of r-proteins (Allas et al., 2003). Namely, naked 23S rRNA is cleaved at position '-7' while 23S rRNA assembled into an RNP is cleaved at position '-3'. Such a straightforward specificity allows one to easily determine whether the 23S rRNA has been processed as a protein-free RNA transcript or as a ribosomal particle. It is interesting to note that while free 50S subunits from both wt and *deaD*-minus cells exhibited nearly identical amount of different pre-23S rRNA species, 40S particles of *deaD*-minus cells contained ~10% more of '-7' form, with a concomitant decrease in '-3' and mature form (IV, Table 1). This in turn suggests strongly that 40S particles are incompletely assembled 50S subunits, due to the lack of RNA helicase DeaD. Similar to our results, Charollais et al had previously determined that 40S particles accumulate in *deaD*-minus cells but there were no indications whether they are true precursors to 50S subunits or so-called 'assembly dead-end' products (Charollais et al., 2004).

It was known previously that the over-expression of DeaD protein can rescue ribosome assembly defects seen in an RNA helicase SrmB-deficient cells but not vice versa (Charollais et al., 2004), indicating that 40S particles from SrmB-deficient cells can be matured into ribosome 50S subunits. Whether similar was true for the 40S particles from DeaD-deficient cells, was not known. Therefore, we decided to solve this question by analyzing the time course of ribosome assembly in *deaD*-minus cells by using an RNA pulse-labeling strategy. Exponentially growing cells were labeled with [^{3}H]uridine for 5 minutes, after which transcription was blocked with rifampicin and time-points were taken for ribosome profile analysis by radioactive counting and UV absorbance. This strategy allowed us to monitor the incorporation of freshly synthesized and labeled rRNA into ribosomes, thereby following the fate of precursor particles.

In wt cells grown at 25 °C, at the 0 minute time point the majority of radioactively labeled RNA was found in the 50S and 30S fractions, indicating fast rate of ribosomal subunit assembly. About half of the radioactively labeled rRNA was incorporated into 70S ribosomes within 5 minutes at 25 °C and after 10 minutes, most of the radioactively labeled rRNA had already been incorporated into the 70S ribosomes (IV, Figure 3A). From thereafter, no changes in the ribosome pattern were observed, leading us to a conclusion that ribosome assembly is completed in 10 minutes in wt cells growing at 25 °C. It is worth to mention here that ribosome subunit assembly is thought ot be completed in the 70S ribosomes that can actively participate in translation.

In contrast, the time course and radioactively labeled RNA pattern was radically different in *deaD*-minus cells. At the 0 minute time point the majority of radioactively labeled RNA was found in the 40S and 30S fractions and it moved very slowly to the 70S ribosomes (IV, Figure 3B). After 20 minutes,

only about 50% of radioactively labeled RNA was incorporated into 70S ribosomes and that process was not completed even after 40 minutes. The fact that radioactive signal shifts from ribosomal precursor particles towards mature 70S ribosomes shows that the 40S particles of *deaD*-minus cells can indeed be matured to form functional ribosomes. The rate of ribosome maturation is approximately four-fold slower than in wt cells, a conclusion that can be explained by the observed deficiency in the 50S subunit assembly. Indeed, the maturation of 30S subunits proceeds without the accumulation of any apparent precursor particles, suggesting that 30S subunit assembly is not affected by the absence of DeaD. This conclusion is supported by the findings of Charollais et al (Charollais et al., 2004), who failed to detect either precursor 16S rRNA or missing r-proteins from 30S subunits of *deaD*-minus cells. This in turn means that the observed accumulation of 30S subunits is solely due to the slow maturation of 50S subunits that leads to the deficit of 50S subunits.

Finally, we analyzed the functional activity of 50S and 40S particles from wt and *deaD*-minus cells by using puromycin-based peptidyltransferase assay. For this purpose, free 50S and 40S particles as well as 50S subunits dissociated from the 70S ribosomes (designated as 50S*) were collected and checked for their catalytic activity. The 50S subunits derived from 70S ribosomes from both *deaD*-minus and wt cells exhibited almost identical level of activity (IV, Table 2). Free 50S subunits from wt cells on the other hand exhibited 2.5-fold lower activity than the mature 50S* subunits derived from 70S ribosomes, suggesting that ~60% of free 50S subunits are in fact precursor particles that sediment at 50S but are incapable of peptidyltransferase reaction. This result agrees with the observation that 50S particles acquire their sedimentation value rapidly and the limiting step of ribosome large subunit assembly is the activation step of 50S particles (Lindahl, 1975). Moreover, the 50S particles of *deaD*-minus strain are incompletely modified (II, Figure 5). Interestingly, free 50S particles from *deaD*-minus cells are approximately three-fold less active than their wt counterparts and about seven-fold less active compared to mature 50S* subunits. Such a big decrease in their catalytic activity suggests that the rate of 50S subunit assembly is reduced not only in the primary assembly step as observed by pulse-labeling experiment, but also during the final maturation step. Expectedly, 40S particles from *deaD*-cells had no activity in peptidyl-transferase assay.

In conclusion, we have shown that the absence of an RNA helicase DeaD results in defects in ribosome large subunit assembly, characterized by the disrupted ribosome profile and appearance of a new 40S precursor particle. While being initially catalytically inactive, 40S particles can be matured into functional 50S subunits, although at much slower rate than that seen in wt cells. Furthermore, the absence of DeaD does not only slow down the 50S particle formation, it also slows down the final maturation step of 50S subunits during which they become catalytically active. We have shown previously that pseudouridine synthase RluD specific pseudouridines are either missing or

present in trace amounts in 50S and 40S particles from *deaD*-minus cells (II, Figure 5) but whether this is the reason of low catalytic activity observed (IV), needs to be confirmed.

In this context it is interesting to note that while DbpA has been characterized as the most specific of RNA helicases towards its substrate in *E. coli*, its absence does not lead to any observable defects in ribosome biogenesis (Iost and Dreyfus, 2006; Peil et al., 2008). In contrast, the absence of an RNA helicase DeaD that has been shown to take part in many processes in the cell leads to major defects in ribosome assembly, especially at lower growth temperatures (Charollais et al., 2004), (IV, Figure 1 and 2). Based on the substrate specificity and known roles in the cell, one could postulate that the wider array of roles a ribosome specific RNA helicase has in the cell, the bigger the defects seen in ribosome biogenesis. While seemingly true in case of DbpA, SrmB and DeaD, it remains to be seen whether such a stepwise recruitment of an RNA helicase is just a coincidence. So far there is evidence of ribosome assembly defects for only two of the RNA helicases – SrmB and DeaD (Charollais et al., 2004; Charollais et al., 2003; Peil et al., 2008) although third helicase RhlE seems to be involved too (Jain, 2008). Based on the facts that over-expression of DeaD can suppress ribosome assembly defects in *srmB*-minus background while not vice versa and that the deletion of *dbpA* gene does not cause any visible defects in ribosome biogenesis despite its high specificity towards 23S rRNA, one could place SrmB between DbpA and DeaD in its action in ribosome biogenesis.

CONCLUSIONS

Ribosome assembly is a highly complex and coordinated process, which starts shortly after the transcription initiation of ribosomal RNA and during which many ribosomal and extra-ribosomal factors participate in it. Extra-ribosomal factors include proteins with many different activities, for example RNA and protein modification enzymes, RNA helicases, ribosome-dependent GTPases, heat-shock proteins and RNA chaperones. The involvement of RNA modification enzymes and corresponding modifications in the ribosome assembly process has been of high interest for the last decades, as has been the involvement of other extra-ribosomal factors. Under normal conditions, ribosome assembly is finished within minutes after the start of ribosomal RNA transcription whereas *in vitro* reconstitution from purified ribosomal components requires numerous long incubations and heating steps. In a way, this suggests that other (extra-ribosomal) factors are required for the efficiency of the ribosome assembly process.

In this study the main focus was on the characterization of roles of pseudouridine synthase RluD and RNA helicase DeaD in the ribosome assembly process. Furthermore, we wanted to identify and characterize the enzyme responsible for the synthesis of an only known modified pseudouridine residue in *Escherichia coli* ribosomal RNA.

Following conclusions can be drawn from the current thesis:

1. The deletion of pseudouridine synthase RluD in *Escherichia coli* causes major defects in the assembly of both small and large ribosomal subunits, resulting in the accumulation of abnormal ribosomal particles in *rluD*-minus cells. At the same time, the amount of free 70S ribosomes is severely reduced and accumulated abnormal 39S ribosomal particles contain incompletely processed precursor 23S rRNA. In addition, RNA helicase DeaD is found in RluD-deficient 39S ribosomal particles, thereby linking the involvement of both RluD and Dead in in the ribosome assembly process.
2. Pseudouridine synthase RluD acts in the late step of ribosome large subunit assembly and is highly specific to pseudouridines at positions 1911 and 1917 in *Escherichia coli* 23S rRNA.
3. Highly conserved SPOUT-family methyltransferase RlmH (previously YbeA) which is present in all kingdoms of life (Bacteria, Archaea, Eukaryota), is responsible for the methylation of pseudouridine at position 1915 in *Escherichia coli* 23S rRNA.
4. The deletion of RNA helicase DeaD in *Escherichia coli* causes major defects in the assembly of large ribosomal subunits and accumulation of the 40S particles. Free 50S and 40S particles contain incompletely processed and modified 23S rRNA. Furthermore, 40S particles can mature into the 70S ribosomes *in vivo*, although at much slower rate than in wt cells.
5. Free ribosome large subunits from RNA helicase DeaD-deficient cells are only partially active in *in vitro* peptidyltransferase assay whereas 40S particles are catalytically inactive.

REFERENCES

Al Refaii, A., and Alix, J. (2009). Ribosome biogenesis is temperature-dependent and delayed in Escherichia coli lacking the chaperones DnaK or DnaJ. Mol Microbiol *71*, 748–762.

Ali, I., Lancaster, L., Feinberg, J., Joseph, S., and Noller, H. (2006). Deletion of a conserved, central ribosomal intersubunit RNA bridge. Mol Cell *23*, 865–874.

Alix, J., and Guérin, M. (1993). Mutant DnaK chaperones cause ribosome assembly defects in Escherichia coli. Proc Natl Acad Sci U S A *90*, 9725–9729.

Alix, J., and Nierhaus, K. (2003). DnaK-facilitated ribosome assembly in Escherichia coli revisited. RNA *9*, 787–793.

Allas, U., Liiv, A., and Remme, J. (2003). Functional interaction between RNase III and the Escherichia coli ribosome. BMC Mol Biol *4*, 8.

Apirion, D., and Lassar, A. (1978). A conditional lethal mutant of Escherichia coli which affects the processing of ribosomal RNA. J Biol Chem *253*, 1738–1742.

Arigoni, F., Talabot, F., Peitsch, M., Edgerton, M., Meldrum, E., Allet, E., Fish, R., Jamotte, T., Curchod, M., and Loferer, H. (1998). A genome-based approach for the identification of essential bacterial genes. Nat Biotechnol *16*, 851–856.

Arnold, R., and Reilly, J. (1999). Observation of Escherichia coli ribosomal proteins and their posttranslational modifications by mass spectrometry. Anal Biochem *269*, 105–112.

Awano, N., Xu, C., Ke, H., Inoue, K., Inouye, M., and Phadtare, S. (2007). Complementation analysis of the cold-sensitive phenotype of the Escherichia coli csdA deletion strain. J Bacteriol *189*, 5808–5815.

Basturea, G., and Deutscher, M. (2007). Substrate specificity and properties of the Escherichia coli 16S rRNA methyltransferase, RsmE. RNA *13*, 1969–1976.

Bharat, A., Jiang, M., Sullivan, S., Maddock, J., and Brown, E. (2006). Cooperative and critical roles for both G domains in the GTPase activity and cellular function of ribosome-associated Escherichia coli EngA. J Bacteriol *188*, 7992–7996.

Bizebard, T., Ferlenghi, I., Iost, I., and Dreyfus, M. (2004). Studies on three E. coli DEAD-box helicases point to an unwinding mechanism different from that of model DNA helicases. Biochemistry *43*, 7857–7866.

Butland, G., Krogan, N., Xu, J., Yang, W., Aoki, H., Li, J., Krogan, N., Menendez, J., Cagney, G., Kiani, G.*, et al.* (2007). Investigating the in vivo activity of the DeaD protein using protein-protein interactions and the translational activity of structured chloramphenicol acetyltransferase mRNAs. J Cell Biochem *100*, 642–652.

Butland, G., Peregrín-Alvarez, J., Li, J., Yang, W., Yang, X., Canadien, V., Starostine, A., Richards, D., Beattie, B., Krogan, N.*, et al.* (2005). Interaction network containing conserved and essential protein complexes in Escherichia coli. Nature *433*, 531–537.

Bylund, G., Persson, B., Lundberg, L., and Wikström, P. (1997). A novel ribosome-associated protein is important for efficient translation in Escherichia coli. J Bacteriol *179*, 4567–4574.

Bylund, G., Wipemo, L., Lundberg, L., and Wikström, P. (1998). RimM and RbfA are essential for efficient processing of 16S rRNA in Escherichia coli. J Bacteriol *180*, 73–82.

Böddeker, N., Stade, K., and Franceschi, F. (1997). Characterization of DbpA, an Escherichia coli DEAD box protein with ATP independent RNA unwinding activity. Nucleic Acids Res *25*, 537–545.

Bügl, H., Fauman, E., Staker, B., Zheng, F., Kushner, S., Saper, M., Bardwell, J., and Jakob, U. (2000). RNA methylation under heat shock control. Mol Cell *6*, 349–360.

Cabezón, T., Herzog, A., Petre, J., Yaguchi, M., and Bollen, A. (1977). Ribosomal assembly deficiency in an Escherichia coli thermosensitive mutant having an altered L24 ribosomal protein. J Mol Biol *116*, 361–374.

Caldas, T., Binet, E., Bouloc, P., Costa, A., Desgres, J., and Richarme, G. (2000a). The FtsJ/RrmJ heat shock protein of Escherichia coli is a 23 S ribosomal RNA methyltransferase. J Biol Chem *275*, 16414–16419.

Caldas, T., Binet, E., Bouloc, P., and Richarme, G. (2000b). Translational defects of Escherichia coli mutants deficient in the Um(2552) 23S ribosomal RNA methyltransferase RrmJ/FTSJ. Biochem Biophys Res Commun *271*, 714–718.

Caldon, C., and March, P. (2003). Function of the universally conserved bacterial GTPases. Curr Opin Microbiol *6*, 135–139.

Campbell, T., and Brown, E. (2008). Genetic interaction screens with ordered overexpression and deletion clone sets implicate the Escherichia coli GTPase YjeQ in late ribosome biogenesis. J Bacteriol *190*, 2537–2545.

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

Charollais, J., Dreyfus, M., and Iost, I. (2004). CsdA, a cold-shock RNA helicase from Escherichia coli, is involved in the biogenesis of 50S ribosomal subunit. Nucleic Acids Res *32*, 2751–2759.

Charollais, J., Pflieger, D., Vinh, J., Dreyfus, M., and Iost, I. (2003). The DEAD-box RNA helicase SrmB is involved in the assembly of 50S ribosomal subunits in Escherichia coli. Mol Microbiol *48*, 1253–1265.

Comartin, D., and Brown, E. (2006). Non-ribosomal factors in ribosome subunit assembly are emerging targets for new antibacterial drugs. Curr Opin Pharmacol *6*, 453–458.

Connolly, K., Rife, J., and Culver, G. (2008). Mechanistic insight into the ribosome biogenesis functions of the ancient protein KsgA. Mol Microbiol *70*, 1062–1075.

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

Czyz, A., and Wegrzyn, G. (2005). The Obg subfamily of bacterial GTP-binding proteins: essential proteins of largely unknown functions that are evolutionarily conserved from bacteria to humans. Acta Biochim Pol *52*, 35–43.

Dahlberg, A., Dahlberg, J., Lund, E., Tokimatsu, H., Rabson, A., Calvert, P., Reynolds, F., and Zahalak, M. (1978). Processing of the 5' end of Escherichia coli 16S ribosomal RNA. Proc Natl Acad Sci U S A *75*, 3598–3602.

Daigle, D., and Brown, E. (2004). Studies of the interaction of Escherichia coli YjeQ with the ribosome in vitro. J Bacteriol *186*, 1381–1387.

Dammel, C., and Noller, H. (1995). Suppression of a cold-sensitive mutation in 16S rRNA by overexpression of a novel ribosome-binding factor, RbfA. Genes Dev *9*, 626–637.

Datta, P., Wilson, D., Kawazoe, M., Swami, N., Kaminishi, T., Sharma, M., Booth, T., Takemoto, C., Fucini, P., Yokoyama, S.*, et al.* (2007). Structural aspects of RbfA action during small ribosomal subunit assembly. Mol Cell *28*, 434–445.

Deutscher, M., Marlor, C., and Zaniewski, R. (1985). RNase T is responsible for the end-turnover of tRNA in Escherichia coli. Proc Natl Acad Sci U S A *82*, 6427–6430.

Diges, C., and Uhlenbeck, O. (2001). Escherichia coli DbpA is an RNA helicase that requires hairpin 92 of 23S rRNA. EMBO J *20*, 5503–5512.

Diges, C., and Uhlenbeck, O. (2005). Escherichia coli DbpA is a 3' --> 5' RNA helicase. Biochemistry *44*, 7903–7911.

Doyle, S., and Wickner, S. (2009). Hsp104 and ClpB: protein disaggregating machines. Trends Biochem Sci *34*, 40–48.

Ejby, M., Sørensen, M., and Pedersen, S. (2007). Pseudouridylation of helix 69 of 23S rRNA is necessary for an effective translation termination. Proc Natl Acad Sci U S A *104*, 19410–19415.

El Hage, A., and Alix, J. (2004). Authentic precursors to ribosomal subunits accumulate in Escherichia coli in the absence of functional DnaK chaperone. Mol Microbiol *51*, 189–201.

El Hage, A., Sbaï, M., and Alix, J. (2001). The chaperonin GroEL and other heat-shock proteins, besides DnaK, participate in ribosome biogenesis in Escherichia coli. Mol Gen Genet *264*, 796–808.

Elkins, P., Watts, J., Zalacain, M., van Thiel, A., Vitazka, P., Redlak, M., Andraos-Selim, C., Rastinejad, F., and Holmes, W. (2003). Insights into catalysis by a knotted TrmD tRNA methyltransferase. J Mol Biol *333*, 931–949.

Elles, L., and Uhlenbeck, O. (2008). Mutation of the arginine finger in the active site of Escherichia coli DbpA abolishes ATPase and helicase activity and confers a dominant slow growth phenotype. Nucleic Acids Res *36*, 41–50.

Ero, R., Peil, L., Liiv, A., and Remme, J. (2008). Identification of pseudouridine methyltransferase in Escherichia coli. RNA *14*, 2223–2233.

Feunteun, J., Jordan, B., and Monier, R. (1972). Study of the maturation of 5 s RNA precursors in Escherichia coli. J Mol Biol *70*, 465–474.

Franceschi, F., and Nierhaus, K. (1988). Ribosomal protein L20 can replace the assembly-initiator protein L24 at low temperatures. Biochemistry *27*, 7056–7059.

Fuller-Pace, F., Nicol, S., Reid, A., and Lane, D. (1993). DbpA: a DEAD box protein specifically activated by 23s rRNA. EMBO J *12*, 3619–3626.

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

Gross, C.A. (1996). Function and regulation of the heat shock proteins. In Escherichia coli and Salmonella, Cellular and Molecular Biology, N.C. Neidhardt, R. Curtiss III, J.L. Ingraham, E.C.C. Lin, K.B. Low, B. Magasanik, W.S. Reznikoff, M. Riley, M. Schaechter, and H.E. Umbarger, eds. (Washington DC, American Society for Microbiology), pp. 217–261.

Gutgsell, N., Del Campo, M., Raychaudhuri, S., and Ofengand, J. (2001). A second function for pseudouridine synthases: A point mutant of RluD unable to form pseudouridines 1911, 1915, and 1917 in Escherichia coli 23S ribosomal RNA restores normal growth to an RluD-minus strain. RNA *7*, 990–998.

Gutgsell, N., Deutscher, M., and Ofengand, J. (2005). The pseudouridine synthase RluD is required for normal ribosome assembly and function in Escherichia coli. RNA *11*, 1141–1152.

Gutgsell, N., Englund, N., Niu, L., Kaya, Y., Lane, B., and Ofengand, J. (2000). Deletion of the Escherichia coli pseudouridine synthase gene truB blocks formation of pseudouridine 55 in tRNA in vivo, does not affect exponential growth, but confers a strong selective disadvantage in competition with wild-type cells. RNA *6*, 1870–1881.

Hage, A., and Tollervey, D. (2004). A surfeit of factors: why is ribosome assembly so much more complicated in eukaryotes than bacteria? RNA Biol *1*, 10–15.

Hager, J., Staker, B., Bugl, H., and Jakob, U. (2002). Active site in RrmJ, a heat shock-induced methyltransferase. J Biol Chem *277*, 41978–41986.
Hager, J., Staker, B., and Jakob, U. (2004). Substrate binding analysis of the 23S rRNA methyltransferase RrmJ. J Bacteriol *186*, 6634–6642.
Hartmann, R., Gössringer, M., Späth, B., Fischer, S., and Marchfelder, A. (2009). The making of tRNAs and more - RNase P and tRNase Z. Prog Mol Biol Transl Sci *85*, 319–368.
Hayes, F., and Vasseur, M. (1976). Processing of the 17-S Escherichia coli precursor RNA in the 27-S pre-ribosomal particle. Eur J Biochem *61*, 433–442.
Henn, A., Medalia, O., Shi, S., Steinberg, M., Franceschi, F., and Sagi, I. (2001). Visualization of unwinding activity of duplex RNA by DbpA, a DEAD box helicase, at single-molecule resolution by atomic force microscopy. Proc Natl Acad Sci U S A *98*, 5007–5012.
Himeno, H., Hanawa-Suetsugu, K., Kimura, T., Takagi, K., Sugiyama, W., Shirata, S., Mikami, T., Odagiri, F., Osanai, Y., Watanabe, D.*, et al.* (2004). A novel GTPase activated by the small subunit of ribosome. Nucleic Acids Res *32*, 5303–5309.
Huang, L., Ku, J., Pookanjanatavip, M., Gu, X., Wang, D., Greene, P., and Santi, D. (1998). Identification of two Escherichia coli pseudouridine synthases that show multisite specificity for 23S RNA. Biochemistry *37*, 15951–15957.
Hwang, J., and Inouye, M. (2001). An essential GTPase, der, containing double GTP-binding domains from Escherichia coli and Thermotoga maritima. J Biol Chem *276*, 31415–31421.
Hwang, J., and Inouye, M. (2006). The tandem GTPase, Der, is essential for the biogenesis of 50S ribosomal subunits in Escherichia coli. Mol Microbiol *61*, 1660–1672.
Hwang, J., and Inouye, M. (2008). RelA functionally suppresses the growth defect caused by a mutation in the G domain of the essential Der protein. J Bacteriol *190*, 3236–3243.
Iggo, R., Picksley, S., Southgate, J., McPheat, J., and Lane, D. (1990). Identification of a putative RNA helicase in E.coli. Nucleic Acids Res *18*, 5413–5417.
Inoue, K., Alsina, J., Chen, J., and Inouye, M. (2003). Suppression of defective ribosome assembly in a rbfA deletion mutant by overexpression of Era, an essential GTPase in Escherichia coli. Mol Microbiol *48*, 1005–1016.
Inoue, K., Basu, S., and Inouye, M. (2007). Dissection of 16S rRNA methyltransferase (KsgA) function in Escherichia coli. J Bacteriol *189*, 8510–8518.
Inoue, K., Chen, J., Tan, Q., and Inouye, M. (2006). Era and RbfA have overlapping function in ribosome biogenesis in Escherichia coli. J Mol Microbiol Biotechnol *11*, 41–52.
Iost, I., and Dreyfus, M. (2006). DEAD-box RNA helicases in Escherichia coli. Nucleic Acids Res *34*, 4189–4197.
Jain, C. (2008). The E. coli RhlE RNA helicase regulates the function of related RNA helicases during ribosome assembly. RNA *14*, 381–389.
Jiang, M., Datta, K., Walker, A., Strahler, J., Bagamasbad, P., Andrews, P., and Maddock, J. (2006). The Escherichia coli GTPase CgtAE is involved in late steps of large ribosome assembly. J Bacteriol *188*, 6757–6770.
Jiang, M., Sullivan, S., Wout, P., and Maddock, J. (2007). G-protein control of the ribosome-associated stress response protein SpoT. J Bacteriol *189*, 6140–6147.
Johnstone, B., Handler, A., Chao, D., Nguyen, V., Smith, M., Ryu, S., Simons, E., Anderson, P., and Simons, R. (1999). The widely conserved Era G-protein contains

an RNA-binding domain required for Era function in vivo. Mol Microbiol *33*, 1118–1131.

Jones, P., and Inouye, M. (1996). RbfA, a 30S ribosomal binding factor, is a cold-shock protein whose absence triggers the cold-shock response. Mol Microbiol *21*, 1207–1218.

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

Jordan, B., Feuteun, J., and Monier, R. (1970). Identification of a 5 s RNA precursor in exponentially growing Escherichia coli cells. J Mol Biol *50*, 605–615.

Jordan, B., Forget, B., and Monier, R. (1971). A low molecular weight ribonucleic acid synthesized by Escherichia coli in the presence of chloramphenicol: characterization and relation to normally synthesized 5 s ribonucleic acid. J Mol Biol *55*, 407–421.

Justesen, J., Lund, T., Skou Pedersen, F., and Kjeldgaard, N. (1986). The physiology of stringent factor (ATP:GTP 3'-diphosphotransferase) in Escherichia coli. Biochimie *68*, 715–722.

Kaczanowska, M., and Rydén-Aulin, M. (2007). Ribosome biogenesis and the translation process in Escherichia coli. Microbiol Mol Biol Rev *71*, 477–494.

Karbstein, K. (2007). Role of GTPases in ribosome assembly. Biopolymers *87*, 1–11.

Kelly, K., Reuven, N., Li, Z., and Deutscher, M. (1992). RNase PH is essential for tRNA processing and viability in RNase-deficient Escherichia coli cells. J Biol Chem *267*, 16015–16018.

Khemici, V., Toesca, I., Poljak, L., Vanzo, N., and Carpousis, A. (2004). The RNase E of Escherichia coli has at least two binding sites for DEAD-box RNA helicases: functional replacement of RhlB by RhlE. Mol Microbiol *54*, 1422–1430.

Kimura, T., Takagi, K., Hirata, Y., Hase, Y., Muto, A., and Himeno, H. (2008). Ribosome-small-subunit-dependent GTPase interacts with tRNA-binding sites on the ribosome. J Mol Biol *381*, 467–477.

King, T., and Schlessinger, D. (1983). S1 nuclease mapping analysis of ribosomal RNA processing in wild type and processing deficient Escherichia coli. J Biol Chem *258*, 12034–12042.

King, T.C., Sirdeshmukh, R., and Schlessinger, D. (1984). RNase III cleavage is obligate for maturation but not for function of Escherichia coli pre-23S rRNA. Proc Natl Acad Sci U S A *81*, 185–188.

Kossen, K., Karginov, F., and Uhlenbeck, O. (2002). The carboxy-terminal domain of the DExDH protein YxiN is sufficient to confer specificity for 23S rRNA. J Mol Biol *324*, 625–636.

Kowalak, J., Bruenger, E., Hashizume, T., Peltier, J., Ofengand, J., and McCloskey, J. (1996). Structural characterization of U*-1915 in domain IV from Escherichia coli 23S ribosomal RNA as 3-methylpseudouridine. Nucleic Acids Res *24*, 688–693.

Laughrea, M., and Moore, P. (1978). On the relationship between the binding of ribosomal protein S1 to the 30 S subunit of Escherichia coli and 3' terminus of 16 S RNA. J Mol Biol *121*, 411–430.

Leppik, M., Peil, L., Kipper, K., Liiv, A., and Remme, J. (2007). Substrate specificity of the pseudouridine synthase RluD in Escherichia coli. FEBS J *274*, 5759–5766.

Lewicki, B., Margus, T., Remme, J., and Nierhaus, K. (1993). Coupling of rRNA transcription and ribosomal assembly in vivo. Formation of active ribosomal subunits in Escherichia coli requires transcription of rRNA genes by host RNA polymerase which cannot be replaced by bacteriophage T7 RNA polymerase. J Mol Biol *231*, 581–593.

Li, Z., and Deutscher, M. (1994). The role of individual exoribonucleases in processing at the 3' end of Escherichia coli tRNA precursors. J Biol Chem *269*, 6064–6071.
Li, Z., and Deutscher, M. (1995). The tRNA processing enzyme RNase T is essential for maturation of 5S RNA. Proc Natl Acad Sci U S A *92*, 6883–6886.
Li, Z., and Deutscher, M. (1996). Maturation pathways for E. coli tRNA precursors: a random multienzyme process in vivo. Cell *86*, 503–512.
Li, Z., Pandit, S., and Deutscher, M. (1998). 3' exoribonucleolytic trimming is a common feature of the maturation of small, stable RNAs in Escherichia coli. Proc Natl Acad Sci U S A *95*, 2856–2861.
Li, Z., Pandit, S., and Deutscher, M. (1999a). Maturation of 23S ribosomal RNA requires the exoribonuclease RNase T. RNA *5*, 139–146.
Li, Z., Pandit, S., and Deutscher, M. (1999b). RNase G (CafA protein) and RNase E are both required for the 5' maturation of 16S ribosomal RNA. EMBO J *18*, 2878–2885.
Liiv, A. (1998). Ribosomal large subunit assembly in vivo. In Department of Molecular Biology, Institute of Molecular and Cell Biology (Tartu, University of Tartu).
Liiv, A., Karitkina, D., Maiväli, U., and Remme, J. (2005). Analysis of the function of E. coli 23S rRNA helix-loop 69 by mutagenesis. BMC Mol Biol *6*, 18.
Lindahl, L. (1975). Intermediates and time kinetics of the in vivo assembly of Escherichia coli ribosomes. J Mol Biol *92*, 15–37.
Lu, J., Aoki, H., and Ganoza, M. (1999). Molecular characterization of a prokaryotic translation factor homologous to the eukaryotic initiation factor eIF4A. Int J Biochem Cell Biol *31*, 215–229.
Lu, Q., and Inouye, M. (1998). The gene for 16S rRNA methyltransferase (ksgA) functions as a multicopy suppressor for a cold-sensitive mutant of era, an essential RAS-like GTP-binding protein in Escherichia coli. J Bacteriol *180*, 5243–5246.
Lövgren, J., Bylund, G., Srivastava, M., Lundberg, L., Persson, O., Wingsle, G., and Wikström, P. (2004). The PRC-barrel domain of the ribosome maturation protein RimM mediates binding to ribosomal protein S19 in the 30S ribosomal subunits. RNA *10*, 1798–1812.
Maguire, B. (2009). Inhibition of bacterial ribosome assembly: a suitable drug target? Microbiol Mol Biol Rev *73*, 22–35.
Maki, J., Schnobrich, D., and Culver, G. (2002). The DnaK chaperone system facilitates 30S ribosomal subunit assembly. Mol Cell *10*, 129–138.
Maki, J., Southworth, D., and Culver, G. (2003). Demonstration of the role of the DnaK chaperone system in assembly of 30S ribosomal subunits using a purified in vitro system. RNA *9*, 1418–1421.
Misra, T., and Apirion, D. (1979). RNase E, an RNA processing enzyme from Escherichia coli. J Biol Chem *254*, 11154–11159.
Moazed, D., and Noller, H. (1989). Interaction of tRNA with 23S rRNA in the ribosomal A, P, and E sites. Cell *57*, 585–597.
Moll, I., Grill, S., Gründling, A., and Bläsi, U. (2002). Effects of ribosomal proteins S1, S2 and the DeaD/CsdA DEAD-box helicase on translation of leaderless and canonical mRNAs in Escherichia coli. Mol Microbiol *44*, 1387–1396.
Myler, P., Venkataraman, G., Lodes, M., and Stuart, K. (1994). A frequently amplified region in Leishmania contains a gene conserved in prokaryotes and eukaryotes. Gene *148*, 187–193.
Nicol, S., and Fuller-Pace, F. (1995). The "DEAD box" protein DbpA interacts specifically with the peptidyltransferase center in 23S rRNA. Proc Natl Acad Sci U S A *92*, 11681–11685.
Nierhaus, K. (1991). The assembly of prokaryotic ribosomes. Biochimie *73*, 739–755.

Nishi, K., Morel-Deville, F., Hershey, J., Leighton, T., and Schnier, J. (1988). An eIF-4A-like protein is a suppressor of an Escherichia coli mutant defective in 50S ribosomal subunit assembly. Nature *336*, 496–498.

Nishi, K., and Schnier, J. (1986). A temperature-sensitive mutant in the gene rplX for ribosomal protein L24 and its suppression by spontaneous mutations in a 23S rRNA gene of Escherichia coli. EMBO J *5*, 1373–1376.

O'Farrell, H., Scarsdale, J., and Rife, J. (2004). Crystal structure of KsgA, a universally conserved rRNA adenine dimethyltransferase in Escherichia coli. J Mol Biol *339*, 337–353.

Ofengand, J. (2002). Ribosomal RNA pseudouridines and pseudouridine synthases. FEBS Lett *514*, 17–25.

Ofengand, J., Bakin, A., Wrzesinski, J., Nurse, K., and Lane, B. The pseudouridine residues of ribosomal RNA. Biochem Cell Biol *73*, 915–924.

Ofengand, J., and Del Campo, M. (2004). Modified nucleotides of Escherichia coli ribosomal RNA. In EcoSal—Escherichia coli and Salmonella: Cellular and molecular biology, R. Curtiss, ed. (Washington DC, American Society for Microbiology).

Ofengand, J., Malhotra, A., Remme, J., Gutgsell, N., Del Campo, M., Jean-Charles, S., Peil, L., and Kaya, Y. (2001). Pseudouridines and pseudouridine synthases of the ribosome. Cold Spring Harb Symp Quant Biol *66*, 147–159.

Ohmori, H. (1994). Structural analysis of the rhlE gene of Escherichia coli. Jpn J Genet *69*, 1–12.

Peil, L., Virumäe, K., and Remme, J. (2008). Ribosome assembly in Escherichia coli strains lacking the RNA helicase DeaD/CsdA or DbpA. FEBS J *275*, 3772–3782.

Persson, B., Gustafsson, C., Berg, D., and Björk, G. (1992). The gene for a tRNA modifying enzyme, m5U54-methyltransferase, is essential for viability in Escherichia coli. Proc Natl Acad Sci U S A *89*, 3995–3998.

Prud'homme-Généreux, A., Beran, R., Iost, I., Ramey, C., Mackie, G., and Simons, R. (2004). Physical and functional interactions among RNase E, polynucleotide phosphorylase and the cold-shock protein, CsdA: evidence for a 'cold shock degradosome'. Mol Microbiol *54*, 1409–1421.

Pugh, G., Nicol, S., and Fuller-Pace, F. (1999). Interaction of the Escherichia coli DEAD box protein DbpA with 23 S ribosomal RNA. J Mol Biol *292*, 771–778.

Purta, E., Kaminska, K., Kasprzak, J., Bujnicki, J., and Douthwaite, S. (2008). YbeA is the m3Psi methyltransferase RlmH that targets nucleotide 1915 in 23S rRNA. RNA *14*, 2234–2244.

Ray, B., and Apirion, D. (1981). Transfer RNA precursors are accumulated in Escherichia coli in the absence of RNase E. Eur J Biochem *114*, 517–524.

Raychaudhuri, S., Conrad, J., Hall, B., and Ofengand, J. (1998). A pseudouridine synthase required for the formation of two universally conserved pseudouridines in ribosomal RNA is essential for normal growth of Escherichia coli. RNA *4*, 1407–1417.

Raynal, L., and Carpousis, A. (1999). Poly(A) polymerase I of Escherichia coli: characterization of the catalytic domain, an RNA binding site and regions for the interaction with proteins involved in mRNA degradation. Mol Microbiol *32*, 765–775.

Redko, Y., Li de Lasierra-Gallay, I., and Condon, C. (2007). When all's zed and done: the structure and function of RNase Z in prokaryotes. Nat Rev Microbiol *5*, 278–286.

Reuven, N., and Deutscher, M. (1993). Multiple exoribonucleases are required for the 3' processing of Escherichia coli tRNA precursors in vivo. FASEB J *7*, 143–148.
Robertson, H.D., Webster, R.E., and Zinder, N.D. (1968). Purification and properties of ribonuclease III from Escherichia coli. J Biol Chem *243*, 82–91.
Roy, M., Singh, B., Ray, B., and Apirion, D. (1983). Maturation of 5-S rRNA: ribonuclease E cleavages and their dependence on precursor sequences. Eur J Biochem *131*, 119–127.
Sato, A., Kobayashi, G., Hayashi, H., Yoshida, H., Wada, A., Maeda, M., Hiraga, S., Takeyasu, K., and Wada, C. (2005). The GTP binding protein Obg homolog ObgE is involved in ribosome maturation. Genes Cells *10*, 393–408.
Sayed, A., Matsuyama, S., and Inouye, M. (1999). Era, an essential Escherichia coli small G-protein, binds to the 30S ribosomal subunit. Biochem Biophys Res Commun *264*, 51–54.
Schumann, W. (1999). FtsH--a single-chain charonin? FEMS Microbiol Rev *23*, 1–11.
Sirdeshmukh, R., and Schlessinger, D. (1985). Ordered processing of Escherichia coli 23S rRNA in vitro. Nucleic Acids Res *13*, 5041–5054.
Sirum-Connolly, K., and Mason, T. (1993). Functional requirement of a site-specific ribose methylation in ribosomal RNA. Science *262*, 1886–1889.
Sirum-Connolly, K., and Mason, T. (1995). The role of nucleotide modifications in the yeast mitochondrial ribosome. Nucleic Acids Symp Ser, 73–75.
Srivastava, A., and Schlessinger, D. (1988). Coregulation of processing and translation: mature 5' termini of Escherichia coli 23S ribosomal RNA form in polysomes. Proc Natl Acad Sci U S A *85*, 7144–7148.
Srivastava, A., and Schlessinger, D. (1990). Mechanism and regulation of bacterial ribosomal RNA processing. Annu Rev Microbiol *44*, 105–129.
Szeberényi, J., Tomcsányi, T., and Apirion, D. (1985). Maturation of the 3' end of 5-S ribosomal RNA from Escherichia coli. Eur J Biochem *149*, 113–118.
Talkington, M., Siuzdak, G., and Williamson, J. (2005). An assembly landscape for the 30S ribosomal subunit. Nature *438*, 628–632.
Tan, J., Jakob, U., and Bardwell, J. (2002). Overexpression of two different GTPases rescues a null mutation in a heat-induced rRNA methyltransferase. J Bacteriol *184*, 2692–2698.
Tatusov, R., Fedorova, N., Jackson, J., Jacobs, A., Kiryutin, B., Koonin, E., Krylov, D., Mazumder, R., Mekhedov, S., Nikolskaya, A.*, et al.* (2003). The COG database: an updated version includes eukaryotes. BMC Bioinformatics *4*, 41.
Tatusov, R., Koonin, E., and Lipman, D. (1997). A genomic perspective on protein families. Science *278*, 631–637.
Tkaczuk, K., Dunin-Horkawicz, S., Purta, E., and Bujnicki, J. (2007). Structural and evolutionary bioinformatics of the SPOUT superfamily of methyltransferases. BMC Bioinformatics *8*, 73.
Toone, W., Rudd, K., and Friesen, J. (1991). deaD, a new Escherichia coli gene encoding a presumed ATP-dependent RNA helicase, can suppress a mutation in rpsB, the gene encoding ribosomal protein S2. J Bacteriol *173*, 3291–3302.
Tsu, C., Kossen, K., and Uhlenbeck, O. (2001). The Escherichia coli DEAD protein DbpA recognizes a small RNA hairpin in 23S rRNA. RNA *7*, 702–709.
Tsu, C., and Uhlenbeck, O. (1998). Kinetic analysis of the RNA-dependent adenosinetriphosphatase activity of DbpA, an Escherichia coli DEAD protein specific for 23S ribosomal RNA. Biochemistry *37*, 16989–16996.
Turner, A., Love, C., Alexander, R., and Jones, P. (2007). Mutational analysis of the Escherichia coli DEAD box protein CsdA. J Bacteriol *189*, 2769–2776.

Vaidyanathan, P., Deutscher, M., and Malhotra, A. (2007). RluD, a highly conserved pseudouridine synthase, modifies 50S subunits more specifically and efficiently than free 23S rRNA. RNA *13*, 1868–1876.

Wachi, M., Umitsuki, G., and Nagai, K. (1997). Functional relationship between Escherichia coli RNase E and the CafA protein. Mol Gen Genet *253*, 515–519.

Wachi, M., Umitsuki, G., Shimizu, M., Takada, A., and Nagai, K. (1999). Escherichia coli cafA gene encodes a novel RNase, designated as RNase G, involved in processing of the 5' end of 16S rRNA. Biochem Biophys Res Commun *259*, 483–488.

Watanabe, K., Nureki, O., Fukai, S., Ishii, R., Okamoto, H., Yokoyama, S., Endo, Y., and Hori, H. (2005). Roles of conserved amino acid sequence motifs in the SpoU (TrmH) RNA methyltransferase family. J Biol Chem *280*, 10368–10377.

Widerak, M., Kern, R., Malki, A., and Richarme, G. (2005). U2552 methylation at the ribosomal A-site is a negative modulator of translational accuracy. Gene *347*, 109–114.

Wilson, D., and Nierhaus, K. (2005). Ribosomal proteins in the spotlight. Crit Rev Biochem Mol Biol *40*, 243–267.

Wilson, D., and Nierhaus, K. (2007). The weird and wonderful world of bacterial ribosome regulation. Crit Rev Biochem Mol Biol *42*, 187–219.

Wout, P., Pu, K., Sullivan, S., Reese, V., Zhou, S., Lin, B., and Maddock, J. (2004). The Escherichia coli GTPase CgtAE cofractionates with the 50S ribosomal subunit and interacts with SpoT, a ppGpp synthetase/hydrolase. J Bacteriol *186*, 5249–5257.

Wrzesinski, J., Bakin, A., Ofengand, J., and Lane, B. (2000). Isolation and properties of Escherichia coli 23S-RNA pseudouridine 1911, 1915, 1917 synthase (RluD). IUBMB Life *50*, 33–37.

Xia, B., Ke, H., Shinde, U., and Inouye, M. (2003). The role of RbfA in 16S rRNA processing and cell growth at low temperature in Escherichia coli. J Mol Biol *332*, 575–584.

Xu, Z., O'Farrell, H., Rife, J., and Culver, G. (2008). A conserved rRNA methyltransferase regulates ribosome biogenesis. Nat Struct Mol Biol *15*, 534–536.

Young, R., and Steitz, J. (1978). Complementary sequences 1700 nucleotides apart form a ribonuclease III cleavage site in Escherichia coli ribosomal precursor RNA. Proc Natl Acad Sci U S A *75*, 3593–3597.

Zhang-Akiyama, Q., Morinaga, H., Kikuchi, M., Yonekura, S., Sugiyama, H., Yamamoto, K., and Yonei, S. (2009). KsgA, a 16S rRNA adenine methyltransferase, has a novel DNA glycosylase/AP lyase activity to prevent mutations in Escherichia coli. Nucleic Acids Res *37*, 2116–2125.

Zhou, Z., and Deutscher, M. (1997). An essential function for the phosphate-dependent exoribonucleases RNase PH and polynucleotide phosphorylase. J Bacteriol *179*, 4391–4395.

SUMMARY IN ESTONIAN

Ribosoomide kokkupakkimise faktorid soolekepikeses *Escherichia coli*

Ribosoomide süntees ja kokkupakkimine on äärmiselt keeruline tegevus, kus osaleb mitmeid ribosomaalseid ja mitte-ribosomaalseid faktoreid. Bakteriaalne 70S ribosoom koosneb kahest alamühikust – suurest (50S) ja väiksest (30S) alamühikust, kus 50S alamühik koosneb 23S ja 5S ribosomaalsest RNA-st (vastavalt 2904 ja 120 nukleotiidi) ning 33-st ribosomaalsest valgust; 30S alamühik aga 16S ribosomaalsest RNA-st (1542 nukleotiidi) ja 21-st ribosomaalsest valgust.

Ribosoomide kokkupakkimine algab samaaegselt ribosomaalse RNA transkriptsiooniga ning sisaldab endas nii ribosomaalse RNA kui ka ribosomaalsete valkude protsessimist, modifitseerimist ning ruumilist voltumist; samuti nende komponentide omavahelist assotsieerumist funktsionaalseteks ribosomaalseteks alamühikuteks. *Escherichia coli* ribosomaalses RNA-s on kokku teada 26 modifitseeritud nukleotiidi (neist 11 asuvad 16S ribosomaalses RNA-s ja 25 asuvad 23S ribosomaalses RNA-s), samuti on translatsioonijärgselt modifitseeritud 11 ribosomaalset valku. Normaalsetes tingimustes toimub ribosoomide kokkupakkimine mõne minuti jooksul, samas kui *in vitro* tingimustes nõuab see mitmeid pikki inkubatsioone ja tempearatuurimuutusi. See kõik viitab aga faktile, et lisaks ribosomaalsetele faktoritele osalevad ribosoomide kokkupakkimises ka mitmed mitte-ribosomaalsed faktorid, mis on vajalikud ribosoomide kokkupakkimise efektiivseks toimumiseks. Selliste mitte-ribosomaalsete faktorite hulka kuuluvad näiteks RNA modifikatsiooniensüümid, RNA helikaasid, kuumaehmatusvastuse valgud (*heat-shock proteins*), ribosoomsõltuvad GTPaasid ning RNA abipakkijad (*RNA chaperones*). Päristuumsetes organismides on praeguseks hetkeks teada rohkem kui 200 mitte-ribosomaalse faktori osalemine ribosoomide kokkupakkimises, bakterites on see number enam kui kümme korda väiksem. Käesoleva töö kirjanduse ülevaates on kirjeldatud ribosomaalse RNA protsessimist ning hetkel teadaolevaid mitte-ribosomaalseid faktoreid soolekepikeses *Escherichia coli* ning nende osalust ribosoomide kokkupakkimises, eriline rõhk on ribosomaalse RNA modifikatsiooniensüümidel ning RNA helikaasidel.

Ribosomaalse RNA modifikatsioonid on sünteesitud koht-spetsiifiliste ensüümide poolt, kusjuures enamasti vastab ühele modifikatsioonile üks kindel ensüüm. Kõikidest senini avastatud ning iseloomustatud rRNA modifikatsiooniensüümidest tekitab vaid kahe ensüümi puudumine olulisi häireid rakkude elutegevuses ja ribosoomide kokkupakkimises – nendeks on pseudouridiini süntaas RluD (katalüüsib kolme uridiini isomerisatsiooni pseudouridiinideks 23S rRNA positsioonides 1911, 1915 ja 1917) ning metüültransferaas RlmE (katalüüsib 2'-O-metüüluridiini sünteesi 23S rRNA positsioonis 2552). RNA helikaasidest on praeguseks hetkeks näidatud nelja valgu otsene või kaudne osalemine ribosoomide kokkupakkimises – nendeks on helikaasid DeaD, SrmB,

DbpA ja RhlE. RNA helikaaside DeaD ja SrmB puudumine tekitab mitmeid häireid rakkude elutegevuses ja ribosoomide kokkupakkimises, seda eriti madalatel temperatuuridel. DbpA valgu puudumine rakkude elutegevuses häireid ei põhjusta, RNA helikaasi RhlE puudumine tekitab häireid aga vaid juhul, kui samaaegselt puudub ka RNA helikaas DeaD või SrmB. Huvitaval kombel on mitmeid teatud mitte-ribosomaalsete faktorite puudumisest tingitud defekte ribosoomide kokkupakkimises võimalik parandada teiste valkude üle-ekspressiooniga, kusjuures üle-ekspresseeritavaks valguks ei pea ilmtingimata olema puuduvale valgule sarnase funktsionaalsusega valk. Siiani on arvatud, et ribosoomide kokkupakkimine toimub mööda kindlaid radu, mis lõppevad translatsiooniks valmisolevate ribosoomidega. Et aga viimasel ajal on tuvastatud mitmete sarnaste, kuid samas siiski erinevate ribosoomi eellaspartiklite esinemist ribosoomide kokkupakkimise suhtes defektsetes bakteritüvedes, siis tekkis õigustatud küsimus – kas sellised partiklid on ikka tegelikud eellaspartiklid või on tegu mitte-funktsionaalsete tupikpartiklitega? Lisaks ülaltoodule on ribosomaalses RNA-s esinevate modifitseeritud nukleotiidide avastamisest alates toimunud elav diskussioon nende ja nende sünteesi eest vastutavate ensüümide osalusest ja olulisusest ribosoomide kokkupakkimises. Sellest tulenevalt said püstitatud järgnevad eesmärgid:

1. Iseloomustada pseudouridiini süntaasi RluD osalust ribosoomide kokkupakkimises soolekepikeses *Escherichia coli* ning kirjeldada ribosoomide kokkupakkimise defekte RluD valgu suhtes defektsetes rakkudes.
2. Iseloomustada detailselt *Escherichia coli* pseudouridiini süntaasi RluD substraadi spetsiifikat.
3. Tuvastada ja iseloomustada *Escherichia coli* pseudouridiini metüültransferaas, mis katalüüsib 23S ribosomaalse RNA positsioonis 1915 asuva pseudouridiini metüleerimist.
4. Iseloomustada *Escherichia coli* RNA helikaaside DeaD ja DbpA rolli ribosoomide kokkupakkimises, kirjeldada ribosoomide kokkupakkimise defekte DeaD valgu suhtes defektsetes rakkudes ning analüüsida ribosomaalse eellaspartiklite küpsemist ja funktsionaalseid omadusi DeaD valgu suhtes defektsetes rakkudes.

Käesolevas dissertatsioonis esitatud tulemuste põhjal võib järeldada, et:

1. Pseudouridiini süntaasi RluD puudumine *Escherichia coli* rakkudes põhjustab olulisi häireid nii ribosoomi väikese kui ka suure alamühiku kokkupakkimises, mis kajastub vähenenud 70S ribosoomide koguses ja ebanormaalsete 39S ning 27S ribosomaalsete partiklite kuhjumises. Tekkinud 39S ribosomaalsed partiklid sisaldavad mittetäielikult protsessitud 23S ribosomaalset RNA-d, samuti esineb nendes partiklites märkimisväärses koguses RNA helikaasi DeaD.
2. Pseudouridiini süntaas RluD osaleb ribosoomide kokkupakkimise viimases etapis ning on ülimalt spetsiifiline *Escherichia coli* 23S ribosomaalse RNA positsioonides 1911 ja 1917 asuvate pseudouridiinide suhtes.

3. Kõrgelt konserveerunud SPOUT-perekonna metüültransferaas RlmH (YbeA), mis on esindatud kõigis kolmes loomariigis (bakterid, ürgid ning päristuumsed), katalüüsib *Escherichia coli* 23S ribosomaalse RNA positsioonis 1915 asuva pseudouridiini metüleerimist.
4. RNA helikaasi DeaD puudumine *Escherichia coli* rakkudes põhjustab olulisi häireid ribosoomi suure alamühiku kokkupakkimises ja 40S ribosomaalsete partiklite teket. Tekkinud vabad 50S ja 40S ribosomaalsed partiklid sisaldavad mittetäielikult protsessitud ning modifitseeritud 23S ribosomaalset RNA-d. Lisaks on 40S partiklid võimelised küpsema 70S ribosoomideks *in vivo*, kuid see toimub oluliselt madalama kiirusega kui metsik-tüüpi rakkudes.
5. RNA helikaasi Dead suhtes defektsest *Escherichia coli* tüvest eraldatud vabad ribosoomi 50S alamühikud on *in vitro* peptidüültransferaasi katses ainult osaliselt aktiivsed, 40S partiklitel katalüütiline aktiivsus puudub.

ACKNOWLEDGEMENTS

First of all, I thank my wife Kadri and daughter Kristin for the all-time support, whether I needed it or not. Much of my success I owe to my mother, father and sister, who were always there when I needed them and who didn't force me to change biology for a 'proper job'. My interest towards nature, fishing, biology and later science was mostly due to my grandfather Arno and godfather Bruno, summer holidays in Koila, Rohu and Voka will not be forgotten, not to mention the great tales of 'good ol' times'.

I want to sincerely thank my supervisor Jaanus Remme for the greatest of all guidances and for injecting healthy scientific mentality into me throughout those years, even when all hope seemed lost. I will not forget the white bread, garlic, hot tea and fruitful discussions on long summer nights when fishing was over or hadn't begun yet. I also want to thank Tõnu Margus and Tanel Tenson for the first guidances in the lab, along with 'lab-oldies' Aivar Liiv, Ülo Maiväli, Kai Virumäe and 'lab-youngsters' Ülar Allas, Arto Pulk, Margus Leppik, Triinu Siibak and Rya Ero. You all have been of tremendous help, thank you.

I am very grateful to late James Ofengand and his group in Miami, those five months of hard work and Miami winter sun are hard to forget. I missed the hurricanes but long walks through the unknown territory in the middle of the night were more-or-less equal replacements. Special thanks go to Mark Del Campo for taking care of me throughout my stay (one for you, two for me, remember?).

Special thanks go to my teachers throughout the school and university years. Helen Kaasik took care of us throughout the mad years of high school, teaching math and pointing our minds towards the future at the same time. Tiit Laasberg was the most charismatic and influential of all teachers – the way (molecular) biology and great art of Salvador Dali was presented by him decided my future. Agu Laisk was the first person to make me realize that in the end physics is easy – you just have to remember numbers 1 (one as for one), 3 (three as for an approximate value of e (2.71828...), three as for an approximate value of pi (3.14159...), three as for an approximate value for half of an order of magnitude (3.16227...)) and 10 (ten as for an order of magnitude); rest will come along at its one pace.

I also want to thank those many people I have worked, eaten, organized events or spent lab or free time together with – Anne Kalling (coffee anyone?), Meelis Kadaja, Toomas Silla, Ivar Ilves, Arvi Jõers, Viljar Jaks (can you get me more of these papers please?), Martin Lepiku, Indrek Ots, Peeter Hõrak and many others. It is true that discussions are useful, it's just not so clear why sometimes the discussions tend to end early in the morning in or in the neighborhood of Zavood.

Similar thanks go to present and former members of different labs in the Institute of Molecular and Cell Biology and in the Institute of Technology. Tiiu

Rootslane and Merike Petuhhov have helped me a lot to get through the long and dark corridors of bureaucracy, thank you for that.

The Music, Muse, Pulp and Jarvis Cocker, The Verve, The KLF, The Smiths and Morrissey, Manic Street Preachers, Bloc Party, Supremesounds boys and many-many others deserve my gratitude for the inspiration throughout the years. Counting fractions and washing filters would have been much more complicated without you.

Finally, I would like to thank Mart Ustav Sr. for trusting me enough to let me be responsible for one of the most unique and expensive machines in Estonia that can make 'ping'. The amount of knowledge collected throughout these years is vast and hopefully fruitful for the future of proteomics on this side of the Baltic Sea.

PUBLICATIONS

CURRICULUM VITAE

I. General

1. Name: Lauri Peil
   Time and place of birth: 09.03.1977, Tallinn, Estonia
   Citizenship: Estonian
   Marital status: married
   Address, telephone, *e-mail*: University of Tartu Institute of Technology
   Proteomics core facility
   Nooruse 1
   50411 Tartu
   tel: +372 737 4865
   email: lauri.peil@ut.ee

2. Current position: University of Tartu Institute of Technology, researcher
   Education:
   - Tallinn Secondary Science School, 1993–1995
   - University of Tartu, 1995–1999, BSc, molecular biology
   - University of Tartu, 1999–2001, MSc, molecular biology
   - University of Tartu, 2001–..., doctorate studies, molecular biology

3. Language skills: estonian, english, finnish, russian
   Working experience:
   - University of Tartu Institute of Technology, 2009–..., researcher
   - University of Tartu Institute of Technology, 2006–2008, Proteomics core facility manager
   - University of Tartu, 2005–2006, Graduate School in Biomedicine and Biotechnology, project manager

II. Scientific and research activity

Main research interests:

My main scientific interest has been to study the process of ribosome assembly in *Escherichia coli* and to characterize some of the extra-ribosomal factors involved in it in depth. At the same time, the involvement of ribosomal RNA modifications and corresponding enzymes in ribosome assembly has been of great interest.

List of publications:

- **Ofengand, J; Malhotra, A; Remme, J; Gutgsell, NS; Del Campo, M; Jean-Charles, S; Peil, L; Kaya, Y:** Pseudouridines and pseudouridine synthases of the ribosome. Cold Spring Harb Symp Quant Biol 2001, 66:147–59.
- **Leppik, M; Peil, L; Kipper, K; Liiv, A; Remme, J:** Substrate specificity of the pseudouridine synthase RluD in *Escherichia coli*. FEBS J 2007, 274:5759–66.
- **Peil, L; Virumäe, K; Remme, J**: Ribosome assembly in *Escherichia coli* strains lacking the RNA helicase DeaD/CsdA or DbpA. FEBS J 2008, 275:3772–82.
- **Ero, R; Peil, L; Liiv, A; Remme, J**: Identification of pseudouridine methyltransferase in *Escherichia coli*. RNA 2008, 14:2223–33.
- **Siibak T, Peil L, Xiong L, Mankin A, Remme J, Tenson T**: Erythromycin- and chloramphenicol-induced ribosomal assembly defects are secondary effects of protein synthesis inhibition. Antimicrob Agents Chemother. 2009 Feb;53(2):563–71.

Fellowships:

Membership in organizations:

- Estonian Proteomics Society, board member

CURRICULUM VITAE

I. Üldandmed

1. Ees- ja perekonnanimi: Lauri Peil
   Sünniaeg ja koht: 09.03.1977, Tallinn, Eesti
   Kodakondsus: Eesti
   Perekonnaseis: abielus
   Aadress, telefon, *e-mail*: Tartu Ülikooli Tehnoloogiainstituut,
   proteoomika tuumiklabor
   Nooruse 1
   50411 Tartu
   tel: +372 737 4865
   email: lauri.peil@ut.ee

2. Praegune töökoht, amet: Tartu Ülikooli Tehnoloogiainstituudi proteoomika tuumiklabor, erakorraline teadur
   Haridus (lõpetatud õppeasutused, lõpetamise aastad, omandatud kraadid, kvalifikatsioonid):
   - Tallinna Reaalkool, 1993–1995, keskharidus
   - Tartu Ülikool, 1995–1999, BSc molekulaarbioloogia erialal
   - Tartu Ülikool, 1999–2001, MSc molekulaarbioloogia erialal
   - Tartu Ülikool, 2001–..., doktoriõpe molekulaarbioloogia erialal

   Keelteoskus: eesti, inglise, soome, vene

Töökogemus (teenistuskäik):
- Tartu Ülikooli Tehnoloogiainstituut, 2009–..., erakorraline teadur
- Tartu Ülikooli Tehnoloogiainstituut, 2006–2008, proteoomika tuumiklabori juhataja kt.
- Tartu Ülikool, 2005–2006, biomeditsiini ja biotehnoloogia doktorikooli projektijuht

II. Teaduslik ja arendustegevus

Peamised uurimisvaldkonnad:

Peamisteks uurimisvaldkondadeks on olnud ribosoomide kokkopakkimise protsessi uurimine bakteris *Escherichia coli* ning selles protsessis osalevate mitte-ribosomaalsete faktorite täpsem iseloomustamine. Samuti on huvi-orbiidis olnud ribosomaalse RNA modifikatsioonide ja modifikatsiooni-ensüümide olulisus ribosoomide assambleerumisprotsessis.

4. Publikatsioonide loetelu:

- **Ofengand, J; Malhotra, A; Remme, J; Gutgsell, NS; Del Campo, M; Jean-Charles, S; Peil, L; Kaya, Y:** Pseudouridines and pseudouridine

synthases of the ribosome. Cold Spring Harb Symp Quant Biol 2001, 66:147–59.

- **Leppik, M; Peil, L; Kipper, K; Liiv, A; Remme, J:** Substrate specificity of the pseudouridine synthase RluD in *Escherichia coli*. FEBS J 2007, 274:5759–66.
- **Peil, L; Virumäe, K; Remme, J**: Ribosome assembly in *Escherichia coli* strains lacking the RNA helicase DeaD/CsdA or DbpA. FEBS J 2008, 275:3772–82.
- **Ero, R; Peil, L; Liiv, A; Remme, J**: Identification of pseudouridine methyltransferase in *Escherichia coli*. RNA 2008, 14:2223–33.
- **Siibak T, Peil L, Xiong L, Mankin A, Remme J, Tenson T**: Erythromycin- and chloramphenicol-induced ribosomal assembly defects are secondary effects of protein synthesis inhibition. Antimicrob Agents Chemother. 2009 Feb;53(2):563–71.

Saadud uurimistoetused ja stipendiumid:

Muu teaduslik organisatsiooniline ja erialane tegevus (konverentside ettekanded, osalemine erialastes seltsides, seadusloome jms.):

- Eesti Proteoomika Ühing, juhatuse liige

DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS

1. **Toivo Maimets**. Studies of human oncoprotein p53. Tartu, 1991, 96 p.
2. **Enn K. Seppet**. Thyroid state control over energy metabolism, ion transport and contractile functions in rat heart. Tartu, 1991, 135 p.
3. **Kristjan Zobel**. Epifüütsete makrosamblike väärtus õhu saastuse indikaatoritena Hamar-Dobani boreaalsetes mägimetsades. Tartu, 1992, 131 lk.
4. **Andres Mäe**. Conjugal mobilization of catabolic plasmids by transposable elements in helper plasmids. Tartu, 1992, 91 p.
5. **Maia Kivisaar**. Studies on phenol degradation genes of *Pseudomonas* sp. strain EST 1001. Tartu, 1992, 61 p.
6. **Allan Nurk**. Nucleotide sequences of phenol degradative genes from *Pseudomonas sp.* strain EST 1001 and their transcriptional activation in *Pseudomonas putida.* Tartu, 1992, 72 p.
7. **Ülo Tamm**. The genus *Populus* L. in Estonia: variation of the species biology and introduction. Tartu, 1993, 91 p.
8. **Jaanus Remme**. Studies on the peptidyltransferase centre of the *E.coli* ribosome. Tartu, 1993, 68 p.
9. **Ülo Langel**. Galanin and galanin antagonists. Tartu, 1993, 97 p.
10. **Arvo Käärd**. The development of an automatic online dynamic fluorescense-based pH-dependent fiber optic penicillin flowthrought biosensor for the control of the benzylpenicillin hydrolysis. Tartu, 1993, 117 p.
11. **Lilian Järvekülg**. Antigenic analysis and development of sensitive immunoassay for potato viruses. Tartu, 1993, 147 p.
12. **Jaak Palumets**. Analysis of phytomass partition in Norway spruce. Tartu, 1993, 47 p.
13. **Arne Sellin**. Variation in hydraulic architecture of *Picea abies* (L.) Karst. trees grown under different enviromental conditions. Tartu, 1994, 119 p.

13. **Mati Reeben**. Regulation of light neurofilament gene expression. Tartu, 1994, 108 p.
14. **Urmas Tartes**. Respiration rhytms in insects. Tartu, 1995, 109 p.
15. **Ülo Puurand**. The complete nucleotide sequence and infections *in vitro* transcripts from cloned cDNA of a potato A potyvirus. Tartu, 1995, 96 p.
16. **Peeter Hõrak**. Pathways of selection in avian reproduction: a functional framework and its application in the population study of the great tit (*Parus major*). Tartu, 1995, 118 p.
17. **Erkki Truve**. Studies on specific and broad spectrum virus resistance in transgenic plants. Tartu, 1996, 158 p.
18. **Illar Pata**. Cloning and characterization of human and mouse ribosomal protein S6-encoding genes. Tartu, 1996, 60 p.
19. **Ülo Niinemets**. Importance of structural features of leaves and canopy in determining species shade-tolerance in temperature deciduous woody taxa. Tartu, 1996, 150 p.

20. **Ants Kurg**. Bovine leukemia virus: molecular studies on the packaging region and DNA diagnostics in cattle. Tartu, 1996, 104 p.
21. **Ene Ustav**. E2 as the modulator of the BPV1 DNA replication. Tartu, 1996, 100 p.
22. **Aksel Soosaar**. Role of helix-loop-helix and nuclear hormone receptor transcription factors in neurogenesis. Tartu, 1996, 109 p.
23. **Maido Remm**. Human papillomavirus type 18: replication, transformation and gene expression. Tartu, 1997, 117 p.
24. **Tiiu Kull**. Population dynamics in *Cypripedium calceolus* L. Tartu, 1997, 124 p.
25. **Kalle Olli**. Evolutionary life-strategies of autotrophic planktonic microorganisms in the Baltic Sea. Tartu, 1997, 180 p.
26. **Meelis Pärtel**. Species diversity and community dynamics in calcareous grassland communities in Western Estonia. Tartu, 1997, 124 p.
27. **Malle Leht**. The Genus *Potentilla* L. in Estonia, Latvia and Lithuania: distribution, morphology and taxonomy. Tartu, 1997, 186 p.
28. **Tanel Tenson**. Ribosomes, peptides and antibiotic resistance. Tartu, 1997, 80 p.
29. **Arvo Tuvikene**. Assessment of inland water pollution using biomarker responses in fish *in vivo* and *in vitro*. Tartu, 1997, 160 p.
30. **Urmas Saarma**. Tuning ribosomal elongation cycle by mutagenesis of 23S rRNA. Tartu, 1997, 134 p.
31. **Henn Ojaveer**. Composition and dynamics of fish stocks in the gulf of Riga ecosystem. Tartu, 1997, 138 p.
32. **Lembi Lõugas**. Post-glacial development of vertebrate fauna in Estonian water bodies. Tartu, 1997, 138 p.
33. **Margus Pooga**. Cell penetrating peptide, transportan, and its predecessors, galanin-based chimeric peptides. Tartu, 1998, 110 p.
34. **Andres Saag**. Evolutionary relationships in some cetrarioid genera (Lichenized Ascomycota). Tartu, 1998, 196 p.
35. **Aivar Liiv**. Ribosomal large subunit assembly *in vivo*. Tartu, 1998, 158 p.
36. **Tatjana Oja**. Isoenzyme diversity and phylogenetic affinities among the eurasian annual bromes (*Bromus* L., Poaceae). Tartu, 1998, 92 p.
37. **Mari Moora**. The influence of arbuscular mycorrhizal (AM) symbiosis on the competition and coexistence of calcareous crassland plant species. Tartu, 1998, 78 p.
38. **Olavi Kurina**. Fungus gnats in Estonia (*Diptera: Bolitophilidae, Keroplatidae, Macroceridae, Ditomyiidae, Diadocidiidae, Mycetophilidae*). Tartu, 1998, 200 p.
39. **Andrus Tasa**. Biological leaching of shales: black shale and oil shale. Tartu, 1998, 98 p.
40. **Arnold Kristjuhan.** Studies on transcriptional activator properties of tumor suppressor protein p53. Tartu, 1998, 86 p.

41. **Sulev Ingerpuu.** Characterization of some human myeloid cell surface and nuclear differentiation antigens. Tartu, 1998, 163 p.
42. **Veljo Kisand.** Responses of planktonic bacteria to the abiotic and biotic factors in the shallow lake Võrtsjärv. Tartu, 1998, 118 p.
43. **Kadri Põldmaa.** Studies in the systematics of hypomyces and allied genera (Hypocreales, Ascomycota). Tartu, 1998, 178 p.
44. **Markus Vetemaa.** Reproduction parameters of fish as indicators in environmental monitoring. Tartu, 1998, 117 p.
45. **Heli Talvik.** Prepatent periods and species composition of different *Oesophagostomum* spp. populations in Estonia and Denmark. Tartu, 1998, 104 p.
46. **Katrin Heinsoo.** Cuticular and stomatal antechamber conductance to water vapour diffusion in *Picea abies* (L.) karst. Tartu, 1999, 133 p.
47. **Tarmo Annilo.** Studies on mammalian ribosomal protein S7. Tartu, 1998, 77 p.
48. **Indrek Ots.** Health state indicies of reproducing great tits (*Parus major*): sources of variation and connections with life-history traits. Tartu, 1999, 117 p.
49. **Juan Jose Cantero.** Plant community diversity and habitat relationships in central Argentina grasslands. Tartu, 1999, 161 p.
50. **Rein Kalamees.** Seed bank, seed rain and community regeneration in Estonian calcareous grasslands. Tartu, 1999, 107 p.
51. **Sulev Kõks.** Cholecystokinin (CCK) — induced anxiety in rats: influence of environmental stimuli and involvement of endopioid mechanisms and erotonin. Tartu, 1999, 123 p.
52. **Ebe Sild.** Impact of increasing concentrations of O_3 and CO_2 on wheat, clover and pasture. Tartu, 1999, 123 p.
53. **Ljudmilla Timofejeva.** Electron microscopical analysis of the synaptonemal complex formation in cereals. Tartu, 1999, 99 p.
54. **Andres Valkna.** Interactions of galanin receptor with ligands and G-proteins: studies with synthetic peptides. Tartu, 1999, 103 p.
55. **Taavi Virro.** Life cycles of planktonic rotifers in lake Peipsi. Tartu, 1999, 101 p.
56. **Ana Rebane.** Mammalian ribosomal protein S3a genes and intron-encoded small nucleolar RNAs U73 and U82. Tartu, 1999, 85 p.
57. **Tiina Tamm.** Cocksfoot mottle virus: the genome organisation and translational strategies. Tartu, 2000, 101 p.
58. **Reet Kurg.** Structure-function relationship of the bovine papilloma virus E2 protein. Tartu, 2000, 89 p.
59. **Toomas Kivisild.** The origins of Southern and Western Eurasian populations: an mtDNA study. Tartu, 2000, 121 p.
60. **Niilo Kaldalu.** Studies of the TOL plasmid transcription factor XylS. Tartu 2000. 88 p.

61. **Dina Lepik.** Modulation of viral DNA replication by tumor suppressor protein p53. Tartu 2000. 106 p.
62. **Kai Vellak.** Influence of different factors on the diversity of the bryophyte vegetation in forest and wooded meadow communities. Tartu 2000. 122 p.
63. **Jonne Kotta.** Impact of eutrophication and biological invasionas on the structure and functions of benthic macrofauna. Tartu 2000. 160 p.
64. **Georg Martin.** Phytobenthic communities of the Gulf of Riga and the inner sea the West-Estonian archipelago. Tartu, 2000. 139 p.
65. **Silvia Sepp.** Morphological and genetical variation of *Alchemilla L.* in Estonia. Tartu, 2000. 124 p.
66. **Jaan Liira.** On the determinants of structure and diversity in herbaceous plant communities. Tartu, 2000. 96 p.
67. **Priit Zingel.** The role of planktonic ciliates in lake ecosystems. Tartu 2001. 111 p.
68. **Tiit Teder.** Direct and indirect effects in Host-parasitoid interactions: ecological and evolutionary consequences. Tartu 2001. 122 p.
69. **Hannes Kollist.** Leaf apoplastic ascorbate as ozone scavenger and its transport across the plasma membrane. Tartu 2001. 80 p.
70. **Reet Marits.** Role of two-component regulator system PehR-PehS and extracellular protease PrtW in virulence of *Erwinia Carotovora* subsp. *Carotovora*. Tartu 2001. 112 p.
71. **Vallo Tilgar.** Effect of calcium supplementation on reproductive performance of the pied flycatcher *Ficedula hypoleuca* and the great tit *Parus major,* breeding in Nothern temperate forests. Tartu, 2002. 126 p.
72. **Rita Hõrak.** Regulation of transposition of transposon Tn*4652* in *Pseudomonas putida*. Tartu, 2002. 108 p.
73. **Liina Eek-Piirsoo.** The effect of fertilization, mowing and additional illumination on the structure of a species-rich grassland community. Tartu, 2002. 74 p.
74. **Krõõt Aasamaa.** Shoot hydraulic conductance and stomatal conductance of six temperate deciduous tree species. Tartu, 2002. 110 p.
75. **Nele Ingerpuu.** Bryophyte diversity and vascular plants. Tartu, 2002. 112 p.
76. **Neeme Tõnisson.** Mutation detection by primer extension on oligonucleotide microarrays. Tartu, 2002. 124 p.
77. **Margus Pensa.** Variation in needle retention of Scots pine in relation to leaf morphology, nitrogen conservation and tree age. Tartu, 2003. 110 p.
78. **Asko Lõhmus.** Habitat preferences and quality for birds of prey: from principles to applications. Tartu, 2003. 168 p.
79. **Viljar Jaks.** p53 — a switch in cellular circuit. Tartu, 2003. 160 p.
80. **Jaana Männik.** Characterization and genetic studies of four ATP-binding cassette (ABC) transporters. Tartu, 2003. 140 p.
81. **Marek Sammul.** Competition and coexistence of clonal plants in relation to productivity. Tartu, 2003. 159 p

82. **Ivar Ilves.** Virus-cell interactions in the replication cycle of bovine papillomavirus type 1. Tartu, 2003. 89 p.
83. **Andres Männik.** Design and characterization of a novel vector system based on the stable replicator of bovine papillomavirus type 1. Tartu, 2003. 109 p.
84. **Ivika Ostonen.** Fine root structure, dynamics and proportion in net primary production of Norway spruce forest ecosystem in relation to site conditions. Tartu, 2003. 158 p.
85. **Gudrun Veldre.** Somatic status of 12–15-year-old Tartu schoolchildren. Tartu, 2003. 199 p.
86. **Ülo Väli.** The greater spotted eagle *Aquila clanga* and the lesser spotted eagle *A. pomarina*: taxonomy, phylogeography and ecology. Tartu, 2004. 159 p.
87. **Aare Abroi.** The determinants for the native activities of the bovine papillomavirus type 1 E2 protein are separable. Tartu, 2004. 135 p.
88. **Tiina Kahre.** Cystic fibrosis in Estonia. Tartu, 2004. 116 p.
89. **Helen Orav-Kotta.** Habitat choice and feeding activity of benthic suspension feeders and mesograzers in the northern Baltic Sea. Tartu, 2004. 117 p.
90. **Maarja Öpik.** Diversity of arbuscular mycorrhizal fungi in the roots of perennial plants and their effect on plant performance. Tartu, 2004. 175 p.
91. **Kadri Tali.** Species structure of *Neotinea ustulata*. Tartu, 2004. 109 p.
92. **Kristiina Tambets.** Towards the understanding of post-glacial spread of human mitochondrial DNA haplogroups in Europe and beyond: a phylogeographic approach. Tartu, 2004. 163 p.
93. **Arvi Jõers.** Regulation of p53-dependent transcription. Tartu, 2004. 103 p.
94. **Lilian Kadaja.** Studies on modulation of the activity of tumor suppressor protein p53. Tartu, 2004. 103 p.
95. **Jaak Truu.** Oil shale industry wastewater: impact on river microbial community and possibilities for bioremediation. Tartu, 2004. 128 p.
96. **Maire Peters.** Natural horizontal transfer of the *pheBA* operon. Tartu, 2004. 105 p.
97. **Ülo Maiväli.** Studies on the structure-function relationship of the bacterial ribosome. Tartu, 2004. 130 p.
98. **Merit Otsus.** Plant community regeneration and species diversity in dry calcareous grasslands. Tartu, 2004. 103 p.
99. **Mikk Heidemaa.** Systematic studies on sawflies of the genera *Dolerus, Empria,* and *Caliroa* (Hymenoptera: Tenthredinidae). Tartu, 2004. 167 p.
100. **Ilmar Tõnno.** The impact of nitrogen and phosphorus concentration and N/P ratio on cyanobacterial dominance and N_2 fixation in some Estonian lakes. Tartu, 2004. 111 p.
101. **Lauri Saks.** Immune function, parasites, and carotenoid-based ornaments in greenfinches. Tartu, 2004. 144 p.

102. **Siiri Rootsi.** Human Y-chromosomal variation in European populations. Tartu, 2004. 142 p.
103. **Eve Vedler.** Structure of the 2,4-dichloro-phenoxyacetic acid-degradative plasmid pEST4011. Tartu, 2005. 106 p.
104. **Andres Tover.** Regulation of transcription of the phenol degradation *pheBA* operon in *Pseudomonas putida*. Tartu, 2005. 126 p.
105. **Helen Udras.** Hexose kinases and glucose transport in the yeast *Hansenula polymorpha*. Tartu, 2005. 100 p.
106. **Ave Suija.** Lichens and lichenicolous fungi in Estonia: diversity, distribution patterns, taxonomy. Tartu, 2005. 162 p.
107. **Piret Lõhmus.** Forest lichens and their substrata in Estonia. Tartu, 2005. 162 p.
108. **Inga Lips.** Abiotic factors controlling the cyanobacterial bloom occurrence in the Gulf of Finland. Tartu, 2005. 156 p.
109. **Kaasik, Krista.** Circadian clock genes in mammalian clockwork, metabolism and behaviour. Tartu, 2005. 121 p.
110. **Juhan Javoiš.** The effects of experience on host acceptance in ovipositing moths. Tartu, 2005. 112 p.
111. **Tiina Sedman.** Characterization of the yeast *Saccharomyces cerevisiae* mitochondrial DNA helicase Hmi1. Tartu, 2005. 103 p.
112. **Ruth Aguraiuja.** Hawaiian endemic fern lineage *Diellia* (Aspleniaceae): distribution, population structure and ecology. Tartu, 2005. 112 p.
113. **Riho Teras.** Regulation of transcription from the fusion promoters generated by transposition of Tn*4652* into the upstream region of *pheBA* operon in *Pseudomonas putida*. Tartu, 2005. 106 p.
114. **Mait Metspalu.** Through the course of prehistory in india: tracing the mtDNA trail. Tartu, 2005. 138 p.
115. **Elin Lõhmussaar.** The comparative patterns of linkage disequilibrium in European populations and its implication for genetic association studies. Tartu, 2006. 124 p.
116. **Priit Kupper.** Hydraulic and environmental limitations to leaf water relations in trees with respect to canopy position. Tartu, 2006. 126 p.
117. **Heili Ilves.** Stress-induced transposition of Tn*4652* in *Pseudomonas Putida.* Tartu, 2006. 120 p.
118. **Silja Kuusk.** Biochemical properties of Hmi1p, a DNA helicase from *Saccharomyces cerevisiae* mitochondria. Tartu, 2006. 126 p.
119. **Kersti Püssa.** Forest edges on medium resolution landsat thematic mapper satellite images. Tartu, 2006. 90 p.
120. **Lea Tummeleht.** Physiological condition and immune function in great tits (*Parus major* l.): Sources of variation and trade-offs in relation to growth. Tartu, 2006. 94 p.
121. **Toomas Esperk.** Larval instar as a key element of insect growth schedules. Tartu, 2006. 186 p.

122. **Harri Valdmann.** Lynx (*Lynx lynx*) and wolf (*Canis lupus*) in the Baltic region: Diets, helminth parasites and genetic variation. Tartu, 2006. 102 p.
123. **Priit Jõers.** Studies of the mitochondrial helicase Hmi1p in *Candida albicans* and *Saccharomyces cerevisia*. Tartu, 2006. 113 p.
124. **Kersti Lilleväli.** Gata3 and Gata2 in inner ear development. Tartu, 2007. 123 p.
125. **Kai Rünk.** Comparative ecology of three fern species: *Dryopteris carthusiana* (Vill.) H.P. Fuchs, *D. expansa* (C. Presl) Fraser-Jenkins & Jermy and *D. dilatata* (Hoffm.) A. Gray (Dryopteridaceae). Tartu, 2007. 143 p.
126. **Aveliina Helm.** Formation and persistence of dry grassland diversity: role of human history and landscape structure. Tartu, 2007. 89 p.
127. **Leho Tedersoo.** Ectomycorrhizal fungi: diversity and community structure in Estonia, Seychelles and Australia. Tartu, 2007. 233 p.
128. **Marko Mägi.** The habitat-related variation of reproductive performance of great tits in a deciduous-coniferous forest mosaic: looking for causes and consequences. Tartu, 2007. 135 p.
129. **Valeria Lulla.** Replication strategies and applications of Semliki Forest virus. Tartu, 2007. 109 p.
130. **Ülle Reier**. Estonian threatened vascular plant species: causes of rarity and conservation. Tartu, 2007. 79 p.
131. **Inga Jüriado**. Diversity of lichen species in Estonia: influence of regional and local factors. Tartu, 2007. 171 p.
132. **Tatjana Krama.** Mobbing behaviour in birds: costs and reciprocity based cooperation. Tartu, 2007.
133. **Signe Saumaa.** The role of DNA mismatch repair and oxidative DNA damage defense systems in avoidance of stationary phase mutations in *Pseudomonas putida.* Tartu, 2007. 172 p.
134. **Reedik Mägi**. The linkage disequilibrium and the selection of genetic markers for association studies in european populations. Tartu, 2007. 96 p.
135. **Priit Kilgas.** Blood parameters as indicators of physiological condition and skeletal development in great tits (*Parus major*): natural variation and application in the reproductive ecology of birds. Tartu, 2007. 129 p.
136. **Anu Albert**. The role of water salinity in structuring eastern Baltic coastal fish communities. Tartu, 2007. 95 p.
137. **Kärt Padari.** Protein transduction mechanisms of transportans. Tartu, 2008. 128 p.
138. **Siiri-Lii Sandre.** Selective forces on larval colouration in a moth. Tartu, 2008. 125 p.
139. **Ülle Jõgar.** Conservation and restoration of semi-natural floodplain meadows and their rare plant species. Tartu, 2008. 99 p.
140. **Lauri Laanisto.** Macroecological approach in vegetation science: generality of ecological relationships at the global scale. Tartu, 2008. 133 p.
141. **Reidar Andreson**. Methods and software for predicting PCR failure rate in large genomes. Tartu, 2008. 105 p.

142. **Birgot Paavel.** Bio-optical properties of turbid lakes. Tartu, 2008. 175 p.
143. **Kaire Torn.** Distribution and ecology of charophytes in the Baltic Sea. Tartu, 2008, 98 p.
144. **Vladimir Vimberg.** Peptide mediated macrolide resistance. Tartu, 2008, 190 p.
145. **Daima Örd.** Studies on the stress-inducible pseudokinase TRB3, a novel inhibitor of transcription factor ATF4. Tartu, 2008, 108 p.
146. **Lauri Saag.** Taxonomic and ecologic problems in the genus *Lepraria* (*Stereocaulaceae*, lichenised *Ascomycota*). Tartu, 2008, 175 p.
147. **Ulvi Karu.** Antioxidant protection, carotenoids and coccidians in greenfinches – assessment of the costs of immune activation and mechanisms of parasite resistance in a passerine with carotenoid-based ornaments. Tartu, 2008, 124 p.
148. **Jaanus Remm.** Tree-cavities in forests: density, characteristics and occupancy by animals. Tartu, 2008, 128 p.
149. **Epp Moks.** Tapeworm parasites *Echinococcus multilocularis* and *E. granulosus* in Estonia: phylogenetic relationships and occurrence in wild carnivores and ungulates. Tartu, 2008, 82 p.
150. **Eve Eensalu.** Acclimation of stomatal structure and function in tree canopy: effect of light and CO_2 concentration. Tartu, 2008, 108 p.
151. **Janne Pullat**. Design, functionlization and application of an *in situ* synthesized oligonucleotide microarray. Tartu, 2008, 108 p.
152. **Marta Putrinš.** Responses of *Pseudomonas putida* to phenol-induced metabolic and stress signals. Tartu, 2008, 142 p.
153. **Marina Semtšenko.** Plant root behaviour: responses to neighbours and physical obstructions. Tartu, 2008, 106 p.
154. **Marge Starast.** Influence of cultivation techniques on productivity and fruit quality of some *Vaccinium* and *Rubus* taxa. Tartu, 2008, 154 p.
155. **Age Tats.** Sequence motifs influencing the efficiency of translation. Tartu, 2009, 104 p.
156. **Radi Tegova.** The role of specialized DNA polymerases in mutagenesis in *Pseudomonas putida.* Tartu, 2009, 124 p.
157. **Tsipe Aavik.** Plant species richness, composition and functional trait pattern in agricultural landscapes – the role of land use intensity and landscape structure. Tartu, 2008, 112 p.
158. **Kaja Kiiver.** Semliki forest virus based vectors and cell lines for studying the replication and interactions of alphaviruses and hepaciviruses. Tartu, 2009, 104 p.
159. **Meelis Kadaja.** Papillomavirus Replication Machinery Induces Genomic Instability in its Host Cell. Tartu, 2009, 126 p.
160. **Pille Hallast.** Human and chimpanzee Luteinizing hormone/Chorionic Gonadotropin beta (*LHB/CGB*) gene clusters: diversity and divergence of young duplicated genes. Tartu, 2009, 168 p.

161. **Ain Vellak.** Spatial and temporal aspects of plant species conservation. Tartu, 2009, 86 p.
162. **Triinu Remmel.** Body size evolution in insects with different colouration strategies: the role of predation risk. Tartu, 2009, 168 p.
163. **Jaana Salujõe.** Zooplankton as the indicator of ecological quality and fish predation in lake ecosystems. Tartu, 2009, 129 p.
164. **Ele Vahtmäe.** Mapping benthic habitat with remote sensing in optically complex coastal environments. Tartu, 2009, 109 p.
165. **Liisa Metsamaa.** Model-based assessment to improve the use of remote sensing in recognition and quantitative mapping of cyanobacteria. Tartu, 2009, 114 p.
166. **Pille Säälik.** The role of endocytosis in the protein transduction by cell-penetrating peptides. Tartu, 2009, 155 p.