DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
218

DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
218

RYA ERO

Modifier view of the bacterial ribosome

Institute of Molecular and Cell Biology, Faculty of Science and Technology, University of Tartu, Estonia.

Dissertation was accepted for the commencement of the degree of *Doctor philosophiae* in molecular biology on June 18th, 2012 by the Council of Institute of Molecular and Cell Biology, Faculty of Science and Technology, University of Tartu.

Supervisors: Prof. Jaanus Remme, PhD
Institute of Molecular and Cell Biology,
University of Tartu, Estonia

Dr. Aivar Liiv, PhD
Institute of Molecular and Cell Biology,
University of Tartu, Estonia

Opponent: Prof. Roland K. Hartmann, PhD
Institut für Pharmazeutische Chemie,
Philipps-Universität Marburg, Marburg, Germany

Commencement: Room 217, 23 Riia street, Tartu, on 24th of August in 2012, at 14.00.

Publication of this thesis is granted by the Institute of Molecular and Cell Biology, University of Tartu and by the Graduate School in Biomedicine and Biotechnology created under the auspices of European Social Fund

ISSN 1024–6479
ISBN 978–9949–32–071–4 (trükis)
ISBN 978–9949–32–072–1 (pdf)

Tartu Ülikooli Kirjastus
www.tyk.ee
Tellimuse nr. 361

TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS

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

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

II **Ero, R; Leppik, M; Liiv, A; Remme, J:** Specificity and kinetics of 23S rRNA modification enzymes RlmH and RluD. RNA 2010, 11:2075–84.

III **Leppik, M; Ero, R; Liiv, A; Kipper, K; Remme, J:** Different sensitivity of H69 modification enzymes RluD and RlmH to mutations in *Escherichia coli* 23S rRNA. Biochimie 2012, 94(5):1080–89.

My contribution to the publications is as follows:

Ref. I – I constructed the *rlmH* and *rlmH/rluD* deletion strains, and the recombinant RlmH protein expression plasmid. I screened various rRNAs for $m^3\Psi$ methylation by primer extension analysis; affinity purified the recombinant RlmH and RluD proteins as well as ribosomes, and conducted the *in vitro* methylation assays. I prepared Figures 1-4 and drafted the manuscript.

Ref. II – I constructed the native RlmH protein expression plasmid and purified the native RlmH protein as well as ribosomes. I conducted the *in vitro* methylation and pseudouridylation assays and determined the kinetic parameters of RlmH and RluD proteins. I prepared Tables, Figures 1-4, and drafted the manuscript.

Ref. III – I share the first authorship. I conducted the *in vitro* methylation and ribosome association assays, prepared Figure 4, and participated in writing of the manuscript.

LIST OF ABBREVIATIONS

A-site –	acceptor site for tRNA on the ribosome
B2a –	inter-subunit bridge
D –	dihydrouridine
DC –	decoding center
EF –	translation elongation factor
E-site –	exit site for tRNA on the ribosome
GTPase –	guanosine triphosphatase
h44 –	helix 44 of the small ribosomal subunit
H69 –	stem-loop 69 of the large ribosomal subunit
H-bond –	hydrogen bond
IF –	translation initiation factor
L-proteins –	ribosomal large subunit proteins
LSU –	ribosomal large subunit(s)
ME –	modification enzyme(s)
MN –	modified nucleoside(s)
MT –	methyltransferase(s)
nt –	nucleotides
PAGE –	polyacrylamide gel electrophoresis
PCR –	polymerase chain reaction
PS –	pseudouridine synthase(s)
P-site –	peptidyl site for tRNA on the ribosome
PTC –	peptidyltransferase center
RF –	release factor
RFMT –	Rossmann-fold methyltransferase(s)
RNase –	ribonuclease
r-proteins –	ribosomal proteins
rRNA –	ribosomal RNA
S –	sedimentation coefficient (Svedberg)
SAM –	S-adenosyl-L-methionine
SDS –	sodium dodecyl sulfate
SPOUT –	SpoU-TrmD-like
S-proteins –	ribosomal small subunit proteins
SSU –	ribosomal small subunit(s)
TCA –	trichloroacetic acid
WT –	wild-type
Ψ –	pseudouridine

INTRODUCTION

Ribosomes are large ribonucleoprotein particles responsible for carrying out protein synthesis – a fundamental process in every cell of every organism. Likewise, the synthesis of ribosomes themselves is a fundamental process. Historically, eubacterium *Escherichia coli* has served as the model organism for studying the structure, functioning, and synthesis of ribosomes.

Synthesis of bacterial ribosomes is a complex process that starts with ribosomal RNA transcription, includes a plethora of chemical and conformational alterations to both its RNA and protein components, and through interwoven assembly pathways ultimately yields functional ribosomes capable of participating in protein synthesis (for comprehensive reviews see: Kaczanowska & Ryden-Aulin, 2007; Wilson & Nierhaus, 2007; Shajani *et al.*, 2011). Multiple rare genetic disorders have been attributed to defects in ribosome biogenesis (Freed *et al.*, 2010).

Post-transcriptional enzymatic modification of ribosomal RNA is an integral and conserved part of ribosome synthesis. Most modified nucleosides are located near the functionally important regions of the ribosome (Decatur & Fournier, 2002; Ofengand & Del Campo, 2004). Notwithstanding their conservation and strategic localization, the function of the modified nucleosides remains a mystery for the most part. Understanding of the substrate recognition and catalysis mechanisms of the ribosomal RNA modification enzymes is an essential stepping stone for creating a complete picture of the processes that govern the synthesis of ribosomes in cells.

First part of this thesis gives an overview of the bacterial ribosome structure and synthesis with special attention being paid to ribosomal RNA modifications, their synthesis and significance. The catalysis and the substrate recognition mechanism of two main types of modification enzymes, the pseudouridine synthases and the methyltransferases, is covered in more detail.

The experimental part of the thesis focuses on two ribosomal RNA modification enzymes, the pseudouridine synthase RluD and the methyltransferase RlmH, both of which modify a functionally important part of the ribosome, the stem-loop 69. RlmH was first identified by us as the pseudouridine specific methyltransferase modifying 70S ribosomes exclusively (Ref. I and Ref. II). The kinetic parameters of RlmH and RluD were determined (Ref. II) and will be discussed in the overall context of the ribosome biosynthesis. Finally, the sensitivity of RlmH to mutations in its substrate stem-loop 69 (Ref. III) and in RlmH protein itself will be discussed in respect to substrate recognition and modification catalysis mechanism.

REVIEW OF LITERATURE

Ribosome is the macromolecular machine responsible for a key process in every organism - decoding of the genetic information stored in the genome and mediated via messenger RNAs into the amino acid sequence of proteins. Ribosomes of eukaryotes are larger and structurally more complex than bacterial and archaeal ribosomes. However, ribosomes from all three domains of life exhibit extraordinary phylogenetic conservation of the core structural and functional features. All ribosomes are made up of two unequal subunits containing ribosomal ribonucleic acid (rRNA) and ribosomal proteins (r-proteins), with rRNA featuring the decoding and the peptidyltransferase activity and r-proteins playing a supporting role (Noller *et al.*, 1992; Cech, 2000; Nissen *et al.*, 2000; Steitz & Moore, 2003; Zhang *et al.*, 2009; Ben-Shem *et al.*, 2011).

Rod-shaped Gram-negative bacterium *Escherichia coli* (commonly abbreviated *E. coli*) is due to its long history of laboratory culture and the ease of manipulation the most extensively studied prokaryotic model organism and serves also as the reference organism for ribosome research. However, the thermophilic bacterium *Thermus thermophilus* (Schluenzen *et al.*, 2000; Wimberly *et al.*, 2000; Yusupov *et al.*, 2001; Korostelev *et al.*, 2006; Selmer *et al.*, 2006; Jenner *et al.*, 2010), the poly-extremophilic bacterium *Deinococcus radiodurans* (Harms *et al.*, 2001), and the halophilic archaeon *Haloarcula marismortui* (Ban *et al.*, 2000; Nissen *et al.*, 2000) have also contributed significantly to the understanding of the various structural aspects of prokaryotic ribosomes. On the other hand, atomic resolution structures of the first eukaryotic ribosomes, those of yeast *Saccharomyces cerevisiae* and *Tetrahymena thermophila*, were determined not long ago (Ben-Shem *et al.*, 2010; Ben-Shem *et al.*, 2011; Klinge *et al.*, 2012).

I. STRUCTURE AND FUNCTION OF THE BACTERIAL RIBOSOME

Bacterial ribosome with its molecular weight of approximately 2.5 megadaltons and a diameter of about 210 Å sediments as a 70S particle and is formed of the large subunit (LSU) and the small subunit (SSU), sedimenting as 50S and 30S particles, respectively (Tissieres & Watson, 1958; Ramakrishnan, 2002) (Figure 1 and 3). S is the Svedberg unit for the sedimentation velocity when subjected to a centrifugal force and is defined as 10^{-13} seconds. Based on the numerous available atomic resolution structures (Schluenzen *et al.*, 2000; Wimberly *et al.*, 2000; Harms *et al.*, 2001; Yusupov *et al.*, 2001; Schuwirth *et al.*, 2005; Korostelev *et al.*, 2006; Selmer *et al.*, 2006; Zhang *et al.*, 2009; Jenner *et al.*, 2010), all bacterial ribosomes consist of roughly two-thirds of RNA and one-third of protein. *E. coli* LSU is composed of two rRNA molecules, 23S (2904 nt) and 5S (120 nt) rRNA, and 33 L-proteins (L1-L36), while SSU is composed of 16S (1542 nt) rRNA and 21 S-proteins (S1-S21) (Figure 1) (for reviews see: Kaczanowska & Ryden-Aulin, 2007; Wilson & Nierhaus, 2007; Steitz, 2008; Shajani *et al.*, 2011). Each component is present as a single copy, with the exception of two copies of the L12 protein and two copies of its N-terminally acetylated derivative, L7 (Hardy, 1975).

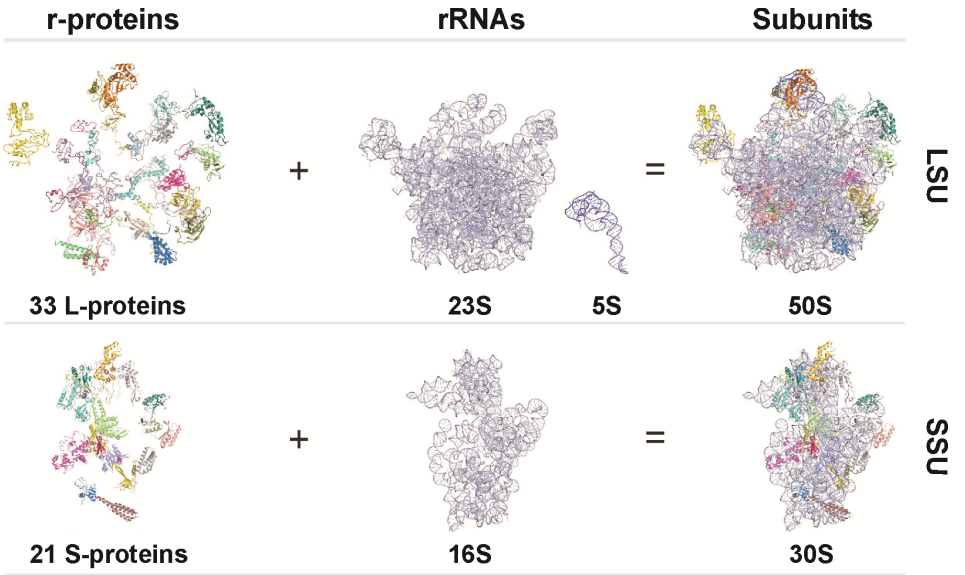

Figure 1. Components of the bacterial ribosome. R-proteins (assortment of colors) and rRNAs (grey) are depicted separately and in the context of the large subunit (LSU) and the small subunit (SSU). Illustration adapted from Wilson Lab Homepage (http://www.lmb.uni-muenchen.de/wilson/).

The sequences of the rRNAs from thousands of organisms are available by now (Cole *et al.*, 2003; Wuyts *et al.*, 2004; Pruesse *et al.*, 2007) and although the size and the primary sequences vary considerably, the secondary structures are comparable in all organisms. The secondary structure of rRNAs is made up of short helical segments connected by single-stranded regions of loops and bulges (Figure 2). Stem-loop (hairpin) structures are common motifs in rRNA secondary structure (Figure 2 and 4). 16S rRNA can be divided into four domains (5', central, 3' major, and 3'minor) and 23S rRNA into six domains (I-VI) (Noller *et al.*, 1981; Noller & Woese, 1981) (Figure 2). The 3' end of 16S rRNA contains the anti-Shine-Dalgarno sequence that base-pairs with the Shine-Dalgarno sequence present in many messenger RNAs. 5S rRNA is considered the seventh domain of LSU.

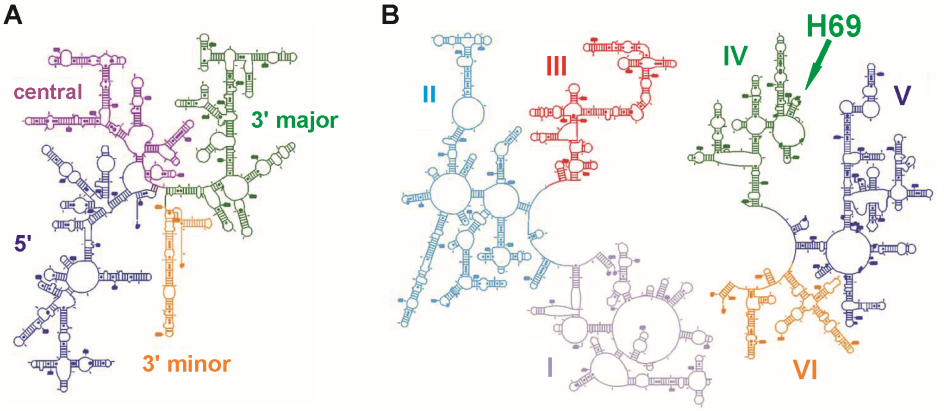

Figure 2. Secondary structure of *E. coli* rRNAs. 16S rRNA (A) 5', central, 3' major, and 3' minor domains are indicated by dark blue, pink, green, and yellow, respectively. 23S rRNA (B) domains I-VI are also indicated by different colors. Location of 23S rRNA stem-loop 69 (H69) is indicated by an arrow. 5S rRNA is not shown. Illustration adapted from (Decatur & Fournier, 2002).

The secondary structure motifs of rRNA are tightly packed together and stabilized by a myriad of tertiary interactions into a complex three-dimensional rRNA scaffold (Holbrook, 2008). Coaxial stacking plays a major role in dictating the overall architecture of the rRNA scaffold. In addition, the 2'-hydroxyl groups of riboses, A-minor interactions, pseudoknots, and more than 20 types of non-canonical base pairs are involved in stabilization of the rRNA tertiary structure (Noller, 2005). Magnesium ions (Mg^{2+}) play an important part by interacting with phosphate groups from remote secondary structure elements bringing them together in the tertiary structure. There are also numerous monovalent cations bound to specific locations neutralizing the negative charge of the rRNA backbone phosphate groups (Klein *et al.*, 2004a).

While the domains of SSU are able to fold (Weitzmann *et al.*, 1993; Samaha *et al.*, 1994; Agalarov *et al.*, 1998; Yusupov *et al.*, 2001) and move (Frank & Agrawal, 2000; Schuwirth *et al.*, 2005; Zhang *et al.*, 2009) relatively independently of each other, the domains of the 23S rRNA are intricately interwoven with the L-proteins as well as each other to form a more monolithic LSU particle (Schluenzen *et al.*, 2000; Wimberly *et al.*, 2000; Harms *et al.*, 2001). Majority of the r-proteins are located on the surface of the subunits, they are relatively small, basic (average isoelectric point is ~10), and either globular in structure or contain extensions that penetrate into the interior of the subunits to fill the gaps between the rRNA secondary structure elements (Schluenzen *et al.*, 2000; Wimberly *et al.*, 2000; Harms *et al.*, 2001; Yusupov *et al.*, 2001; Schuwirth *et al.*, 2005; Korostelev *et al.*, 2006; Selmer *et al.*, 2006). Therefore, the primary function of the r-proteins seems to be the stabilization of highly compact rRNA structures (Klein *et al.*, 2004b; Wilson & Nierhaus, 2005).

The translation process is not within the scope of this thesis and is thus reviewed only briefly from the structural aspects to provide the necessary background. The two ribosomal subunits perform different functions in protein synthesis (reviews of translation: Ramakrishnan, 2002; Moore & Steitz, 2003; Bashan & Yonath, 2008; Steitz, 2008; Schmeing & Ramakrishnan, 2009). The SSU is responsible for the association with the messenger RNA (mRNA) during translation initiation and contains the decoding center (DC), where interactions between codons in the mRNA and the anticodons of transport RNAs (tRNAs) determine which amino acid is to be incorporate into the polypeptide chain next (Figure 3C). The peptidyltransferase center (PTC) of the LSU (Figure 3B) catalyzes two reactions: i) the peptide bond formation between the amino acid attached to the tRNA in the A-site (aminoacyl-tRNA) and the nascent peptide chain attached to the tRNA in P-site (peptidyl-tRNA) during translation elongation, and ii) the hydrolysis of nascent peptide from P-site tRNA during translation termination. Just below the PTC begins the polypeptide exit tunnel, which provides the nascent peptides with a stable path through the LSU.

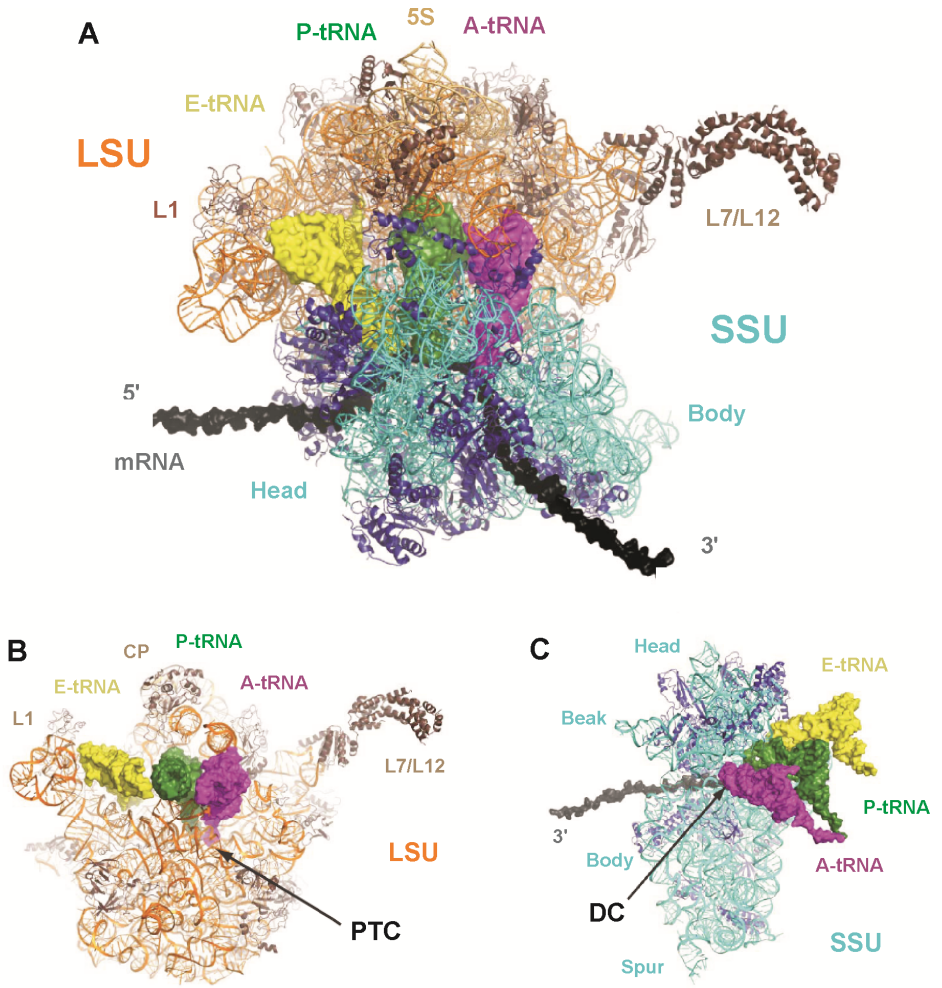

Figure 3. Structure of the bacterial ribosome. 70S ribosome with mRNA and A-, P-, and E-site tRNAs (A). "Split" view of the LSU (B) and SSU (C) from the subunit interface side displaying the mRNA, tRNAs, decoding center (DC), peptidyltransferase center (PTC), and the structural landmarks (in brown for LSU and in blue for SSU). Illustration adapted from (Frank *et al.*, 1995; Schmeing & Ramakrishnan, 2009).

Upon translation initiation, the two subunits associate - become connected via a complex network of molecular interactions between the protruding structures of the subunits, termed inter-subunit bridges (Frank *et al.*, 1995; Yusupov *et al.*, 2001; Maiväli & Remme, 2004; Hennelly *et al.*, 2005; Schuwirth *et al.*, 2005). Some of the inter-subunit bridges are composed entirely of RNA, while others have at least one protein component. First type of bridges are for the most part located in the central region of the subunit interface, while the latter type of

bridges are located mostly peripherally (Yusupov *et al.*, 2001; Schuwirth *et al.*, 2005; Korostelev *et al.*, 2006; Selmer *et al.*, 2006). The interface between the two subunits is relatively free of r-proteins and provides the binding surface for various substrates and ligands, including the three binding sites (A-, P-, and E-sites) for tRNAs (Figure 3A). Rearrangement of the inter-subunit bridges during the translation elongation cycle is integral to the coordinated action between the DC of SSU and PTC of the LSU as well as to translocation of the mRNA/tRNA complex precisely one codon at the time relative to the ribosome (Yusupov *et al.*, 2001; Chan *et al.*, 2006; Zhang *et al.*, 2009; Jenner *et al.*, 2010). Protein synthesis is hence a cyclic and dynamic process of the ribosome undergoing a series of coordinated motions (Frank & Agrawal, 2000; Zhang *et al.*, 2009; Jenner *et al.*, 2010; Dunkle *et al.*, 2011). Subunits remain together during translation elongation cycles and finally separate in conjunction with the release of the finished polypeptide (Ramakrishnan, 2002; Bashan & Yonath, 2008; Steitz, 2008; Schmeing & Ramakrishnan, 2009).

rRNA is the key component of the ribosome, being crucial for all aspects of translation. For example, the 23S rRNA has a central role in peptide bond formation as a number of its residues serve to fix the tRNA substrates for the reaction (entropic catalysis) and participate in the proton shuttle network during the peptidyl group transfer (Cech, 2000; Nissen *et al.*, 2000; Steitz & Moore, 2003; Moore & Steitz, 2010). Nonetheless, in addition to being important for the stabilization of rRNA tertiary structure as mentioned above, r-proteins are known to assist various steps of translation such as mRNA binding (S1), decoding and fidelity of translation (S4, S5, and S12), and fixing the tRNAs in orientation facilitating peptide bond formation (S7, S11, L5, L16, and L27), just to name a few (comprehensive review of the functions of r-proteins in Wilson & Nierhaus, 2005). Some r-proteins have an essential function in the biogenesis of ribosomal subunits (discussed in chapter 2.1.), but are dispensable for function after the ribosome is fully assembled (Wilson & Nierhaus, 2005). Taken together, r-proteins are necessary for the optimal functioning of the ribosome and even small improvements in the speed and accuracy of translation can result in strong selective advantages for the cells.

1.1 Stem-loop 69

Stem-loop 69 (H69) is a universally conserved secondary structure element (23S rRNA nucleotides 1906-1924 according to *E. coli* numbering) in domain IV of the LSU (Figures 2). Furthermore, the sequence of H69 is highly conserved as well (Cannone *et al.*, 2002). Accordingly, many point mutations in H69 cause strong growth defects in *E. coli* and are known to inhibit translation (Liiv *et al.*, 2005), reflecting the functional importance of H69. In fact, H69 has been shown to be involved in various steps of translation such as: subunit association (O'Connor & Dahlberg, 1995; Maiväli & Remme, 2004; Gutgsell *et al.*, 2005; Liiv *et al.*, 2005; Ali *et al.*, 2006; Hirabayashi *et al.*, 2006), initiation

(Hirabayashi *et al.*, 2006; Kipper *et al.*, 2009), ensuring translational fidelity (O'Connor & Dahlberg, 1995; Liiv *et al.*, 2005; Hirabayashi *et al.*, 2006; O'Connor, 2007; Kipper *et al.*, 2009; O'Connor, 2009; Ortiz-Meoz & Green, 2011), elongation (Liiv *et al.*, 2005; Kipper *et al.*, 2009), translocation (Bashan *et al.*, 2003), termination (Ali *et al.*, 2006; O'Connor, 2009; Kipper *et al.*, 2011; Ortiz-Meoz & Green, 2011), and ribosome recycling (Agrawal *et al.*, 2004; Wilson *et al.*, 2005; Ali *et al.*, 2006). In addition, H69 has been implicated in ribosome biogenesis (Gutgsell *et al.*, 2005; Liiv *et al.*, 2005). Deleting H69 is lethal to *E. coli in vivo* but, unexpectedly, ribosomes lacking the entire H69 maintain the ability to translate natural mRNAs *in vitro* (Ali *et al.*, 2006).

A curious characteristic of the H69 is its high content of modified nucleosides (MN) (Ofengand & Bakin, 1997). *E. coli* H69 (Figure 4) contains three MN, two pseudouridines (Ψ) at positions 1911 and 1917, and a N3-methylated pseudouridine ($m^3\Psi$) at position 1915 (Bakin & Ofengand, 1993; Kowalak *et al.*, 1996). Ψ1911 is highly but not completely conserved, lacking in archaea and yeast, for example; Ψ1915 has been found in all organisms examined, except for archaeon *Sulfolobus acidocaldarius*; and Ψ1917 is believed to be universally conserved in cytoplasmic ribosomes (Ofengand, 2002), as well as proposed to be important for ribosome functioning (Hirabayashi *et al.*, 2006). Other bases besides Ψ are tolerated at positions 1911 and 1915 (Del Campo *et al.*, 2005; Hirabayashi *et al.*, 2006). Methylation of the N3 position of Ψ located in H69 and corresponding to position 1915 in *E. coli* 23S rRNA, has been described in bacterial and chloroplast ribosomes (Ofengand & Bakin, 1997; Del Campo *et al.*, 2005).

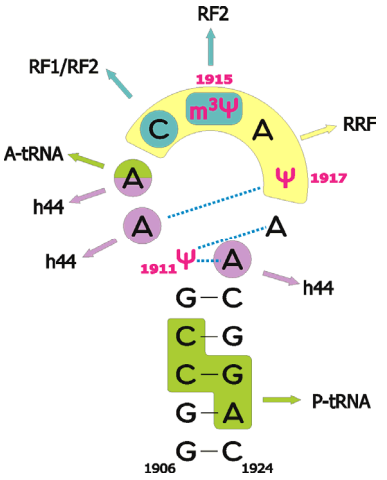

Figure 4. The sequence and secondary structure of the *E. coli* 23S rRNA stem-loop 69 (H69) showing key interactions within H69 itself, with 16S rRNA helix 44 (h44), A- and P-site tRNAs, and protein factors (RFs and RRF). Pseudouridine and N3-methyl-pseudouridine are marked with Ψ and $m^3\Psi$, respectively. Tertiary interactions within H69 are indicated by dotted blue lines. H69 contacts with A- and P-site tRNAs, h44, RFs, and RRF are shown by arrows.

Chow and co-workers have extensively studied the influence of the MN on the stability and the conformation of H69; and conclude that Ψs at positions 1915 and 1917 have a destabilizing effect on H69 structure; whereas Ψ at position 1911 has a stabilizing effect (Meroueh *et al.*, 2000). The N3-methylation of Ψ1915 causes a slight increase in the thermodynamic stability but does not cause any substantial changes in the overall structure of H69 (Chui *et al.*, 2002). Collectively, Ψs lead to increased base stacking in H69 (Desaulniers *et al.*, 2008). Furthermore, Ψ but not U at position 1917 can form the non-canonical reversed Hoogsteen base pair with A1912 (Figure 4) (Sakakibara & Chow, 2012) seen in the crystal structure of *E. coli* 70S ribosomes (Schuwirth *et al.*, 2005). The same might be true for the Ψ at position 1911 and its involvement in the non-canonical reversed Hoogsteen base pair with A1919 (Figure 4) (Sakakibara & Chow, 2012). Therefore, the presence of Ψs is clearly important for the formation and the stability of the tertiary structure of H69. The aforementioned reversed Hoogsteen base pair with A1912 formed by Ψ1917 but not by U1917, is important for interactions between ribosomal subunits (discussed below), implicating Ψ at 1917 as a modification involved in subunit association (Sakakibara & Chow, 2012). Accordingly, ribosomes lacking Ψs in H69 exhibit reduced subunit association *in vitro* (Gutgsell *et al.,* 2005).

H69 extends from the LSU and, upon translation initiation, contacts the tip of 16S rRNA helix 44 (h44) immediately adjacent to the DC of SSU, forming the central inter-subunit bridge, B2a (Mitchell *et al.*, 1992; Yusupov *et al.*, 2001; Hennelly *et al.*, 2005; Schuwirth *et al.*, 2005). Reflecting the high conservation of H69, bridge B2a is present in ribosomes from all three domains of life as well as in the organelle ribosomes (Mears *et al.*, 2002). B2a is among the first inter-subunit bridges to form (Hennelly *et al.*, 2005) and essential for 70S formation (Maiväli & Remme, 2004). Also, B2a plays an important role in maintaining the subunit association as the SSU rotates relative to the LSU during translocation (Frank & Agrawal, 2000; Dunkle *et al.*, 2011).

The orientation of H69 within the 70S ribosome (Yusupov *et al.*, 2001; Schuwirth *et al.*, 2005; Selmer *et al.*, 2006; Zhang *et al.*, 2009) differs from the one seen in the free LSU (Harms *et al.*, 2001). Namely, in the 70S ribosomes, the tip of H69 stretches about 13 Å towards the SSU, whereas in the free LSU, it makes more contacts with the LSU itself. The other notable difference between the H69 structures of LSU and 70S is the positioning of A1913. While A1913 is in a stacked conformation within the loop in the free LSU, it is in a flipped-out conformation in the 70S ribosomes.

In the atomic resolution structure of the *E. coli* vacant 70S ribosomes (Schuwirth *et al.*, 2005), several interactions between the LSU H69 and the SSU h44 can be seen (Figure 4). The widened reversed Hoogsteen base pair between Ψ1911 and A1919, bridged by the 2'-hydroxyl of A1918, allows A1918 and A1919 to form an A-A dinucleotide platform which projects A1919 into the minor groove of h44 near the bases U1406 and U1495, where it also interacts with the base of G1517. N1 position of A1919 directly interacts with the 2'-OH position of U1495 (Figure 4). A1912, which stacks on A1918 and forms a

distorted reversed Hoogsteen base pair with Ψ1917, projects into the minor groove of the base pair C1407 and G1494 in h44 of 16S rRNA (Figure 4). The involvement of H69 positions A1912 and A1918 in the formation of the bridge B2a is in agreement with the interference of 70S formation *in vitro* caused by chemical modifications at the aforementioned positions (Maiväli & Remme, 2004). Also, single base substitutions of A1912 and A1919 have strong negative effects on growth rate of *E. coli* and cell-free translation (Liiv *et al.*, 2005; Kipper *et al.*, 2009). Furthermore, using systematic genetic selection of functional sequences in the loop of H69, A1912 was determined to be essential (Hirabayashi *et al.*, 2006). These findings are in good agreement with the central role of A1912, A1918, and A1919 in tertiary interactions within H69 and with SSU. Noller and coworkers showed that disrupting the bridge B2a by discarding H69 altogether is sufficient to prevent subunit association in the absence of tRNA (Ali *et al.*, 2006).

Compared to the vacant 70S ribosome structure discussed above, in the *Thermus thermophilus* (*T. thermophilus*) 70S ribosomes complexed with mRNA and tRNAs in the classical sites (Selmer *et al.*, 2006), the entire H69 is shifted slightly toward the SSU. A1913 is seen to protrude into a pocket between the h44 of SSU and the A-site tRNA, instead of being inserted into the minor groove of h44, and to form a hydrogen bond (H-bond) between its N1 and the 2'-hydroxyl of ribose 37 of the A-site tRNA anticodon stem-loop (Figure 4) (Selmer *et al.*, 2006). The base of A1913 is oriented toward the 16S rRNA bases A1492 and A1493 that flip out during the decoding to interact with tRNA and mRNA (Ogle *et al.*, 2001). Structures of the ribosome in the intermediated states of ratcheting reveal that A1913 of H69 and A1493 of h44 adopt different conformations depending on the tRNA occupancy in the A-site (Zhang *et al.*, 2009). Also, the H69 itself undergoes at least a 7 Å movement during ratcheting (Valle *et al.*, 2003; Dunkle *et al.*, 2011). Ribosome structures with A-site tRNA in pre- and post-accommodation states show the tip of H69 (positions 1913-1915) contacting A-site tRNA D-stem (Schmeing *et al.*, 2009; Schuette *et al.*, 2009). The stem region of H69 (positions 1908-1909 and 1922–1923) makes minor groove interactions with the D-stem of P-site tRNA (Figure 4) (Korostelev *et al.*, 2006; Selmer *et al.*, 2006). A1913 has been proposed to monitor the incoming aminoacyl tRNAs as it interacts slightly differently with cognate and near-cognate tRNAs (Selmer *et al.*, 2006; Jenner *et al.*, 2010). So, A1913 of H69 likely participates directly in the decoding process and the conformational change in the H69 upon A-site tRNA binding may offer a way to signal the LSU GTPase center before tRNA accommodation (Selmer *et al.*, 2006; Jenner *et al.*, 2010). Indeed, several groups have proposed that H69 forms a part of the signal transmission pathway between the DC of SSU and the PTC of LSU (Rodnina *et al.*, 2002; Bashan *et al.*, 2003; Cochella & Green, 2005; Frank *et al.*, 2005; Ortiz-Meoz & Green, 2011).

Cryo-electron microscopy and X-ray crystallographic studies show that H69 contacts various translation factors: elongation factor G (EF-G), release factors (RFs), ribosome recycling factor (RRF), and ribosome modulation factor (RMF)

(Agrawal *et al.*, 2000; Klaholz *et al.*, 2004; Yoshida *et al.*, 2004; Weixlbaumer *et al.*, 2007; Korostelev *et al.*, 2008; Laurberg *et al.*, 2008; Pai *et al.*, 2008; Weixlbaumer *et al.*, 2008; Korostelev *et al.*, 2010). Also, hydroxyl radical footprinting studies indicate that H69 contacts regions of SSU that overlap with the binding site of the translation initiation factor 3 (IF3) (Dallas & Noller, 2001) suggesting that H69 may be competing with IF3 for binding to the SSU during the translation initiation. The involvement of H69 in translation termination and ribosome recycling has been studied in more detail.

The termination of protein synthesis occurs through a specific recognition of a stop codon in the A-site of the ribosome by a release factor (RF), which then triggers the hydrolysis of the nascent protein chain from the P-site tRNA (Capecchi, 1967). In bacteria, there are two RFs (RF1 and RF2) with overlapping specificity (Scolnick *et al.*, 1968). In the co-crystal structures of *T. thermophilus* 70S ribosomes with RF1 and RF2, H69 is seen to be located between the domains II and III of RFs (Laurberg *et al.*, 2008; Weixlbaumer *et al.*, 2008; Korostelev *et al.*, 2010). The tip of H69, more specifically the 2'-hydroxyl of C1914 and the backbone phosphates of $m^3\Psi$1915 (Figure 4), contact the switch loop of RFs during stop codon recognition (Laurberg *et al.*, 2008; Weixlbaumer *et al.*, 2008). A1913 projects into the minor groove of h44 and base stacks with A1493; this interaction is believed to prevent the extrusion of A1493 involved in decoding and to promote RF binding to the ribosome. The interaction of A1913 with A1493 has been shown to induce a conformational change in RFs, which probably leads to the hydrolysis of the peptidyl-tRNA linkage (Laurberg *et al.*, 2008; Weixlbaumer *et al.*, 2008). Consistent with the structural data, mutations in H69 or the lack of H69 altogether, strongly impair the RF-mediated peptide release (Ali *et al.*, 2006; Korostelev *et al.*, 2010). Interactions around Ψ1917 position appear to be most important for a conformation of H69 that can properly interact with RFs (Kipper *et al.*, 2011), which is in not surprising given the universal conservation of Ψ1917 and its involvement in tertiary interactions within H69 (discussed above). Interestingly, H69 Ψs appear to affect the RF1- and RF2-dependent peptide release differently, stimulating RF2 exclusively (Kipper *et al.*, 2011). Also, the stem of H69 appears to be critical specifically for the recognition of UGA stop codons by RF2 (Ortiz-Meoz & Green, 2011). These findings underline the differences between the RF1- and RF2-dependent stop codon recognition and peptide release.

Pedersen and co-workers (Ejby *et al.*, 2007) showed that the mutant phenotype of *E. coli* associated with the lack of H69 Ψs, namely the slow growth rates *in vivo* and the reduced subunit association *in vitro* (Raychaudhuri et al., 1998), can be rescued by a single point mutation (glutamic acid to lysine substitution at the position 172) in RF2 protein at a site close to H69 in co-crystal structure (Weixlbaumer *et al.*, 2008). Based on this and the finding that the lack of Ψs in H69 causes a defect in RF2-mediated peptide release (Kipper *et al.*, 2011), it seems that at least one of the Ψs in H69 is involved in translation termination by RF2, and that most if not all of the defects seen in the strain lacking Ψs in H69 are mostly due to errors in translation termination (Ejby *et al.*, 2007). However,

a recent study by O'Connor and Gregory demonstrates that the slow growth, impaired subunit association, and increased stop codon read-through phenotypes associated with the lack of H69 Ψs is limited to the *E. coli* K-12 strain already carrying a mutant RF2 protein and does not occur with other *E. coli* strains even when they do lack Ψs in H69 (O'Connor & Gregory, 2011). RF2 in K-12 strain contains a threonine instead of an alanine at position 246 rendering RF2 less active in peptide release (Mora *et al.*, 2007). Also, the SSU r-protein S7 is different in K-12 strain (Schaub & Hayes, 2011). The less efficient RF2-dependent translation termination in K-12 strain due to variant RF2 and S7 proteins is apparently tolerated as long as the cell still possesses all three Ψs in H69. However, when Ψs are lacking from H69, the accumulation of otherwise moderate termination defects apparently passes a threshold and leads to defects in downstream processes that result in significantly reduced cellular viability. In conclusion, although the role of Ψs in translation termination is still enigmatic, H69 itself is indispensable for efficient termination by RFs.

At the end of translation, ribosome recycling factor (RRF) together with elongation factor G (EF-G) disassembles the ribosome post-termination complex into free subunits, mRNA and tRNA, so they can be used in the next round of translation (Hirokawa *et al.*, 2005). Co-crystal structure with 70S ribosomes shows *E. coli* RRF contacting H69 loop nucleotides C1914-Ψ1917 (Pai *et al.*, 2008). Upon RRF binding, the tip of H69 moves away from the SSU by about 8 Å, thereby disrupting the bridge B2a and dissociating the subunits (Pai *et al.*, 2008). Accordingly, the recycling of subunits is affected by the binding of aminoglycoside antibiotics to the H69 thereby preventing the movement of H69 toward RRF (Borovinskaya *et al.*, 2007). Noller and co-workers showed that H69 deletion results in the loss of requirement for RRF in ribosome recycling (Ali *et al.*, 2006). Taken together, these findings implicate H69 in ribosome recycling.

2. RIBOSOME BIOGENESIS IN BACTERIA

In accordance with the vast and intricate molecular architecture, the biogenesis of the ribosome is a highly complex and energy intensive process. All of its components must be transcribed (and r-proteins also translated), processed, modified, folded, and assembled efficiently and accurately into functional ribosomes (Kaczanowska & Ryden-Aulin, 2007; Connolly & Culver, 2009; Shajani *et al.*, 2011).

The rRNA genes are organized into operons (Deutscher, 2009). In *E. coli*, seven rRNA operons are present, all of which have a similar overall structure and nearly identical sequences of the rRNA genes (Nomura & Morgan, 1977; Condon *et al.*, 1995; Kaczanowska & Ryden-Aulin, 2007; Deutscher, 2009). Each rRNA operon is transcribed as a primary transcript containing 16S, 23S, and 5S rRNA sequences in that order together with leader, spacer, trailer, and 1-3 tRNA sequences (Ginsburg & Steitz, 1975; Hayes *et al.*, 1975). The organization of the 16S, 23S, and 5S genes into one operon ensures the production of equimolar amounts of the three rRNA species. Depending on the operon, one or several tRNAs are also part of the primary transcript, located between the 16S and 23S sequences and downstream of the 5S rRNA (Srivastava & Schlessinger, 1990; Kaczanowska & Ryden-Aulin, 2007; Deutscher, 2009). Most of the genes of the r-proteins are also organized into operons (Nomura & Morgan, 1977).

Ribosome assembly, rRNA nucleolytic processing and chemical modification of ribosomal components will be discussed in the following chapters in more detail. However, it should be kept in mind that the various processes of ribosome biogenesis are intimately intertwined and interdependent. Meaning that essentially all of the rRNA maturation reactions occur in the context of the assembling ribosome.

2.1 Assembly

Ribosome assembly is the process of r-protein binding to rRNA molecules leading to conformational changes and to the emergence of ribosome precursor particles of increasing size and compactness.

All of the information required to assemble a functional ribosome is encoded in the sequence of the rRNAs and r-proteins. This is demonstrated most elegantly by the reconstitution of functionally active ribosome subunits *in vitro* from purified rRNA and r-proteins, albeit using conditions far from physiological ones, namely high magnesium ion concentrations and long incubations at elevated temperatures (Traub & Nomura, 1968; Held *et al.*, 1973; Nierhaus & Dohme, 1974; Dohme & Nierhaus, 1976). The extensive *in vitro* reconstitution experiments done mostly in the 70's and 80's by Nomura's group on SSU, and Nierhaus and co-worker on LSU, have provided the corresponding assembly maps that illustrate the hierarchical and cooperative nature of the r-protein binding with rRNAs (Mizushima & Nomura, 1970; Held *et al.*, 1974; Rohl &

Nierhaus, 1982; Herold & Nierhaus, 1987). While the assembly maps illustrate the interdependence of r-proteins for their incorporation into the ribosomal particles, they do not necessarily reflect the temporal sequence of the individual assembly steps, or the physical proximity of r-proteins in the subunits.

Using pulse-chase coupled with quantitative mass spectrometry, Williamson and co-workers determined the binding kinetics of the individual S-proteins during the SSU *in vitro* reconstitution (Talkington *et al.*, 2005). The binding parameters of S-proteins suggest that the SSU assembly proceeds via numerous local transitions, an assembly landscape, rather than through a global rate-limiting step and a small number of discrete intermediates. A few years later, by mapping the changes in the structure of the 16S rRNA after the addition of S-proteins using time-resolved hydroxyl radical footprinting, Woodson and co-workers demonstrated that the early SSU assembly *in vitro* is not strictly cooperative, nucleating simultaneously from different points along the 16S rRNA and yielding many early assembly intermediates which is in good agreement with the assembly landscape model (Adilakshmi *et al.*, 2008). They also showed that the 16S rRNA nucleotides interacting with the same S-protein are protected at different rates, indicating that RNA-protein interactions are reorganized during the assembly (Adilakshmi *et al.*, 2008). More recently, using a high-throughput strategy for capturing electron microscopy images, Williamson and co-workers succeeded to visualize the heterogeneous population of the assembly intermediates arising from SSU reconstitution *in vitro* (Mulder *et al.*, 2010), further illustrating that assembly can proceed through alternative pathways.

Studying the ribosome assembly *in vivo* has proven to be a rather complicated task. First off, assembly *in vivo* occurs within a couple of minutes (Lindahl, 1975), which is a significantly shorter time than is required for the *in vitro* reconstitution. Assembly intermediate particles are not abundant under normal growth conditions (Lindahl, 1975), but can accumulate in response to deletions or mutations in certain genes and in the presence of antibiotics. In a few cases, r-protein content and abundance of the *in vivo* assembly intermediates have been measured (Charollais *et al.*, 2003; Sharpe Elles *et al.*, 2009; Sykes *et al.*, 2010). While many similarities between the r-protein content of the *in vitro* reconstitution and *in vivo* assembly intermediates exist, there also seem to be a fair amount of discrepancies (Shajani *et al.*, 2011). The existence of multiple assembly pathways have also been implied from the *in vivo* studies (Bubunenko *et al.*, 2006; Sykes *et al.*, 2010). Supposedly allowing ribosome biogenesis to bypass steps blocked by mutations or deficiencies of the essential assembly proteins. *E. coli* can survive without the protein S15 (Bubunenko *et al.*, 2006) or L24 (Franceschi & Nierhaus, 1988), central players according to the SSU (Mizushima & Nomura, 1970; Held *et al.*, 1974) and LSU (Rohl & Nierhaus, 1982; Herold & Nierhaus, 1987) assembly maps, respectively. Apparently, *in vivo* assembly displays high plasticity, as might be expected from the assembly landscape model derived from *in vitro* experiments (Talkington *et al.*, 2005). The main difference between *in vitro* reconstitution of ribosomal subunits and

in vivo assembly is that the latter is coupled to the transcription of the primary rRNA transcript (Lewicki *et al.*, 1993).

The overall picture of ribosome assembly emerging from both *in vitro* and *in vivo* studies is the following. The SSU and the LSU rRNAs are synthesized as one primary transcript (Srivastava & Schlessinger, 1990) and the ribosome assembly is initiated before the transcription is completed (Lewicki *et al.*, 1993; Besancon & Wagner, 1999). While the primary rRNA transcript is still being synthesized, local secondary structure motifs start to form, creating binding sites for r-proteins. Unlike DNA-binding proteins, r-proteins bind to their substrate rRNAs by recognizing higher-order structural features rather than the primary sequence and most interactions are formed with the sugar-phosphate backbone (Brodersen *et al.*, 2002; Klein *et al.*, 2004). Each r-protein not only stabilizes the rRNA within its immediate binding site, it also stimulates structural changes in adjacent regions of rRNA which help to recruit other r-proteins to the complex (Shajani *et al.*, 2011; Woodson, 2011). Therefore, r-proteins appear to stage the order of rRNA folding during the ribosome assembly. Nearly one-third of *E. coli* L-proteins display RNA chaperone activity (Semrad *et al.*, 2004; Wilson & Nierhaus, 2005) which is likely necessary to avoid kinetic traps leading to improperly folded ribosomes. Especially the N-terminal extensions of r-proteins are believed to participate in ribosome assembly (Klein *et al.*, 2004; Guillier *et al.*, 2005).

In line with the coupling to transcription, assembly appears to proceeds in the 5'–3' direction (Zimmermann *et al.*, 1972; de Narvaez & Schaup, 1979; Powers *et al.*, 1993; Talkington *et al.*, 2005; Adilakshmi *et al.*, 2008; Bunner *et al.*, 2010; Mulder *et al.*, 2010). However, the ordered transcription of rRNA domains is not obligatory as *E. coli* strains with circularly permuted 16S and 23S genes are viable (Kitahara & Suzuki, 2009). Contrary to the earlier views arising from the assembly maps, it now seems that the assembly of the ribosomal subunits is not restricted to a single rate-limiting step or pathway but rather proceeds through multiple pathways with similar activation barriers (Bubunenko *et al.*, 2006; Shajani *et al.*, 2011; Woodson, 2011). The multiple parallel assembly pathways generate a wide variety of intermediate particles that have distinct r-protein compositions (Sykes *et al.*, 2010).

Besides being coupled to the rRNA transcription, the *in vivo* assembly is intertwined with nucleolytic processing and modification of rRNA and r-proteins, and aided by numerous assembly factors (Kaczanowska & Ryden-Aulin, 2007; Connolly & Culver, 2009; Shajani *et al.*, 2011).

2.2 rRNA processing

rRNA processing is the set of enzymatic nucleolytic events needed to convert the precursor rRNA (pre-rRNA) molecule into SSU and LSU rRNAs with 3' and 5' ends found in native ribosomes. As already mentioned, the SSU and the LSU rRNAs are transcribed together in a single transcript, the pre-rRNA

(Kaczanowska & Ryden-Aulin, 2007; Deutscher, 2009; Shajani *et al.*, 2011). Sequences flanking both the 16S and 23S rRNAs form double helical structures that are recognized and cleaved by ribonuclease III (RNase III) before the transcription of the pre-rRNA is completed, consequently, the complete pre-rRNA transcript is not present in *E. coli* wild-type (WT) cells (Robertson *et al.*, 1968; Dunn & Studier, 1973; Nikolaev *et al.*, 1974; Gegenheimer & Apirion, 1975; Ginsburg & Steitz, 1975; Gegenheimer *et al.*, 1977; Young & Steitz, 1978; Bram *et al.*, 1980). RNase III cleavages of the pre-rRNA result in the release of pre-16S and pre-23S rRNA molecules together with the 3' terminal part of the transcript, that contains the 5S rRNA sequence and additional sequences for one or two tRNAs (Deutscher, 2009). Although not directly dependent on r-protein binding (Birenbaum *et al.*, 1978), the sequence specificity of the RNase III cleavage reaction changes in their presence (Allas *et al.*, 2003). Somewhat surprisingly, RNase III cleavages are not absolutely essential processing steps since *E. coli* strains lacking this endonuclease are still viable, albeit being unable to complete the processing of 23S rRNA (King *et al.*, 1984; King *et al.*, 1986; Srivastava & Schlessinger, 1990).

The pre-16S rRNA contains 115 and 33 additional nt (nucleotides) in its 5' and 3' ends, respectively, whereas the pre-23S rRNA has 3 or 7 additional nt in its 5' and 7 to 9 additional nt in its 3' ends (Srivastava & Schlessinger, 1990; Deutscher, 2009). Final processing of the 5' end of the 16S rRNA requires the combined action of two endonucleases, RNase E and RNase G (Li *et al.*, 1999b). While the processing of the 3' end of the 16S rRNA has not yet been elucidated, it most likely consist of a single endonucleolytic cleavage event (Deutscher, 2009). The exonuclease RNase T is mainly responsible for the final processing of the 23S rRNA 3' end (Li *et al.*, 1999a). The RNase responsible for the 5' end processing of the 23S rRNA has not been identified yet but its action is likely endonucleolytic and independent of the 23S rRNA 3' end processing (Deutscher, 2009). The pre-5S rRNA is released from the 3' terminal fragment of the pre-rRNA transcript by RNase E (Misra & Apirion, 1979; Roy *et al.*, 1983). The additional 3 nt at each end of the pre-5S rRNA are removed by RNase T and by a still unknown RNase (Feunteun *et al.*, 1972; Li & Deutscher, 1995). As is the case with the 23S rRNA, the processing of the 5S rRNA 5' and 3' ends is likely independent of each other (Deutscher, 2009).

SSU containing pre-16S rRNA is inactive in translation (Lindahl, 1973; Wireman & Sypherd, 1974; Lindahl, 1975) indicating that the final processing of 16S rRNA has to occur before the association of SSU with the LSU during translation initiation. In mature SSU the 5' and 3' ends of the 16S rRNA are far apart (Schluenzen *et al.*, 2000; Wimberly *et al.*, 2000) whereas in the pre-16S rRNA the additional nt at the ends are base paired, this additional secondary structure may either directly or indirectly prohibit LSU binding . Furthermore, the 3' end of the 16S rRNA contains the anti-Shine-Dalgarno sequence, blocking its interaction with the Shine-Dalgarno sequence in mRNAs could also inhibit translation initiation. Hence, the final processing of the 16S rRNA ends triggers the activation of SSU. Since the 16S rRNA processing is coupled to

SSU assembly, the final processing steps guarantee that only correctly assembled SSU can associate with LSU and initiate translation (Deutscher, 2009). Final processing of the LSU rRNAs is not required for ribosome activity and appears to take place after the 70S ribosomes are formed, probably during the translation initiation complex formation, or during the first cycles of translation (Kaczanowska & Ryden-Aulin, 2007; Deutscher, 2009). This is based on the observations that the RNase T dependent processing of the pre-23S and pre-5S is likely a very late event in ribosome biogenesis (Li & Deutscher, 1995; Li *et al.*, 1999a), pre-23S and pre-5S rRNAs can be found in polysomes (Ceccarelli *et al.*, 1978; Sirdeshmukh & Schlessinger, 1985; Srivastava & Schlessinger, 1988, 1990), and that the final processing of the 5S rRNA ends is inhibited by protein synthesis inhibiting antibiotic chloramphenicol (Deutscher, 2009).

Thus, as already mentioned, the rRNA maturation events generally occur in the context of the assembling ribosome (King *et al.*, 1986; Deutscher, 2009). The close connection between rRNA processing and ribosome assembly is supported by the isolation of assembly precursor particles containing rRNAs with immature ends from numerous *E. coli* assembly deficient strains (Charollais *et al.*, 2003; Charollais *et al.*, 2004; Kaczanowska & Ryden-Aulin, 2007; Peil *et al.*, 2008).

2.3 rRNA modification

In all organisms, specific sets of standard ribonucleotides in rRNA are covalently modified during ribosome biogenesis. The phylogenetic conservation of the rRNA modifications indicates that they emerged early in the evolution of the translational machinery. Pseudouridylations (Ψs) and various methylations represent the two major types of rRNA modifications and there is a correlation between the overall complexity of an organism and the number of modified nucleosides (MN) in its rRNAs (Maden, 1990; Decatur & Fournier, 2002; Czerwoniec *et al.*, 2009; Cantara *et al.*, 2011). There are 36 naturally occurring rRNA MN in *E. coli*: 16S rRNA contains 11 MN, 10 methylations and one Ψ; 23S rRNA contains 25 MN, 13 methylations, 9 Ψs, one methylated Ψ ($m^3\Psi$), one dihydrouridine (D), and one 5-hydroxycytidine (ho^5C) (Table 1).

Table 1. Modified nucleosides in *E. coli* rRNAs[1]

Position	Modification[2]	Enzyme[3]	Alternative name(s)	*In vitro* substrate[5]	*In vivo* assembly stage[7]
16S rRNA					
516	Ψ	RsuA	YejD	pre-SSU	early, intermediate
527	m^7G	RsmG	GidB	SSU	intermediate
966	m^2G	RsmD	YhhF	SSU	late
967	m^5C	RsmB	YhdB, Fmu, RrmB	16S rRNA	early
1207	m^2G	RsmC	YjjT	SSU	late
1402	m^4Cm	RsmH / RsmI	MraW / YraL	SSU	stochastic
1407	m^5C	RsmF	YebU	SSU	late
1498	m^3U	RsmE	YggJ	SSU	late
1516	m^2G	RsmJ	YhiQ	SSU	late
1518	$m^6{}_2A$	RsmA	KsgA	SSU	late
1519	$m^6{}_2A$	RsmA	KsgA	SSU	late
23S rRNA					
745	m^1G	RlmA	RrmA, YebH	23S rRNA	early
746	Ψ	RluA	YabO	23S rRNA	early
747	m^5U	RlmC	RumB, YbjF		early
955	Ψ	RluC	YceC	23S rRNA	early
1618	m^6A	RlmF	YbiN	pre-LSU	early, intermediate
1835	m^2G	RlmG	YgjO	23S rRNA	early
1911	Ψ	RluD	YfiI, SfhB	LSU	late
1915	$m^3Ψ$	RluD / RlmH[4]	YfiI, SfhB / YbeA[4]	LSU / 70S[6]	late / very late[6]

Position	Modification[2]	Enzyme[3]	Alternative name(s)	*In vitro* substrate[5]	*In vivo* assembly stage[7]
1917	Ψ	RluD	YfiI, SfhB	LSU	late
1939	m^5U	RlmD	RumA, YgcA	23S rRNA	intermediate
1962	m^5C	RlmI	YccW	23S rRNA	early
2030	m^6A	RlmJ	YhiR	23S rRNA	early
2069	m^7G	RlmKL	YcbY		early, intermediate
2251	Gm	RlmB	YjfH		intermediate
2445	m^2G	RlmKL	YcbY	23S rRNA	early
2449	D	RldA			
2457	Ψ	RluE	YmfC	23S rRNA	early
2498	Cm	RlmM	YgdE	23S rRNA	intermediate
2501	ho^5C	RltA			
2503	m^2A	RlmN	YfgB		early
2504	Ψ	RluC	YceC	23S rRNA	early
2552	Um	RlmE	RrmJ, FtsJ, MrsF	LSU, 70S	late
2580	Ψ	RluC	YceC	23S rRNA	early
2604	Ψ	RluF	YjbC	23S rRNA, LSU	early
2605	Ψ	RluB	YciL	23S rRNA	early

[1]Data taken from the RNA Modification Database (Cantara *et al.*, 2011), Modomics – A Database of RNA Modifications (Czerwoniec *et al.*, 2009), and 3D Ribosomal Modification Maps Database (Piekna-Przybylska *et al.*, 2008), unless otherwise indicated.
[2] m^x_yN refers to a methylation (m) of the rRNA nucleotide N at the x of the base position (y is the number of methylations), whereas Nm indicates a methylation of the ribose at the 2' position of nucleotide N. Ψ, D, and ho^5C are pseudouridine, dihydrouridine, and 5-hydroxycytidine, respectively.
[3] According to unified nomenclature (Ofengand & Del Campo, 2004a; Andersen & Douthwaite, 2006). Modification enzymes whose genes have not been identified yet are in *Italic*.
[4] Ref. I
[5] Reviewed in (Ofengand & Del Campo, 2004a) and (Siibak & Remme, 2010).
[6] Ref. II
[7] The *in vivo* assembly stage of the modification synthesis according to (Siibak & Remme, 2010).

In both prokaryotes and eukaryotes, mapping all of the methylations and Ψs onto the three-dimensional structures of the SSU and the LSU reveals that they

concentrate around the active sites of the ribosome, namely, the mRNA and tRNA binding sites on the SSU, the PTC and the entrance of the polypeptide exit tunnel on the LSU, and the sites of subunit-subunit interactions (Figure 5) (Bakin & Ofengand, 1993; Brimacombe *et al.*, 1993; Ofengand *et al.*, 2001b; Decatur & Fournier, 2002; Ofengand & Del Campo, 2004a). This is clearly illustrated by the three conserved MN present in the strategically located H69 of the LSU (chapter 1.1). Based on the clustering around the functionally important regions of the ribosome, MN are believed to be important for ribosome biogenesis, ensuring the stability of the ribosome, and for various aspects of ribosome functioning (discussed in chapter 3). Again, this is illustrated well by the H69 MN as discussed in chapter 1.1 and chapter 3.4.

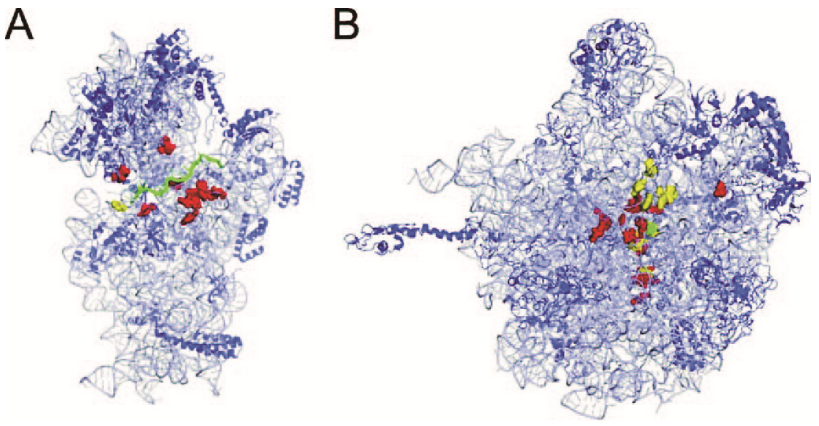

Figure 5. Distribution of modified nucleosides in the bacterial ribosome. Sites of methylation (depicted in red) and pseudouridylation (depicted in yellow) are shown on the *E. coli* SSU (A) and LSU (B). rRNA and r-proteins are shown as ribbons in light and dark blue, respectively. In (A) a green ribbon indicates the path of mRNA through the SSU, whereas in (B) the antibiotic chloramphenicol (green) bound to PTC of the LSU is shown. Adapted from (Wilson & Nierhaus, 2007).

In bacteria, each rRNA MN is made by a specific modification enzyme (ME) – a protein that contains both the catalytic site for a particular modification reaction and the specificity center for a given rRNA substrate. Synthesis of the more complex MN such as m^4Cm and $m^3\Psi$ requires multiple ME and some ME such as RsmA (most familiarly known as KsgA), RluD and RluC are responsible for synthesizing the same MN at more than one position in rRNA (Table 1). Interestingly, in case of the *E. coli* RlmKL protein, the m^7G2069 is first introduced to the 23S rRNA by its RlmK domain, followed by the m^2G2445 synthesis by its RlmL domain (Wang *et al.*, 2012). However, phylogenetic analysis suggests that separated RlmK and RlmL methyltransferases are found in other bacteria (Wang *et al.*, 2012). In contrast, eukaryotes and archaea use a small nucleolar RNA (snoRNA) guided rRNA modification mechanism that allows the use of a limited number of modification (Ψ and ribose 2'-O-methylation)

specific proteins to introduce the majority of the rRNA MN (Kiss-Laszlo *et al.*, 1996; Bachellerie & Cavaille, 1997; Ni *et al.*, 1997; Tollervey & Kiss, 1997; Kiss, 2001; Decatur & Fournier, 2002). The emergence of alternative mechanisms for creating the same type of modifications further underlines their importance.

All of the rRNA pseudouridine synthases (PS) and methyltransferase (MT) have been identified in *E. coli* (Table 1). For the most part, the rRNA ME have been identified by the corresponding gene deletion/complementation analyses and have not been characterized in great detail. However, *in vitro* experiments using purified ME have determined that some MN can be synthesized using the protein-free rRNAs or even rRNA fragments as substrates, while the synthesis of other MN requires the presence of at least some if not all of the r-proteins (Table 1) (Ofengand & Del Campo, 2004a; Siibak & Remme, 2010). It is possible that the ME that require the presence of r-protein(s) actually require a certain rRNA structure that only forms upon the binding of certain r-protein(s). The shortcoming of most of the ME *in vitro* assays is the limited set of substrates analyzed, usually just the naked rRNA *versus* the mature subunits isolated from the ME deletion strains were tested. Furthermore, in many cases the reported activities of the ME were quite low (Hager *et al.*, 2004; Basturea & Deutscher, 2007). It is credible that the true substrates of the ME are in fact the ribosome subunit assembly intermediate particles that have proven to be difficult to test *in vitro*. Also, it is possible that additional proteins such as ribosome assembly factors may facilitate the rRNA modification process in cells. Nonetheless, in general, the substrate specificities of ME determined *in vitro* are in very good agreement with the assembly dependence of the rRNA modification synthesis *in vivo,* as determined by the rRNA modification pattern of ribosome subunits at different assembly stages. Accordingly, ME were divided into three classes: early, intermediate, and late assembly specific enzymes (Table 1) (Siibak & Remme, 2010). Consequently, the modification of the 16S rRNA during the assembly of SSU seems to be mainly a late assembly event, whereas the modification of the 23S rRNA during the LSU assembly seems to be mainly an early event. The explanation for this tendency could be that the SSU is smaller than the LSU and, in the latter case, many substrate nucleotides in rRNA cannot be accessed by the ME later on in the LSU assembly pathway. That in turn would mean that most of the 23S rRNA ME only have a limited time window to modify their targets. All in all, it is believed that the MN are added gradually throughout the assembly of both subunits and that the main criteria for the temporal order of the MN synthesis, is the physical accessibility of the substrate. While the r-proteins can help to create the recognition sites for the ME, they can also inhibit the rRNA modification by blocking the modification site. The dual role of the r-proteins in rRNA modification is illustrated by S7 and S19, which are necessary for RsmD activity but inhibit RsmB activity (Weitzmann *et al.*, 1991). Indisputably, the rRNA modification process is intimately linked to the progression of the ribosome assembly and defects in the assembly process can likely lead to undermodification of rRNAs.

Although the ME are present in low abundance in the cell, the entire process of rRNA modification is astonishingly efficient in spite of the short time window during ribosome biogenesis (Winkler, 1998). This indicates that the ME process their substrates rapidly and/or channel the substrate through multi-enzymatic complexes. The latter may be more relevant for those MN whose synthesis involves several enzymatic events. However, not much is known about the engagement of ME in multi-enzymatic complexes.

All bacterial rRNA ME must be able to specifically recognize their substrate(s) and to catalyze their respective modification reactions, two processes inextricably linked. Energy derived from the binding to the rRNA likely fuels both the recognition or discrimination of substrates and the catalysis of the modification reaction (Garcia & Goodenough-Lashua, 1998). Most rRNA ME have a modular structure made up of RNA binding domain(s) and a catalytic core domain (Byrne *et al.,* 2009). The RNA binding domains are used to target certain rRNA regions through sequence and/or structure specificity and bring them in the correct orientation and proximity to catalytic domains for modification reactions to occur. The overall structures, catalytic mechanisms, and substrate specificities of the two main types of rRNA ME, PS and MT, will be discussed in more detail.

2.3.1 Pseudouridine synthases

Pseudouridine (Ψ), also known as the „fifth nucleoside“, was identified over fifty years ago (Davis & Allen, 1957; Cohn, 1960) and is the most abundant MN found in a number of RNA species (tRNA, rRNA, tmRNA, snRNA, and snoRNA) in all three domains of life (Czerwoniec *et al.*, 2009; Cantara *et al.*, 2011). Ψs result from the enzymatic isomerization of genetically encoded uridines in RNA molecules and the enzymes responsible for catalyzing the isomerization reaction are called pseudouridine synthases (PS). *E. coli* RNA PS fall into five families, called RsuA, RluA, TruA, TruB, and TruD, after the first identified members (Gustafsson *et al.*, 1996; Koonin, 1996; Kaya & Ofengand, 2003; Ofengand & Del Campo, 2004a). Despite minimal sequence similarities, they all share a common core fold found exclusively in PS, as well as similar active site structures (Ferre-D'Amare, 2003; Kaya *et al.*, 2004; Ofengand & Del Campo, 2004a). Thus, all PS have probably evolved from a common ancestor (Mueller, 2002). The 11 Ψs in *E. coli* rRNAs are made by seven PS belonging to RluA and RsuA families (Table 2), two of the most closely related families of PS (Ofengand *et al.*, 2001b; Mueller, 2002; Ofengand, 2002; Ofengand & Del Campo, 2004a). The RsuA family contains RsuA, RluB, RluE, and RluF, each responsible for a single Ψ in either 16S rRNA or 23S rRNA; RluA, RluC, and RluD belong to the RluA family (Table 2). RluC and RluD each make three Ψs in the 23S rRNA, whereas RluA, in addition to introducing one Ψ to the 23S rRNA, also synthesizes Ψs in four tRNAs (Table 2) (Huang *et al.*, 1998; Ofengand, 2002).

Table 2. *E. coli* rRNA pseudouridine synthases[1].

PS family	PS	Substrate RNA and base	Catalytic aspartic acid	N-terminal domains	C-terminal domains
RsuA	RsuA	16S rRNA U516	102	S4-like	
	RluE	23S rRNA U2457	69	S4-like	
	RluF	23S rRNA U2604	107	S4-like	CT domain
	RluB	23S rRNA U2605	110	S4-like	CT domain
RluA	RluA	23S rRNA U746 and tRNA U32	64		
	RluC	23S rRNA U955, U2504, and U2580	144	S4-like	CT subdomain
	RluD	23S rRNA U1911, U1915, and U1917	139	S4-like	CT subdomain

[1] Data taken from the RNA Modification Database (Cantara *et al.*, 2011) and Modomics – A Database of RNA Modifications (Czerwoniec *et al.*, 2009).

RsuA and RluA family PS share three conserved sequence motifs: I, II, and III (Koonin, 1996; Ofengand & Del Campo, 2004a). Motif II forms the active site loop and contains the only universally conserved amino acid residue, aspartic acid, among all PS (Koonin, 1996; Huang *et al.*, 1998; Conrad *et al.*, 1999; Raychaudhuri *et al.*, 1999; Del Campo *et al.*, 2001; Gutgsell *et al.*, 2001; Ofengand & Del Campo, 2004a). Site-directed mutagenesis experiments have established that the aforementioned aspartic acid residue is essential for the catalytic activity of all rRNA PS (Table 2) (Ramamurthy *et al.*, 1999a; Ferre-D'Amare, 2003; Ofengand & Del Campo, 2004a). Various RNA-binding domains are linked to the conserved catalytic core domain resulting in PS with different substrate specificities. Most of the rRNA PS have N-terminal domains that resembles the one found in r-protein S4 and several PS exhibit the presence of C-terminal (CT) (sub)domains (Table 2) (Aravind & Koonin, 1999; Ofengand & Del Campo, 2004b).

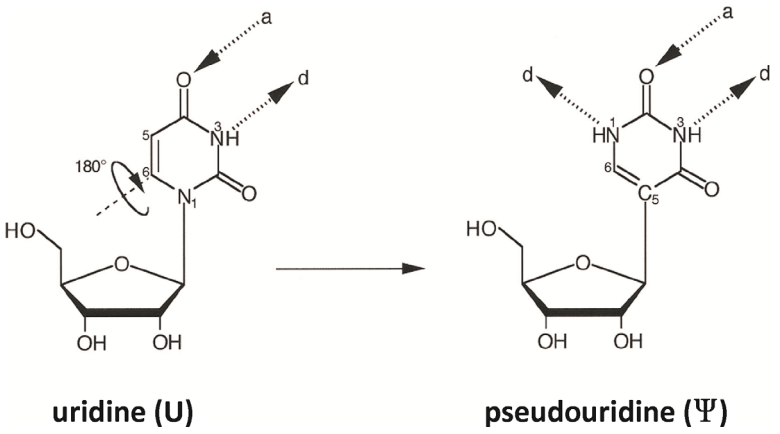

Figure 6. Pseudouridine synthesis and chemical differences between U and Ψ. The uracil base in U is linked through its N1 position to the C1' position of ribose and U possesses one H-bond acceptor (a) and one donor (d). Isomerization results in the uracil base being rotated 180° through the N3-C6 diagonal axis. In Ψ, the C5 position of uracil is linked to the C1' position of ribose and Ψ possesses one more H-bond donor at the N1 position compared to U. Illustration adapted from (Charette & Gray, 2000).

Ψ synthesis is energetically favored and does not require any cofactors (Lane *et al.*, 1995; Ferre-D'Amare, 2003; Hamma & Ferre-D'Amare, 2006). Given the high degree of structural conservation, it is likely that all PS share the same catalytic mechanism, involving the breakage of the standard C1'-N1 glycosyl bond between the uracil base and the ribose, rotation of the base by 180° through the N3-C6 axis with respect to the ribose while still enzyme-bound, and formation of the non-canonical C1'-C5 glycosyl bond (Figure 6). The exact chemical mechanism and the function of the conserved aspartic acid are still not conclusively established (Mueller & Ferre-D'Amare, 2009).

2.3.2 Methyltransferases

All *E. coli* rRNA MT use S-adenosyl-L-methionine (SAM) as the methyl group donor and belong to either class I or class IV of the five classes of SAM-dependent MT (Schubert *et al.*, 2003; Ofengand & Del Campo, 2004a). There is no significant sequence or structural conservation across all five MT classes that likely result from a convergent evolution (Schubert *et al.*, 2003). Class I MT (RFMT) that feature the ancient Rossmann-fold in their SAM binding domain, dominate among the rRNA MT (Table 3), as well as among all known SAM-dependent MT methylating a wide variety of substrates (DNA, RNA, proteins, and small molecules) (Schubert *et al.*, 2003). Much less conservation is noticed at the sequence level, where only a few conserved motifs are present, most of them being a part of the SAM binding pocket located in the N-terminus of the catalytic domain (Schubert *et al.*, 2003). Many RFMT have additional N- and

C-terminal domains (Table 3) presumably used for substrate recognition (Ofengand & Del Campo, 2004a).

The remainder of the known rRNA MT (called SPOUT MT based on their homology to the SpoU and TrmD MT) belong to the class IV (Table 3). The unique characteristic of the SPOUT MT is a structural core made up of six parallel β-strands, with the final three (~30 amino acids in length) folded into a very deep topological knot, known as the deep trefoil knot (Schubert *et al.*, 2003; Tkaczuk *et al.*, 2007). The trefoil knot structure contains catalytic residues confirming its structural importance (Michel *et al.*, 2002; Schubert *et al.*, 2003). The other intriguing feature of this group of MT is that the active site is located at the interface of the homodimer with both monomers making substantial contributions to both the active site and the SAM binding cleft (Schubert *et al.*, 2003; Tkaczuk *et al.*, 2007). Consequently, dimerization is believed to be essential for the MT activity, which is also supported by the finding that all of the SPOUT MT are dimers (Tkaczuk *et al.*, 2007). Yet, different SPOUT MT exhibit different modes of dimerization, in the dimers formed by RlmB and RsmE, the two monomers are oriented in a nearly perpendicular way, while in the case of RlmH, they are antiparallel (Tkaczuk *et al.*, 2007). The conserved SAM-binding loop is located in the C-terminal trefoil knot region of the catalytic domain (Tkaczuk *et al.*, 2007). Additionally, RlmB and RsmE have r-protein L30-like and PUA subdomains, respectively, linked to the N-terminus of the catalytic domains (Table 3) (Tkaczuk *et al.*, 2007). These N-terminal additions are believed to be required for substrate binding. Crystal structures in complex with co-factors show that the two classes of rRNA MT differ in the mode of SAM binding: the RFMT bind SAM in an extended conformation, whereas the SPOUT MT bind SAM in a bent conformation (Schubert *et al.*, 2003; Hou & Perona, 2010). Also, the ribose of SAM adopts the 2'-*endo* conformation in complex with RFMT and the 3'-*endo* conformation in complex with SPOUT MT (Schubert *et al.*, 2003).

Table 3. *E. coli* rRNA methyltransferases[1].

MT family	MT	Substrate RNA and base	Substrate atom[2]	N-terminal domain	C-terminal domain
RFMT	RsmA	A1518, A1519	exocyclic N6		α-helical
	RsmB	C967	exocyclic C5	NusB-like, N1 central	
	RsmC	G1207	exocyclic N2	RFD	
	RsmD	G966	exocyclic N2		
	RsmF	C1407	exocyclic C5		
	RsmG	G527	endocyclic N7		
	RsmH	C1402	exocyclic N4		

MT family	MT	Substrate RNA and base	Substrate atom[2]	N-terminal domain	C-terminal domain
	RsmI	C1402	2'O		
	RsmJ	G1516	exocyclic N2		
	RlmA	G745	endocyclic N1	Zn-finger	
	RlmC	U747	exocyclic C5		
	RlmD	U1939	exocyclic C5	OB and Fe_4S_4 domain	
	RlmE	U2552	2'O		
	RlmF	A1618	exocyclic N6		
	RlmG	G1835	exocyclic N2		
	RlmI	C1962	exocyclic C5		
	RlmKL	G2069, G2445	endocyclic N7 exocyclic N2	THUMP	
	RlmM	C2498	2'O		
	RlmN	A2503	endocyclic C2		
SPOUT	RsmE	U1498	endocyclic N3	PUA	
	RlmB	G2251	2'O	L30e-like	
	RlmH[3]	Ψ1915[4]	endocyclic N3		

[1]Data taken from the RNA Modification Database (Cantara *et al.*, 2011) and Modomics – A Database of RNA Modifications (Czerwoniec *et al.*, 2009).
[2] Carbon (C), nitrogen (N), and oxygen (O).
[3] Ref. I
[4] Ref. II

The driving force for the methyl transfer reaction is the electrophilic character of the methyl group attached to the positively charged sulfur atom of the SAM. The catalytic mechanisms proposed for the various rRNA MT share a common theme involving a nucleophilic attack on the methyl group of SAM by a negatively polarized methyl-acceptor atom of the substrate. However, the activation of the nucleophiles prior to the nucleophilic attack can be achieved in a variety of ways. The predominant difference between the various methylation reactions catalyzed by rRNA MT is the chemical nature of the atom being methylated (endo- and exocyclic carbon and nitrogen atoms, and ribose 2' oxygen atoms) (Table 3) (Schubert *et al.*, 2003). Thus, the main question is: how do the rRNA MT activate the diverse substrate atoms for the nucleophilic attack? In many cases, the atom being methylated is sufficiently nucleophilic that binding of both the substrate nucleotide and the co-factor SAM, and the correct orientation of the two in respect to each other probably suffices to catalyze the reaction

(Garcia & Goodenough-Lashua, 1998). In other cases, a general base in the MT is required to deprotonate the potential nucleophile (Schubert *et al.*, 2003). Methylation of the endocyclic carbon atom in case of 23S rRNA m^2A2503 by RlmN is chemically more challenging and requires the radical-SAM mechanism (Toh *et al.*, 2008; Yan *et al.*, 2010).

S-adenosylhomocysteine (SAH) is formed when the methyl group of SAM is transferred to an acceptor and SAH product inhibition is a general characteristic of the SAM dependent MT (Garcia & Goodenough-Lashua, 1998). It has been suggested that the activities of various MT may be regulated by the relative levels of SAM and SAH in the cell (Garcia & Goodenough-Lashua, 1998).

2.3.3 Substrate recognition

High-resolution crystal structures of several rRNA ME (RluA, RluF, and RlmD) in complex with their rRNA substrates have been determined and enlighten the mechanisms of substrate recognition employed by rRNA ME (Lee *et al.*, 2005; Hoang *et al.*, 2006; Alian *et al.*, 2009). The observed rRNA-ME interactions correlate well with the general principles known to be sheared by other RNA binding proteins such as tRNA ME.

Firstly, the binding site for the negatively charged RNA is usually a positively charged concave on protein surface enriched in arginine and largely devoid of aspartic acid and glutamic acid residues (Byrne *et al.,* 2009). Electrostatic interactions are effective over longer distances than other intermolecular interactions and are therefore likely involved in the initial attraction of the oppositely-charged molecules in solution.

Secondly, non-polar van der Waals interactions and hydrogen bonds (H-bonds) are a crucial part of ME-rRNA interactions (Byrne *et al.,* 2009). Although the energy provided by a single van der Waals interaction is relatively small, significant stabilization is achieved by numerous van der Waals interactions along the entire protein-RNA interface. Non-polar cavities on protein surfaces are often employed to tightly fit the substrate bases (Byrne *et al.,* 2009). The specificity of such cavities is enhanced by distinct patterns of H-bonding groups and by steric exclusion. The bases of RNA are rich in H-bond donors and acceptors allowing the identification of any given base by its H-bonding properties (Byrne *et al.,* 2009). In case of RNA, the sugar phosphate backbone (2'-OH of ribose, in particular) provides a possibility to stabilize the interactions with proteins through H-bonding in a RNA sequence-independent manner (Byrne *et al.,* 2009). From the protein side, amide and carboxyl groups of the polypeptide main chain, as well as various groups present in the side chains of most of the amino acids can be involved in the H-bonding interactions with RNA (Byrne *et al.,* 2009). In addition, water-mediated H-bonds provide a means to partially shield the negatively charged amino acid side chains located at the protein-RNA interfaces (Byrne *et al.,* 2009). On the other hand, aromatic amino acids (tyrosine, tryptophan, and phenylalanine) located at the interface

can interact with the unpaired RNA bases through stacking interactions (Byrne *et al.,* 2009).

Thirdly, while both the induced fit and the rigid body docking mechanisms are common among the RNA binding proteins, structural studies on the rRNA-ME complexes have revealed that while the rRNA substrates are extensively remodeled, the ME themselves are not and display only minor structural changes upon the complex formation (Lee *et al.*, 2005; Hoang *et al.*, 2006; Ellis & Jones, 2008; Alian *et al.*, 2009). Conformational changes in rRNA are likely needed to increase the affinity for ME and for the ME to gain access the substrate base that is often found to be buried within the RNA molecule (Byrne *et al.,* 2009). Indeed, the base flipping mechanism has been shown for all of the rRNA ME whose co-crystal structures with their substrates have been solved to rotate the target base around its flanking phosphodiester bonds such that the base projects into the catalytic pocket of the ME (Lee *et al.*, 2005; Hoang *et al.*, 2006; Alian *et al.*, 2009). Binding to ME presumably provides the energy for the conformational changes in rRNA. Hence, rRNA ME most likely rely on the dynamics of the substrate as a method of substrate recognition (Lee *et al.*, 2005; Hoang *et al.*, 2006). As the ability of RNA to adopt a certain conformation depends upon its sequence, substrate recognition in case ME can occur without a large number of direct contacts to conserved bases in rRNA (Byrne *et al.*, 2009). Alternatively, given the flexibility of RNA molecules, it is possible that an ensemble of RNA conformers exists and that the ME bind a conformer which is complementary to their active site. However, this mechanism requires fast conformational transitions in rRNA.

The two aspects of the substrate recognition mechanisms are: how do the ME recognize the correct nucleotide within the rRNA molecule; and how do they recognize the correct atom within that nucleotide? Recognition of the target atom within the substrate nucleotide is likely to be inextricably linked to the mechanism of the specific modification reaction. Binding and orientation of the substrate base will likely dictate which atom will be suitably positioned for the attack on the methyl group of SAM (Garcia & Goodenough-Lashua, 1998).

Particularly intriguing is the structural basis for the substrate nucleotide selection that is, elucidating the mechanism by which the specificity of a ME is limited to a single site in a particular RNA among the myriad of different RNAs in the cell, or broadened to multiple sites in the same or different RNAs. For instance, while RluA modifies two different RNAs (23S rRNA and tRNA) at positions that share local sequence and structural similarity (Wrzesinski *et al.*, 1995; Raychaudhuri *et al.*, 1999), RluC substrate uridines (955, 2504, and 2580) in 23S rRNA are neither in a common sequence and/or structural context nor are they close in the tertiary structure of the LSU (Ofengand & Del Campo, 2004a). RluD, on the other hand, is specific to three uridines (1911, 1915, and 1917) in the loop region of H69 of 23S rRNA (Leppik *et al.*, 2007). RluF and RluB recognize 23S rRNA adjacent uridines (U2604 and U2605, respectively) that reside in a stem region and carry out the same modification reaction, yet are specific for their respective sites (Del Campo *et al.*, 2001). The structural fea-

tures of RluF and RluB responsible for the substrate selectivity must be highly specialized, since they are two of the most closely related PS in the RsuA family. RsmA, on the other hand, methylates two adjacent adenosines in the 16S rRNA and is able to methylate either of the two in the absence of the other, ruling out any obligate order of methylation (Cunningham *et al.*, 1990). In accordance, the active site of RsmA appears to be able to accommodate only one adenosine at the time (O'Farrell *et al.*, 2004). The specificity of the RlmKL for G2069 in order to synthesize m^7G and for G1225 in order to synthesize m^2G in 23S rRNA comes from its modular structure consisting of two RFMT domains. The two different methylation reactions are catalyzed by different domains and occur independently of each other (Wang *et al.*, 2012).

Given the fact that the target nucleotides of ME are found in a wide variety of structural contexts in both single- and double-stranded regions of rRNA, the mode of substrate recognition is more than likely to be idiosyncratic for each ME. Evolution appears to have taken the path of modularizing rRNA ME so that rather universal catalytic domains perform very specific reactions by being combined with specific RNA recognition and binding domains (Byrne *et al.,* 2009). While some ME seem to have a strict requirement for the sequence/ structure near its target nucleotide others do not and likely recognize sequence and/or structural features outside the immediate vicinity of its target nucleotide. The requirements for an RNA substrate to be able to "refold" into the unusual conformation and to form specific H-bonds with the ME are powerful constraints that help explain the high specificity of the rRNA ME. Though the dynamics of the ME itself is also likely important for rRNA-ME complex formation.

2.4 r-protein modification

As is the case with rRNA, r-protein modifications have been observed in all three domains of life. To date, eleven r-proteins are known to be post-translationally modified in *E. coli* (Arnold & Reilly, 1999; Polevoda & Sherman, 2007) (Table 4). R-protein modifications consist mainly of methylations and acetylations. R-protein L7 is in fact the acetylated version of L12 and the ratio between the two of them depends on the growth rate (Ramagopal & Subramanian, 1975). The pattern of r-protein methylation appears to be highly similar in bacteria (Polevoda & Sherman, 2007). Furthermore, L3 and L12 are methylated in both prokaryotes and eukaryotes, though the methylation sites differ. Methylating the N-terminal amino acids of r-proteins is more common in bacteria (Polevoda & Sherman, 2007). As do *E. coli* rRNA MT the r-protein MT also use SAM as the methyl group donor. Methylation of r-proteins is believed to be irreversible and the extent of methylation can be complete or incomplete (Polevoda & Sherman, 2007).

Table 4. Post-translational modifications of *E. coli* r-proteins[1].

r-protein	Modification	Position of modification	Modification enzyme	References
S5	acetylation	Ala 1	RimJ	
S6	glutamic acid residues	C-terminus	RimK	Kino *et al.,* 2011
S11	methylation	Ala 1		
	isoaspartate[2]			
S12	β-methylthiolation	Asp 88	RimO	Anton *et al.,* 2008; Strader *et al.,* 2011
S18	acetylation	Ala 1	RimI	
L3	methylation	Gln 150	PrmB	
L7/L12	acetylation	Ser 1	RimL	Miao *et al.,* 2007
	methylation	Lys 81		
L11	3 trimethylations	Ala 1, Lys 3, Lys 39	PrmA	
L16	methylation	Met 1		
	unknown	Arg 81		
L33	methylation	Ala 1		

[1] according to (Arnold & Reilly, 1999; Kaczanowska & Ryden-Aulin, 2007; Polevoda & Sherman, 2007) unless otherwise stated
[2] partial modification

R-protein modification may take place either on free r-proteins before their incorporation into ribosomes or during/after the ribosome assembly. The methylation of L3, L7, and L11 likely occurs prior to their incorporation into the ribosome (Polevoda & Sherman, 2007). The substrate recognition and modification mechanism of PrmA, responsible for trimethylating multiple positions in L11 (Table 4), is particularly intriguing. Methyl group addition appears to be sequential and does not require the dissociation of PrmA from L11 (Cameron *et al.*, 2004). Unlike the r-protein methylations mentioned above, the *E. coli* specific β-methylthiolation likely occurs when the S12 is already assembled into the SSU (Polevoda & Sherman, 2007; Strader *et al.*, 2011).

Similarly to rRNA modifications (discussed in chapter 3), the functional role of most of the r-protein modifications is unclear. None of the known r-protein ME (Table 4) appear to be essential as deleting or mutating the corresponding genes usually do not cause major changes in bacterial phenotypes (Kaczanowska & Ryden-Aulin, 2007; Polevoda & Sherman, 2007).

The only modification known to lead to a change in phenotype when absent is the methylation of L3. Namely, the PrmB deficient *E. coli* strain exhibits cold-sensitivity and reduced growth rate, it also accumulates subunit precursors and abnormal ribosomal particles and the native subunits are forming slower (Colson *et al.*, 1979; Lhoest & Colson, 1981). However, once assembled, the ribosomes containing unmodified L3 display protein synthesis parameters similar to native ribosomes. It has been suggested that the PrmB protein may act as a ribosomal assembly factor (Lhoest & Colson, 1981). Interestingly, overexpressing the S5 acetyltransferase RimJ suppresses the growth defects and anomalous ribosome profile exhibited by a S5 mutant strain, however, it is not dependent on the acetyltransferase activity of the Rim J protein (Roy-Chaudhuri *et al.*, 2008). Additionally, RimJ appears to associate with the pre-SSU (Roy-Chaudhuri *et al.*, 2008). These findings suggest that RimJ protein has two functions in ribosome biogenesis in *E. coli*; it is an r-protein ME as well as a ribosome assembly factor.

In summary, the fact that many r-proteins are modified, and that several contain more than one modification, indicates their significance for ribosome structure, assembly, and/or function. Modifications alter the charge distribution, H-bonding, and steric properties of r-proteins, and thus may affect the efficiency and specificity of r-protein binding to the rRNA or optimize the binding sites for various players of translation (Polevoda & Sherman, 2007). Alternatively, r-protein modifications may play an important role in controlling the life span of r-proteins within living cells or be a way of signaling the physiological state of the cell.

2.5 Assembly factors

Functional bacterial ribosome subunits can be reconstituted *in vitro* from purified rRNA and r-proteins without the presence of any additional factors, however, long incubations at above physiological temperatures and unphysiological ion concentrations are needed (chapter 2.1). Long RNA molecules can form in addition to their native structure a multitude of non-native structures (Weeks, 1997). Once misfolded structures form, they are often very stable and the transitions from kinetically trapped intermediates to the native conformations are slow (Weeks, 1997). Regardless, in rapidly growing *E. coli* cells, it only takes a couple of minutes to synthesize new ribosomes (Lindahl, 1975). The surprisingly effective rRNA folding during ribosome biogenesis is likely achieved with the help of an ever increasing number of proteins collectively known as assembly factors, especially in eukaryotic cells where hundreds of assembly factors have been described (Hage & Tollervey, 2004). Assembly factors could limit the number of alternative conformations by facilitating proper rRNA folding and r-protein-rRNA interactions, thereby avoiding the accumulation of kinetically trapped intermediates. Also, assembly factors may reduce the activation energies of rate-limiting reactions in ribosome synthesis (Kaczanowska &

Ryden-Aulin, 2007; Wilson & Nierhaus, 2007; Shajani *et al.*, 2011). Assembly factors contain many classes of functionally different proteins including RNA helicases, ribosome-dependent GTPases, heat-shock proteins, and RNA chaperones. It should be mentioned that most of the assembly factors are not essential, except for under stress conditions, consistent with the notion that ribosome assembly can take different paths, bypassing steps that are inefficient or blocked (discussed in chapter 2.1).

2.6 Regulation

The growth of bacteria directly depends on their capacity for protein synthesis. An increase in total protein synthesis can be achieved only by increasing the number of ribosomes per cell (Kaczanowska & Ryden-Aulin, 2007). The fraction of cellular matter and energy devoted to the synthesis of ribosome is substantial, more so in rapidly growing bacteria that contain more ribosomes per unit cell mass than slowly growing bacteria. For example, a rapidly growing *E. coli* cell can contain up to 70 000 ribosomes, which constitute as much as 50% of its dry mass (Bremer & Dennis, 1996; Nomura, 1999). Correspondingly, approximately 50% of the total energy production of a cell is consumed by ribosome biogenesis (Condon *et al.*, 1995; Bremer & Dennis, 1996). Also, it is known that the transcription of rRNA operons accounts for more than 50% of the total RNA synthesis in rapidly growing cells (even though rRNA operons only account for about 0.5% of the total genome), but only 2 to 5% of total rRNA is contained in assembling ribosomes (Lindahl, 1975; Condon *et al.*, 1995; Kaczanowska & Ryden-Aulin, 2007). Obviously, the synthesis of new ribosomes must be highly coordinated, fast, and efficient. Indeed, it is estimated that ribosome biogenesis in WT *E. coli* takes approximately 2-5 minutes at 37°C, and that most of the time required, is spent at a stage where the precursor particles have already attained their final sedimentation coefficients (Lindahl, 1975). In fact, the final maturation of LSU seems to be the rate limiting step under optimal growth condition of *E. coli* (Peil *et al.*, 2008; Rene & Alix, 2011; Roy-Chaudhuri *et al.*, 2010).

The main aspects of the regulation of ribosome biogenesis are: ensuring the stoichiometry between rRNA and r-proteins (and among r-proteins themselves) as virtually all of the ribosomal components in the cell are found in ribosomes; and adaption of ribosomal synthesis to the nutritional environment (Kaczanowska & Ryden-Aulin, 2007). In bacteria, there is an intricate network of regulatory mechanisms involved in the synthesis of ribosomal components: rRNA synthesis is regulated by stringent control, growth rate control, transcription antitermination, upstream activation, and feedback control; r-protein synthesis in controlled in addition to transcription regulation also by translational feedback (Condon *et al.*, 1995; Kaczanowska & Ryden-Aulin, 2007). For example, stringent control results in immediate shut-down of rRNA and tRNA synthesis in response to amino acid or energy starvation; growth rate control

leads to adjustments in rRNA and tRNA synthesis in response to changes in the nutritional environment; and the antitermination system is thought to maintain the transcription elongation rate and processivity that is optimal for both speed and folding of the pre-rRNA transcript (Gausing, 1977; Heinrich *et al.*, 1995; Pfeiffer & Hartmann, 1997; Kaczanowska & Ryden-Aulin, 2007). A too-high transcription elongation rate has been shown to result in an improper folding of the rRNA transcript and misassembly of the LSU (Lewicki *et al.*, 1993). The rate of r-protein synthesis depends on the availability of rRNA (Condon *et al.*, 1995; Kaczanowska & Ryden-Aulin, 2007). In *E. coli*, most of the r-protein genes are clustered into operons that often include genes for translation elongation factors and for RNA polymerase subunits (Keener & Nomura, 1996). The main mechanism of regulating the stoichiometry of production of individual r-proteins in response to the available amount of rRNA is the translational feedback control, where one of the r-proteins in the operon serves not only as a structural component of the ribosome, but also controls the expression of itself and other genes in the same operon (Zengel & Lindahl, 1994).

3. FUNCTION OF rRNA MODIFICATIONS

The classical way to determine the function of MN is to block their synthesis and monitor the ensuing changes in the phenotypes. The phenotypes of the rRNA MT and PS deletion strains range from severe growth retardation and translational defects to a lack of any observable phenotype. However, growth defects that only appear when the ME deletion strains must compete with *E. coli* WT cells, or lack of phenotypes altogether, are by far prevalent. The only ME whose loss results in major phenotypic defects in *E. coli* are RsmA, RlmE, RlmA, and RluD (discussed below in more detail). In fact, irrespective of the conservation and localization of MN in functionally important regions of the ribosome (discussed in chapter 2.3), not a single one of them has been found to be absolutely essential for viability in *E. coli*. Furthermore, the SSU with significant functional activity can be reconstituted *in vitro* using completely unmodified 16S rRNA, implying that MN are not essential for either the assembly or the activity of the SSU (Krzyzosiak *et al.*, 1987). In *E. coli*, the *in vitro* reconstitution of LSU using completely unmodified 23S rRNA is extremely inefficient (Green & Noller, 1996; Semrad & Green, 2002), although it has been shown that the 23S rRNA MN are not essential for the assembly or the activity of the LSU in other bacteria (Green & Noller, 1996, 1999; Khaitovich *et al.*, 1999). Nonetheless, MN are known to alter the chemical properties and the structures of the RNA regions embedding them, and are believed to be important for fine-tuning the intra- and intermolecular interactions involved in rRNA folding, stability, and/or ribosome functioning.

For instance, base methylations lead to increased hydrophobicity and stacking of the nucleosides, and may also increase the steric hindrance, alter the H-bonding ability and the charge distribution within the purine and pyrimidine rings (Agris, 1996). Methylations of the 2'-O-positions of riboses increase the hydrophobicity and the stability of RNA and may protect sensitive rRNA regions against RNases (Kowalak *et al.*, 1994; Lane *et al.*, 1995; Schroeder *et al.*, 2004).

Although Ψ is a structural isomer of uridine (Cohn, 1960), the presence of an additional imino (NH) group at position 1 of the base (Figure 6) significantly alters the chemical properties of RNA (Newby & Greenbaum, 2001). Conformational studies of free Ψ nucleoside indicate that there is a higher degree of rotational freedom for the base in the C-C glycosyl bond compared to N-C glycosyl bond of uridine and therefore a greater conformational flexibility for Ψ is anticipated (Charette & Gray, 2000). However, when present in helical regions of RNA, while the N3 of Ψ participates in H-bonding interactions with adenosines much like uridine; the extra H-bond donor at N1 of Ψ has been observed to participate in water-mediated H-bonds with the sugar-phosphate backbone 5'-oxygens of both the Ψ itself and the preceding residue (Arnez & Steitz, 1994; Charette & Gray, 2000). This H-bonding to the backbone locks the Ψ base in the *anti* conformation, rigidifies the nearby RNA backbone, and enhances the base stacking interactions by favoring a 3'-*endo* conformation of the

ribose, all in all, contributing to the stability of RNA (Davis, 1995; Schroeder *et al.*, 2004). Ψs in the single stranded regions of rRNA may employ the extra H-bond donor at N1 for tertiary interactions with its phosphate backbone, with other regions of the rRNA or with certain ribosome binding proteins; and have been shown to either stabilize or to destabilize rRNA structures depending on their context (Charette & Gray, 2000; Meroueh *et al.*, 2000). The context-dependent stabilizing/destabilizing effect of Ψ in the 23S rRNA H69 is discussed in chapter 1.1.

Dihydrouridine (D) is known to increase the local RNA flexibility by favoring the inherently more flexible 2'-*endo* sugar conformation and thereby causing a complete destacking of bases in its vicinity (Dalluge *et al.*, 1996). The location of D in the 23S rRNA just two residues away from the putative peptide transfer site (Nissen *et al.*, 2000) suggests that D may be there to provide the necessary flexibility *in vivo* (Ofengand & Del Campo, 2004a).

3.1 Translation

Alterations of local rRNA structures arising from MN could influence the rate and accuracy of decoding and proofreading during translation by facilitating interactions with tRNA and mRNA, and by promoting catalytic efficiency in peptide bond formation. In accordance, majority of the rRNA MN have been found to cluster around the DC and the PTC of the ribosome (discussed in chapter 2.3).

While the reconstituted SSU lacking all rRNA MN is functional in all of the partial reactions of protein synthesis *in vitro*, including codon recognition, it was only 50% as active as the SSU reconstituted from natively modified rRNA suggesting a functional role for at least some of the 16S rRNA MN in translation (Cunningham *et al.*, 1991). Varshney and co-workers have shown that a decrease in the intracellular levels of SAM resulting in the deficiency of rRNA methylations influences the initiator tRNA selection in the P-site of the ribosome (Das *et al.*, 2008). Furthermore, by analyzing initiator tRNA selection in the 16S rRNA MT deletion strains, they showed that the most prominent effect was caused by the lack of dimethylations at the positions A1518 and A1519 in the 3' terminal helix of the 16S rRNA close to the anti-Shine-Dalgarno sequence (Das *et al.*, 2008). Moreover, the efficiency of RRF-mediated ribosome recycling was adversely affected (Seshadri *et al.*, 2009). RsmA, the MT responsible for these methylations, has been shown to interact with the same region of the SSU as LSU and initiation factor 3 (IF3) (Xu *et al.*, 2008). Varshney and co-workers proposed that the aforementioned methylations have a role in the IF3-mediated translation initiation (Das *et al.*, 2008), which is in good agreement with the observation that the SSU lacking RsmA-dependent methylations display decreased association with LSU *in vitro* and need more IF3 for optimal binding of the initiator tRNA (Poldermans *et al.*, 1979b). Based on the atomic resolution structure of the SSU from the *rsmA* deletion strain of

T. thermophilus, it has been suggested that the methylations synthesized by RsmA play a direct role in establishing a fully active SSU conformation by facilitates the formation of a packing interaction between two 16S rRNA helices in the vicinity of the DC required for correct formation of both the A- and P-sites (Boehringer *et al.*, 2012). Also, m^2G966 (RsmD) and m^5C967 (RsmB) in 16S rRNA have been suggested of being involved in IF3 binding and translation initiation (Saraiya *et al.*, 2008). In case of the *rsmD/rsmB* double deletion strain, both the AUU initiator codon usage *in vivo* and initiation efficiency *in vitro* are reduced, in addition, the level of IF3 is decreased (Sergiev *et al.,* 2011). A deletion of RsmH, the MT responsible for m^4C1402, resulted in increased efficiency of non-AUG initiation and decreased UGA read-through rate (Kimura & Suzuki, 2010). Clearly, SSU MN influence both efficiency and fidelity of translation initiation and decoding, most likely by altering interactions with the tRNA and mRNA.

As already mentioned, *in vitro* reconstitution of translationally active LSU using completely unmodified 23S rRNA is inefficient in *E. coli* system (Green & Noller, 1996). On the other hand, the *Thermus aquaticus* LSU reconstituted from unmodified 23S rRNA are active in peptide bond synthesis, however, the activity is significantly reduced compared to LSU reconstituted with native 23S rRNAs (Khaitovich *et al.*, 1999). As no MN in either of the bacterium comes within 10 Å of the catalytic site of the ribosome (Voorhees *et al.*, 2009), their direct participation in peptide bond synthesis is unlikely. However, MN in the surrounding regions could affect peptide bond synthesis indirectly by helping to correctly position the A- and P-site tRNAs for efficient catalysis. Base pairing is predicted to occur between the acceptor end of the P-site tRNA and the universally conserved Gm2251 in 23S rRNA synthesized by RlmB (Voorhees *et al.*, 2009). Gm2251 is essential for the formation of the functional P-site as the dominant lethal phenotype of G2251 substitutions has been attributed to a deficiency in peptidyl transferase activity (Green *et al.*, 1997). However, deletion of *rlmB* gene did not exhibit any observable effect on bacterial growth even when the *rlmB* deletion strain had to compete with WT *E. coli* cells undermining the importance of Gm2251 modification in translation (Lovgren & Wikstrom, 2001). In contrast, the phylogenetically conserved Um2552 in *E. coli* 23S rRNA synthesized by RlmE has been confirmed to alter the conformation of the A-site region of the ribosome (Blanchard & Puglisi, 2001). In accordance, the *E. coli rlmE* deletion strain displays impaired cell growth, decreased protein synthesis rate *in vitro*, and increased translational accuracy, all attributed solely to the lack of Um2552 modification (Bugl *et al.*, 2000; Caldas *et al.*, 2000b; Hager *et al.*, 2002; Tan *et al.*, 2002; Widerak *et al.*, 2005). The exact mechanism of how Um2552 affects the translational accuracy is unknown. However, it is speculated that U2552 in 23S rRNA could be unmethylated under some circumstances when it is advantageous for the cell to improve its translational accuracy at the expense of the translation rate and that RlmE could belong to a protein quality control pathway (Widerak *et al.*, 2005).

Several rRNA MN cluster at the entrance to the polypeptide exit tunnel. The additional hydrophobic and hydrophilic properties of the MN at specific sites of the tunnel wall were proposed to modulate the functional interactions with the nascent polypeptide chain (Decatur & Fournier, 2002; Ofengand & Del Campo, 2004a). Recently, Mankin and co-workers demonstrated that the methylation of A2503 in 23S rRNA is important for nascent peptide recognition and relaying the signal from the exit tunnel to the PTC leading to ribosome stalling (Vazquez-Laslop *et al.*, 2010).

3.2 Antibiotic resistance

Large varieties of antibiotic agents are known to bind to ribosomes and inhibit protein synthesis (Poehlsgaard & Douthwaite, 2005). Post-transcriptional modification, foremost methylation, of specific rRNA nucleotides can interfere with the binding of antibiotics to their target sites on the ribosome and thereby confer antibiotic resistance. Most of the rRNA methylations implicated in antibiotic resistance are synthesized by specific MT often encoded by a plasmid or a transposon and expressed only when the antibiotic is present, in contrast to genome encoded rRNA MT responsible for "house-keeping" MN (Poehlsgaard & Douthwaite, 2005). For instance, dimethylation of A2058 of the 23S rRNA confers macrolide resistance (Kovalic *et al.*, 1994; Vester & Douthwaite, 1994) and methylation of A1408 of the 16S rRNA confers resistance to some specific aminoglycosides (Macmaster *et al.*, 2010). An extra methyl group added by MT Cfr to the C8 position of already naturally modified m^2A2503 renders cells resistant to an array of antibiotics (Kehrenberg *et al.*, 2005). Acquisition of such MT genes by pathogens is one of the major causes of clinical resistance to a number of antibacterial drugs.

However, the "house-keeping" MN can also confer antibiotic resistance or sensitivity as observed in the cases where the lack of modifications affects antibiotic susceptibility of the cells. For example, the lack of the $m^6{}_2$A at position 1518 and 1519 in 16S rRNA confers resistance to kasugamycin (Helser *et al.*, 1972; Poldermans *et al.*, 1979a), the lack of a conserved m^7G527 (RsmG) in 16S rRNA confers moderate streptomycin resistance (Okamoto *et al.*, 2007), and the lack of Ψ2457 (RluE) in 23S rRNA confers moderate resistance to erythromycin (Nichols *et al.*, 2011). In contrast, the lack of Ψ2504 (RluC) in 23S rRNA was found to significantly increase the susceptibility to a number of peptidyl transferase inhibitors (Toh & Mankin, 2008) and the lack of m^2G966 (RsmD) in 16S rRNA to confer high tetracycline sensitivity (Nichols *et al.*, 2011). Hence, modification of rRNAs may have evolved as an intrinsic resistance mechanism protecting bacteria against natural antibiotics. Co-evolution of antibiotic biosynthesis and corresponding resistance mechanisms might have led to the global spread of rRNA ME genes protecting bacteria from antibacterial agents which were in use by bacteria billions of years ago. Modern antibiot-

ics could be the products of evolution driven by the development of resistance caused by rRNA MN (Sergiev *et al.,* 2011).

3.3 Small subunit biogenesis

While functional SSU can be *in vitro* reconstituted using unmodified 16S rRNA, the conditions required are more extreme than those for native 16S rRNA, the SSU particles are more varying in size, and their activity in protein synthesis is only about half that of a modified control SSU (Krzyzosiak *et al.*, 1987; Cunningham *et al.*, 1991) suggesting that MN in 16S rRNA, while not essential, facilitate the assembly of SSU. RsmA dimethylates A1518 and A1519 in the 16S rRNA generating two of the few modifications conserved throughout evolution (Poldermans *et al.*, 1979; Formenoy *et al.*, 1994; O'Farrell *et al.*, 2006). Although RsmA is universally conserved and essential in eukaryotes, it is not essential in *E. coli* (Lafontaine *et al.*, 1994; Connolly *et al.*, 2008). Nevertheless, its deletion in *E. coli* results in a cold-sensitive phenotype, altered ribosome profile displaying virtually no polysomes and more free subunits, and defects in 16S rRNA processing (Connolly *et al.*, 2008). Overexpressing RsmA protein in the *rsmA* deletion strain partially rescues the growth defect at low temperatures, but also leads to an even more pronounced increase in free SSU. However, at 37°C, overexpression of RsmA protein has a negative effect on growth rate and causes an accumulation of pre-16S rRNA in both *E. coli* WT and *rsmA* deletion strains (Connolly *et al.*, 2008). Interestingly, overexpressing a MT-inactive RsmA protein is more deleterious than overexpressing the native RsmA protein, causing a dramatic increase in free SSU (Connolly *et al.*, 2008). Both the native and the MT-inactive RsmA proteins associate with the SSU, but binding with the mutant RsmA is much stronger, implying that the MT activity is required for the release of RsmA (Connolly *et al.*, 2008). RsmA methylates the 16S rRNA in the context of a nearly mature SSU (Desai & Rife, 2006). Culver and co-workers proposed a model whereby methylation activates the release of the RsmA allowing for the SSU assembly to proceed and the final maturation of the 16S rRNA to take place (Connolly *et al.*, 2008). As RsmA interacts with the same region of SSU as LSU and IF3 (Xu *et al.*, 2008), it is conceivable that it inhibits the incorporation of immature SSU into 70S ribosomes. Indeed, cryo-electron microscopy structure of RsmA in complex with SSU reveals that RsmA recognizes the SSU in a translationally inactive conformation and that the dissociation of RsmA is required for the formation of the translationally active conformation (Boehringer *et al.*, 2012). Apparently, 16S rRNA methylation and the subsequent dissociation of RsmA controls conformational changes in pre-SSU required for final rRNA processing and initiation complex formation (Boehringer *et al.*, 2012). The direct role of RsmA-dependent modifications in establishing the active SSU conformation is discussed in chapter 3.1. The RsmA case illustrates how MN in a functionally important region of the ribosome can be linked to its biogenesis. Hence, the methylation

of 16S rRNA by RsmA provides a quality control mechanism of SSU biogenesis sequestering incompletely assembled SSU from being involved in translation. This may explain the high conservation of this particular rRNA modification (Connolly *et al.*, 2008).

3.4 Large subunit biogenesis

Assembly of the LSU depends more on MN as compared to the SSU. Reconstitution of *E. coli* LSU using *in vitro* transcribed unmodified 23S rRNA has very low efficiency (Green & Noller, 1996; Khaitovich *et al.*, 1999; Semrad & Green, 2002). Also, inhibiting methylation by ethionine leads to formation of functionally inactive LSU particles lacking r-protein L16 and showing reduced amounts of several other r-proteins (Alix *et al.*, 1979). Nevertheless, most of the 23S rRNA MN are not essential as only seven MN close to the PTC must be present for *in vitro* reconstitution of functional LSU (Green & Noller, 1996). Furthermore, *in vivo* LSU assembly requires only Um2552 and the three Ψs in H69 (Caldas *et al.*, 2000b; Gutgsell *et al.*, 2005).

Um2552 in *E. coli* 23S rRNA is synthesized by RlmE (Caldas *et al.*, 2000a). Ribosomes isolated from the *rlmE* deletion strain display in addition to the decreased protein synthesis rate *in vitro* and the increased translational accuracy (mentioned in chapter 3.1) also severe ribosome assembly defects with notable increase in free subunits and accumulation of pre-LSU rRNA containing pre-23S rRNA, resulting in a slow growth phenotype (Bugl *et al.*, 2000; Caldas *et al.*, 2000b; Tan *et al.*, 2002; Hager *et al.*, 2004; Widerak *et al.*, 2005). Solely the lack of Um2552 modification is responsible for the observed growth, ribosome assembly, and translational defects as a catalytically inactive RlmE is not able to rescue the *rlmE* deletion strain (Hager *et al.*, 2002). On the other hand, overexpression of two different ribosome-dependent GTPases can rescue the slow-growth phenotype of *rlmE* deletion strain, presumably by overcoming the destabilizing effects of the absent modification by stabilizing the 70S ribosome (Tan *et al.*, 2002). Intriguingly, some second-site mutation(s) in other parts of the genome are also able to rescue the *rlmE* deletion strain without the reappearance of the Um2552 modification (Tan *et al.*, 2002). The essentiality of the Um2552 modification in 23S rRNA for effective translation was one of the first indications that the defects seen in ribosome assembly could be due to the global errors in translational apparatus leading to an imbalance in rRNA and r-protein ratio.

The three highly conserved Ψs in the H69 of 23S rRNA (chapter 1.1) are all synthesized by RluD (Huang *et al.*, 1998; Raychaudhuri *et al.*, 1998; Ofengand, 2002). RluD deficient *E. coli* strain was shown to exhibit severely reduced growth rate and massive defects in ribosome biogenesis, namely, a reduction in 70S ribosomes, accumulation of free subunits, and appearance of pre-LSU and pre-SSU containing pre-23S and pre-16S rRNA, respectively (Gutgsell *et al.*, 2001; Ofengand *et al.*, 2001b; Gutgsell *et al.*, 2005). Given the fact that a

catalytically inactive RluD protein could not alleviate the growth defect and the abnormal ribosome profiles, Ofengand and co-workers proposed that the H69 Ψs themselves are important for ribosome biogenesis (Gutgsell *et al.*, 2005). However, second-site mutation(s) arising elsewhere in the genome can alleviate the growth and assembly defects of *rluD* deletion, without restoring the H69 Ψs (Raychaudhuri *et al.*, 1998; Gutgsell *et al.*, 2005). Current view about the involvement of H69 Ψs in ribosome assembly arose from the findings that inactivation or lack of RluD prompted misreading of stop codons and that a point mutation in RF2 can suppress the growth defects associated with the *rluD* deletion strain (Ejby *et al.*, 2007; O'Connor & Gregory, 2011). These findings point to the function of H69 Ψs in translation termination (discussed in more detail in chapter 1.1). Hence, errors in the biogenesis of the ribosome subunits in the *rluD* deletion strain could be explained as being an indirect consequence of global errors in cellular translation as seems to be the case with the *rlmE* deletion strain discussed above. Moreover, RluD deficient cells seem to spend twice the energy to produce the same amount of biomass and display higher expression of heat-shock proteins compared to WT cells, further suggesting errors in global translation (Ejby *et al.*, 2007). However, as already mentioned in chapter 1.1, the deleterious effect caused by the lack of H69 Ψs is restricted to the *E. coli* K-12 strain carrying variant RF2 and S7 proteins (O'Connor & Gregory, 2011; Schaub & Hayes, 2011). This finding, while further supporting the role of H69 Ψs in translation termination, also illustrates that the initial impact of the lack of H69 Ψs was slightly overestimated. Nonetheless, the strong conservation of the H69 Ψs indicates that their presence may be essential under some more stringent growth conditions.

In some cases however, it appears that the ME themselves play a role in ribosome biogenesis and/or functioning independently of the MN they synthesize (discussed in chapter 2.3). This is exemplified by RlmA responsible for m^1G745 in 23S rRNA, deletion of *rlmA* gene results in the *E. coli* cells growing 40% slower in rich medium, 20% reduction in translation elongation rate, and an increase in free ribosomal subunits (Gustafsson & Persson, 1998). These defects disappeared when the *rlmA* gene was reintroduced from a plasmid and were due to the absence of RlmA protein itself rather than to the lack of the corresponding methylation, since mutating G745 to C, U, or A caused no growth defects (Liu *et al.*, 2004). Furthermore, an as yet unidentified second-site mutation in *rlm*A deletion strain restores nearly WT growth rate without restoring the m^1G745 modification (Liu *et al.*, 2004). Hence, RlmA protein may possess a second function important for cells independent of its MT activity, such as being a part of the ribosome quality control or being an assembly chaperone.

3.5 Fine-tuning ribosome structure and function

Conservation of MN between very distant species and clustering around the functionally important regions of the ribosome, not to mention the energetic and metabolic cost of their synthesis, strongly argues for a functional role of the rRNA MN. Also, it seems unlikely that the two distinct mechanisms in bacteria and eukaryotes for forming the rRNA MN would have evolved unless important for cell survival. In accordance, rRNA ME are believed to be a part of the minimal set of molecules required for life (Koonin, 1996; Anantharaman *et al.*, 2002; Ferre-D'Amare, 2003). The roles of a few rRNA MN in ribosome assembly and functioning in *E. coli* have been demonstrated, yet in the majority of cases, the functional significance of the rRNA MN remains obscure.

The co-clustering of hydrophobic methylations and hydrophilic Ψs at the interface of ribosomal subunits where mRNA, tRNA, and translation factors are bound, suggests their combined role in creating the optimal binding sites for these ligands. It appears that most individual MN confer a subtle and not easily demonstratable benefit, and only by the full ensemble of MN working in cooperation, a significant benefit for the cell is gained. It might even be the case that some of the MN have no function at all. Indeed, due to the relatively broad specificity of certain multi-site ME, it is possible that they catalyze the formation of a MN in several locations of an rRNA molecule, only one of which is functionally relevant and others are tolerated as a benign artifact of accidental homology of the substrate. It is also possible that the real significance of the rRNA MN is not fully revealed under the "mild" growing conditions used in the analyses of most of the ME deletion strains. In nature, a mutant strain that arises would have to compete with WT cells for survival in an environment where nutrients and energy are periodically supplied and exhausted. More strict growing conditions, such as using minimal media and various stress conditions, as well as analyzing the survival of ME deletion strains in stationary phase and in competition studies in mixed cultures with WT strains and other bacterial species, might provide more insight into the biological significance of the rRNA MN.

In conclusion, the variety of MN with diverse chemical properties provides the ribosome with a broader range of possible interactions between different rRNA regions, tRNAs, mRNAs, translation factors, and various ligands by influencing the local rRNA conformation and by fine-tuning the translation process. While most of the rRNA MN are individually dispensable, together they ensure the production of accurate and efficient ribosomes.

RESULTS AND DISCUSSION

Objectives

Modified nucleosides (MN) are found in functionally important regions of all ribosomes (chapter 2.3). However, the physiological role of the MN remains largely enigmatic (chapter 3). One possible way to study the functional roles of the MN is to identify the corresponding modification enzyme (ME) genes and to characterize the phenotypes of the ME deletion strains. In addition, for the characterization of the ME themselves, it is important to determine the features of both the ribosomes and the ME involved in the substrate recognition and the catalytic mechanism. Also, it is interesting to infer how the rRNA modification process fits into the overall ribosome biogenesis and functioning.

In present study we aspired:

1. to identify the gene encoding for the *E. coli* 23S rRNA $m^3\Psi$ methyltransferase and to shed light onto the functional role of the $m^3\Psi$ modification
2. to determine the substrate specificity of the $m^3\Psi$ methyltransferase
3. to determine the kinetic parameters of the stem-loop 69 modification enzymes
4. to describe the substrate recognition and the methyl group transfer reaction mechanisms of the $m^3\Psi$ methyltransferase

I. IDENTIFICATION OF THE PSEUDOURIDINE METHYLTRANSFERASE RlmH (REF. I)

The strategically located stem-loop 69 (H69) of 23S rRNA contains three modified nucleosides in *E. coli*: pseudouridines (Ψs) at positions 1911 and 1917, and a methylated pseudouridine ($m^3\Psi$) at position 1915 (chapter 1.1 and Figure 4). While the pseudouridine synthase (PS) RluD was known to be responsible for the synthesis of all three Ψs in H69 (Huang *et al.*, 1998; Raychaudhuri *et al.*, 1998), the gene encoding for the methyltransferase (MT) responsible for introducing a methyl group to the N3 nitrogen of Ψ at position 1915 was previously not known.

In order to identify the gene encoding for the 23S rRNA $m^3\Psi$1915 MT, we selected as candidates 11 genes (*ybiN*, *ymfD*, *yafE*, *yafS*, *yjhP*, *yjtD*, *yfiF*, *yibK*, *ybeA*, *ygdE*, and *smtA*) annotated as putative RNA MT in *E. coli*. The corresponding deletion strains (Ref. I Figure 2) were ordered from the KEIO collection containing single gene knockouts of all non-essential genes in the *E. coli* K-12 strain background (Baba *et al.*, 2006). Total rRNA was extracted from the putative RNA MT deletion strains and from *E. coli* WT strain (MG1655), and monitored for the methylation status at the 23S rRNA position 1915 by primer extension analysis followed by separation of cDNAs using poly-acrylamide gel electrophoresis (PAGE). Methylation at the N3 position of both uridine (U) and Ψ perturbs Watson–Crick base-pairing and results in a strong reverse transcriptase stop.

In case of the WT strain and all but one putative RNA MT deletion strains, a strong stop signal corresponding to the position 1915 of 23S rRNA was observed (Ref. I Figure 2 lanes 1–12), indicating the presence of a methyl group at N3 of the base. The only analyzed rRNA not exhibiting a strong stop signal at the corresponding position was extracted from the cells lacking the *ybeA* gene (Ref. I Figure 2 lane 9) suggesting that the *ybeA* gene encodes for the $m^3\Psi$1915 MT. In order to exclude the possibility that some unknown second-site mutation(s) in the KEIO collection's *ybeA* deletion strain are responsible for the loss of methylation at 23S rRNA position 1915, the effect of replacing the *ybeA* gene with the kanamycin resistance cassette was also tested in the genetic background of the *E. coli* WT MG1655 strain. The absence of the $m^3\Psi$1915 methylation is evident from both the primer extension analysis (Ref. I Figure 2 lane 18) and the reversed phase high pressure liquid chromatography (RP-HPLC) analysis of the nucleosides of the H69 region (Ref. I Figure 3B and Table 1) of the newly constructed *ybeA* deletion strain. In RP-HPLC analyses, an RNA fragment comprising nucleotides 1777–1922 of the 23S rRNA derived from oligonucleotide-directed RNase H excision was used. Retention times of the MN in 23S rRNA fragment under the conditions used are: 4.9 minutes (Ψ), 11.2 minutes ($m^3\Psi$), 23.2 minutes (m^3U), and 32.2 minutes (m^2G) according to (Gehrke & Kuo, 1989). Obtained amounts of nucleosides were presented in relation to the 100% value of the corresponding nucleosides in the WT probe. As the m^3U was not detected in the WT probe, the fraction of the m^3U in

following experiments was calculated with respect to the maximum theoretical amount of m^3U in the corresponding RNA fragment (Ref. II Figure 1 and Table 1).

It should be noted that the deletion of the *ybeA* gene does not have a detectable effect on the bacterial growth rate in rich medium either at 25°C, 37°C, or 42°C (data not shown). Introducing a recombinant *ybeA* gene (encoding for N-terminally His_6-tagged YbeA protein) from an isopropyl-β-D-thiogalactopyranoside (IPTG) inducible plasmid restored the WT-like methylation pattern in the H69 region of 23S rRNA in the *ybeA* deletion strain as revealed by primer extension analysis, although a slight increase in the read-through past position 1915 was visible (Ref. I Figure 2 lane 19). In good agreement, the RP-HPLC analysis of the nucleoside composition revealed that the plasmid born recombinant YbeA restores the methylation in the *ybeA* deletion strain, introducing the $m^3\Psi$ modification to about half of the ribosomes (Ref. I Figure 3C and Table 1) even though the protein is present in much larger quantities than in the WT cells (data not shown).

All in all, these results unambiguously establish the YbeA protein as the methyltransferase responsible for methylating Ψ1915 in *E. c*oli 23S rRNA to $m^3\Psi$. The involvement of the YbeA protein in the 23S rRNA $m^3\Psi$1915 synthesis was confirmed independently by Purta and co-workers using MALDI mass spectrometry (Purta *et al.*, 2008). In agreement with the accepted convention (Ofengand & Del Campo, 2004a; Andersen & Douthwaite, 2006), we proposed to rename the YbeA protein to RlmH (rRNA large subunit methyltransferase H).

RlmH was the first pseudouridine methyltransferase to be identified and is likely to be the only one existing in bacteria, as $m^3\Psi$1915 is the only methylated Ψ in bacterial RNAs described to date (Czerwoniec *et al.*, 2009; Cantara *et al.*, 2011). In archaea and eukaryotes, on the other hand, $m^1\Psi$, Ψm, and $m^1acp^3\Psi$, but no $m^3\Psi$ have been found (Czerwoniec *et al.*, 2009; Cantara *et al.*, 2011). Since then, it has been established that Nep1 (Emg1) is the MT responsible for the synthesis of $m^1\Psi$ in *S. cerevisiae* SSU 18S rRNA (Leulliot *et al.*, 2008; Taylor *et al.*, 2008) and TrmY is the MT responsible for $m^1\Psi$54 in *Haloferax volcanii* tRNAs (Chatterjee *et al.*, 2012; Wurm *et al.*, 2012). RlmH, Nep1, and TrmY all belong to the intriguing SPOUT superfamily of MT (chapter 2.3.2) (Tkaczuk *et al.*, 2007). The crystal structure of the RlmH protein has been determined and like other SPOUT MT crystallizes as a homodimer and contains the deep C-terminal trefoil knot formed by the threading of the last 35 amino acids through a 45 amino acid long knotting loop (Benach *et al.,* 2003). However, unlike the other two *E. coli* SPOUT MT that methylate rRNA (RlmB and RsmE), RlmH monomers are antiparallel (chapter 2.3.2). Dimerization is thought to be important for the co-factor S-adenosyl-L-methionine (SAM) dependent methyltransferase function of all SPOUT MT (Tkaczuk *et al.*, 2007). RlmH is composed solely of the core catalytic domain characteristic of the SPOUT MT and lacks the extra N-terminal domains exhibited by RlmB and RsmE (Table 3) (Tkaczuk *et al.*, 2007).

Proteins with significant similarity to RlmH exist in virtually all bacteria, and the corresponding gene is also present in the genomes of green plants and in a few archaeal species (Ref. I Figure 5) probably resulting from a horizontal gene transfer (Tkaczuk *et al.*, 2007). In agreement, the RlmH ortholog in *Arabidopsis thaliana* is annotated as a chloroplast protein. The RlmH orthologs likely perform the same function in these organisms. While the $m^3\Psi$ modification in 23S rRNA was experimentally determined in *E. coli* (Kowalak *et al.*, 1996) and *D. radiodurans* (Del Campo *et al.,* 2005); *B. subtilis* and *Z. mays* chloroplast LSU rRNAs likely contain the equivalent of $m^3\Psi$1915 as revealed by a strong reverse transcriptase stop (Ofengand *et al.*, 1995). The exact extent of the conservation of the $m^3\Psi$ modification at the position equivalent to *E. coli* 23S rRNA 1915 is unknown; however, there is no evidence of $m^3\Psi$ methylation within the cytoplasmic ribosomes of eukaryotes.

The distribution of RlmH orthologs among different species and the "uniqueness" of the $m^3\Psi$ modification imply that there must have been a compelling reason for the cells to evolve an enzyme catalyzing this modification. Deletion of the *rlmH* gene resulting in the loss of $m^3\Psi$1915 in H69 does not have a detectable effect on the bacterial growth rate at least as far as rich medium is concerned. However, Purta and co-workers demonstrated that cells lacking functional RlmH protein had a clear competitional growth disadvantage against *E. coli* WT cells (Purta *et al.*, 2008) indicating the functional importance of $m^3\Psi$ either in ribosome biogenesis, stability, or functioning.

Hydrophilic pseudouridylations and hydrophobic methylations rarely occur in the same position of RNA. As discussed in chapter 1.1, Ψ1915 has a destabilizing effect (~0.5 kcal/mol) on the H69 structure (Meroueh *et al.*, 2000), while the following N3-methylation of Ψ1915 causes an increase (~0.5 kcal/mol) in the thermodynamic stability without causing any substantial changes to the overall structure of H69 (Chui *et al.*, 2002). It is tempting to propose that Ψ1915 is synthesized as a "by-product" by RluD whose real purpose is to synthesize the universally conserved and functionally important Ψ1917 (discussed in chapter 1.1). It is conceivable that the destabilizing/hydrophilic effect of the Ψ1915 on H69 structure is compensated by the stabilizing/hydrophobic effect of the $m^3\Psi$1915. The idea that the main role of the RlmH-dependent methylation at position 1915 lies in countering the destabilizing effect of a Ψ at this position and is not essential for other ribosomal functions is in agreement with the observation that replacing the U/Ψ at 1915 with the isosteric C does not affect the growth rate of the *E. coli* cells (Hirabayashi *et al.*, 2006). However, Chow and co-workers have suggested that methylation of Ψ1915 may play a role in stabilizing specific long-range tertiary RNA-RNA or RNA-protein interactions, rather than be involved in the maintenance of the local conformation of H69 (Chui *et al.*, 2002).

2. SUBSTRATE SPECIFICITY OF RlmH (REF. I AND II)

2.1 RlmH modifies 70S ribosomes

RluD was shown to synthesize the H69 Ψs, including the one located at position 1915, using the assembled LSU isolated from the *rluD* deletion strain as a substrate (Leppik *et al.*, 2007; Vaidyanathan *et al.*, 2007) (chapter 2.3). Furthermore, analysis of the pseudouridylation pattern of 23S rRNA isolated from ribosome assembly precursor particles showed that the Ψs in H69 are formed during the late assembly steps (Leppik *et al.*, 2007). RlmH, which was predicted to follow RluD in action (Raychaudhuri *et al.*, 1998) in modifying the U1915 of H69 would therefore also be a late assembly specific rRNA ME acting at the stage of assembled LSU or even later.

In order to determine the substrate of RlmH, incorporation of methyl groups into LSU and 70S ribosomes isolated from the *rlmH* deletion strain was tested *in vitro* using purified RlmH protein. As revealed by both the incorporation of [^{14}C]-methyl groups derived from the [^{14}C]-SAM co-factor into the trichloroacetic acid (TCA) insoluble material and by the RP-HPLC analysis of the nucleoside composition of the H69 region following the *in vitro* methylation assay, both the N-terminally His_6-tagged RlmH protein and the native (non-tagged) RlmH protein were able to methylate the 70S ribosomes, with methylation efficiencies ~60% and ~90%, respectively (Ref. I Figure 3D, Figure 4, and Table 1; Ref. II Figure 1 and Table 1). Unlike the His-tagged RlmH protein, the native RlmH protein was able to methylate the LSU as well (with ~80% efficiency) when excess amounts of protein (~200-fold molar excess) and long incubation times (1 hour) were used (Ref. I Figure 4 and Ref. II Table 1). As these results did not exclusively establish the preferred substrate, the methyltransferase activity of the native RlmH protein using either 70S ribosomes or LSU and SSU isolated from the *rlmH* deletion strain as substrates at various Mg^{2+} concentrations was studied. It is known that 70S ribosomes dissociate at Mg^{2+} concentrations below 2 mM, whereas SSU and LSU associate to form 70S ribosomes at Mg^{2+} concentrations above 6 mM (Blaha *et al.*, 2002). Testing RlmH activity within this range of Mg^{2+} concentrations allows determining whether the LSU or the 70S ribosomes are modified. RlmH dependent incorporation of [^{3}H]-methyl groups was monitored and revealed to follow the same Mg^{2+} dependency pattern as ribosome subunit association/ dissociation (Ref. II Figure 2). This establishes that the RlmH protein needs its substrate base to be presented in the context of the associated 70S ribosomes and not in free LSU for the methylation reaction to take place. The RlmH activity seen on free LSU in the *in vitro* methyltransferase assay (Ref. II Table 1) can be explained by the presence of trace amounts of SSU in the LSU preparation.

While RlmA, RlmE, and RluD have also been shown to be able to modify 70S ribosomes, free LSU is still preferred as substrates by these rRNA ME (Bugl *et al.*, 2000; Caldas *et al.*, 2000a; Vaidyanathan *et al.*, 2007). Hence,

RlmH is the only rRNA ME shown to modify 70S ribosomes exclusively. Requirement for the 70S ribosomes indicates that RlmH is acting during the final stages of ribosome assembly, probably during translation initiation as this is when the 70S ribosomes are formed in the cell. Based on this finding, Purta and co-workers (Purta *et al.*, 2008) docked the crystal structure of the RlmH dimer onto the *E. coli* 70S ribosome (Schuwirth *et al.*, 2005). The docking data suggest that in order to position the methyl group donor SAM adjacent to the substrate Ψ1915, the RlmH dimer would have to bind into the ribosomal A-site at the interface between the SSU and the LSU (Purta *et al.*, 2008). According to this model, RlmH binding to the ribosome A-site would not interfere with tRNA binding to the ribosomal P-site, supporting the notion that the physiological substrate of RlmH is the translation initiation complex with initiator tRNA in the P-site. Remarkably, RlmH seems to recognize its substrate by making simultaneous contacts with both ribosomal subunits, a feature that has not been previously seen for any of the rRNA ME. Moreover, the interactions with the "non-substrate" SSU are expected to be extensive and to contribute considerably to the orientation and the stability of the RlmH-ribosome complex, whereas the interactions with the LSU are mainly confined to the loop region of the H69 (Purta *et al.*, 2008). In good agreement, RlmH protein is known to co-purify primarily with the SSU r-proteins (Sergiev *et al.*, 2012). Thus, RlmH could use almost its entire surface for precise substrate recognition, in contrast to the usual case where a substrate fits into an active-site cleft of the ME, RlmH fits into a cleft of its substrate.

The docking data prompted Purta and co-workers to speculate that RlmH is involved in the quality control of the ribosome biogenesis and that the corresponding methylation may functions as a stamp of approval indicating that LSU has been engaged in translational initiation (Purta *et al.*, 2008). However, the who and the how of the ribosome validation process is enigmatic.

Altogether, RlmH is inclined to be the ultimate rRNA ME acting during the ribosome biogenesis and the first example of rRNA modification process overlapping with translation. The fact that RlmH modifies LSU only in 70S ribosomes demonstrates the dependence of LSU maturation on not just the mere presence of SSU, but also the ability of both LSU and SSU to be incorporated into 70S ribosomes. Final processing of the LSU rRNA ends also appears to take place during the translation initiation complex formation, or during the first cycles of translation as discussed in chapter 2.2.

2.2 RlmH modifies pseudouridine

Ψ results from isomerization of U and the substrate atom of RlmH, the N3-nitrogen, is located at the same position in both of those bases (chapter 2.3.1 and Figure 6). Thus, Ψ and U could be difficult to distinguish by RlmH. However, it was proposed that Ψ would be a better substrate for the following methylation reaction (Raychaudhuri *et al.*, 1998). Furthermore, Purta and co-

workers made an interesting observation that every genome containing an *rlmH* ortholog also contains an *rluD* ortholog, whereas the opposite is not the case (Purta *et al.*, 2008), which is in good agreement with the requirement for the prior Ψ formation for the RlmH dependent methylation to occur.

To check whether ribosomes with U instead of Ψ at position 1915 of 23S rRNA can be methylated by RlmH, 70S ribosomes isolated from the *E. coli* strain lacking both the *rlmH* and *rluD* genes were tested in the *in vitro* methylation assay using purified RlmH and RluD proteins. Our results indicate that RlmH protein was unable to incorporate [^{3}H]-methyl groups into the 70S ribosomes of the *rlmH/rluD* strain unless they were first treated with RluD protein introducing the H69 Ψs (Ref. I, Figure 4). For quantitative assessments, RP-HPLC analysis of the nucleoside composition of the H69 region was performed following the *in vitro* methylation assay. Even when the ribosomes were incubated for as long as 1 hour with ~200-fold molar excess of native RlmH protein, the level of U1915 methylation in *rluD* deletion and *rluD/rlmH* double deletion strain ribosomes was relatively low (~20–30%), whereas under the same conditions, the level of Ψ1915 methylation in *rlmH* deletion strain ribosomes was ~90% (Ref. II Figure 1 and Table 1). This shows that RlmH preferentially methylates Ψ at position 1915 of 23S rRNA *in vitro*. To confirm the substrate nucleotide specificity of the RlmH protein *in vivo*, we analyzed the nucleoside composition of 23S rRNA H69 region isolated from *rluD* deletion and *rluD/rlmH* double deletion strain ribosomes and studied the effect of complementing these strains with the plasmid-encoded RlmH protein. Nucleoside composition analysis by RP-HPLC revealed that a small fraction (<10%) of m^3U was detected in the *rluD* deletion strain but not in the *rluD/rlmH* double deletion strain (Ref. II Figure 1 and Table 1). This finding indicates that in the absence of Ψ1915, RlmH present in amounts normally found in cells, is able to methylate U1915, albeit very inefficiently. Expressing the RlmH protein from a plasmid in the *rluD* and *rluD/rlmH* strains causes a significant amount ~50%–60%) of m^3U formation (Ref. II Figure 1 and Table 1) that can be attributed to the high level of protein overexpression from the arabinose-inducible plasmid construct (data not shown). It should be mentioned that although it is widely accepted that a MN at a given site in rRNA is present in all ribosomes in WT cells, this is not entirely the case for the MN of H69. Purta and co-workers showed that methylation by RlmH was less than stoichiometric and remained constant during the logarithmic growth phase even when the intracellular concentration of RlmH was increased (Purta *et al.*, 2008). A slight increase in the amount of both Ψ and $m^3Ψ$ in the H69 region of 23S rRNA was detected when ribosomes isolated from *E. coli* MG1655 and BL21 stationary phase cells were compared to ribosomes isolated from mid-logarithmic phase cells (data not shown). The explanation that fits best with these observations is that the unmethylated faction in rapidly growing cells reflects recently synthesized 23S rRNA molecules that have not yet reached the late stages of ribosome biogenesis when H69 MN are synthesized.

Our ability to detect the m^3U1915 modification in the 23S rRNA lacking Ψs in H69 indicates that when RlmH protein is in excess and the substrate Ψ is not available, RlmH can also methylate U at the same position both *in vitro* and *in vivo* (Ref. II Figure 1 and Table 1). In contrast to the ribosomes isolated from the *rluD* and *rluD/rlmH* deletion strains, we failed to detect the m^3U1915 modification in the ribosomes isolated from the *E. coli* WT strain (Ref. II Figure 1 and Table 1). The absence of the m^3U1915 modification in the WT strain is most likely explained by the sequential action of the ME RluD and RlmH. Thus, when the functional RluD enzyme is present in the cells, the U at the 1915 position is quantitatively converted into Ψ and there is no U left for RlmH to act upon.

While it remains to be determined what the exact mechanism is that allows RlmH to distinguish between U and Ψ, it very likely involves recognizing the N1 nitrogen atom present only in the latter. Even though much less likely, it can not be excluded that the presence of the other two Ψs in H69 (Ψ1911 and Ψ1917), also synthesized by RluD, are directly or indirectly required for the RlmH dependent methylation.

Taken together, although the activity of RlmH is not uniquely restricted to Ψ, the sequential action of the modification enzymes RluD and RlmH ensures that it is m^3Ψ and not m^3U that is the main modification at the 1915 position of 23S rRNA.

3. KINETIC PARAMETERS (REF. II)

As discussed in chapter 2.1, ribosomal subunits acquire their final sedimentation value in less than a minute after the transcription of the pre-rRNA is completed (Lindahl, 1975). The freshly formed subunits are immature and take additional 1 to 2 minutes at 37°C (Lindahl, 1975) and ~5 minutes at 25°C (Peil *et al.*, 2008) to become able to participate in translation. Therefore, the rate-limiting step in ribosome biogenesis is the final maturation of the subunits after majority, if not all, of the r-proteins have already associated with the rRNA. Conformational rearrangements in rRNA and r-proteins, as well as a subset of rRNA processing and modification events are known to take place during that time (chapter 2). In case of the LSU, only a few of the rRNA MN, namely the three Ψs in the H69 and Um2552, are shown to be introduced at this stage (Bugl *et al.*, 2000; Leppik *et al.*, 2007; Vaidyanathan *et al.*, 2007). Incidentally, the same modifications have been implicated in ribosome assembly (discussed in chapter 3.4). We determined that the H69 $m^3\Psi$1915 MT RlmH uniquely modifies already associated 70S ribosomes (Ref. I and Ref. II) assigning it to the last stages of ribosome biogenesis that probably coincides with the translation initiation. However, the rates of the rRNA modification reactions during the late stages of ribosome biogenesis have remained unknown.

3.1 RlmH

To characterize the kinetics of the RlmH dependent methylation reaction, we determined the apparent K_M and k_{cat} values of the RlmH protein for its substrate and for co-factor SAM. The *rlmH* deletion strain 70S ribosomes, purified N-terminally His_6-tagged or native RlmH proteins, and [^{3}H]-SAM were used to monitor the amount of [^{3}H]-methyl group incorporation into TCA insoluble material. The initial rates of the methylation reaction were calculated from the time-courses of the product formation at different substrate or co-factor concentrations. The apparent K_M and k_{cat} values for the RlmH-catalyzed methylation reaction were estimated by fitting the data to the standard Michaelis-Menten equation. The methylation rates were measured at varying concentrations of the 70S ribosome and at a SAM concentration (100 μM) that is 4-fold above the respective K_M value (Ref. II Figure 3). For co-factor, the initial methylation rates were determined with constant (2 μM) 70S ribosome and varying SAM concentrations (Ref. II Figure 3).

Based on the kinetic constants, the RlmH dependent methylation is a relatively fast process with a k_{cat} value of ~5–6 min^{-1} and apparent K_M values of ~0.5 and ~27 μM for 70S ribosomes and co-factor SAM, respectively (Ref. II Table 2). k_{cat} values determined for both 70S ribosomes and co-factor SAM correlated relatively well (varying <20%), indicating that the kinetic constants were obtained under appropriate conditions. It should be noted that the N-terminally His-tagged RlmH protein is significantly less efficient compared to the

native RlmH protein (Ref. II Table 2). The snug fit of the RlmH dimer between the ribosome subunits as suggested by the docking data (Purta *et al.*, 2008) would explain why the His-tagged RlmH was less active, namely, one or both (one per monomer) of the N-terminal His-tags located far from C-terminal active site of RlmH could hinder substrate recognition and/or RlmH binding to the 70S ribosome by sterically clashing with the SSU. The other aspect that can be explained by the RlmH-ribosome binding model is the lack of additional domains attached to the catalytic core domain of RlmH.

RlmH was the first pseudouridine-specific MT to be enzymatically characterized and was shown to be a relatively fast enzyme (k_{cat} value of ~5–6 min^{-1}) when 70S ribosomes were used as a substrate, yet again confirming that 70S is the true substrate of RlmH. Only a few other rRNA MT (RsmE, RlmD, and RlmE) have been kinetically characterized. The k_{cat} value determined for RlmD (synthesizes m^5U1939 on 23S rRNA) is 3.6 min^{-1} (Agarwalla *et al.*, 2002), which is in good agreement with the early assembly-specific modification enzymes being relatively fast, as the time frame when their substrate is available, is limited in cells (discussed in chapter 2.3). The k_{cat} values for RsmE (synthesizes m^3U1498 on SSU) and RlmE (synthesizes Um2552 on LSU), are 0.078 and 0.064 min^{-1}, respectively (Hager *et al.*, 2004; Basturea & Deutscher, 2007). It is unlikely that RsmE and RlmE enzymes act this slowly *in vivo*. More likely, their physiological substrates are different from the substrates tested *in vitro*, or alternatively, the purified enzymes have lost a co-factor that enhances their activity in cells.

3.2 RluD

Since the conversion of U to Ψ is an isomerization reaction (chapter 2.3.1) and, unlike the methylation reaction, nothing is added to the substrate, there is nothing that can be radioactively tagged and traced. The only rapid quantitative assay for pseudouridylation activity measures the release of [^{3}H] from C5 of U upon Ψ formation (Ramamurthy *et al.*, 1999b) but because this method requires the *in vitro* transcription of the PS substrate in order to incorporate the 5-[^{3}H]-uracil, it sets constraints to the size and the complexity of the substrate that can be used. Consequently, the kinetic parameters were not available for any of the rRNA PS apart from RluA (Ramamurthy *et al.*, 1999b), which in addition to rRNA also modifies tRNA allowing its kinetic characterization at least as far as tRNA is concerned.

The finding that RlmH preferentially methylates Ψ in the context of the 70S ribosomes, whereas the substrate of the RluD protein is the LSU, gave us the opportunity to use a coupled assay with RlmH as the reporter enzyme to determine the kinetic parameters of the U1915 pseudouridylation by RluD. As methylation of Ψ1915 by RlmH was found to be a relatively fast reaction, pseudouridylation of U1915 by RluD was believed to be the rate-limiting step.

It must be noted that the association of ribosomal subunits is a fast reaction and occurs in less than a second (Antoun *et al.*, 2004; Hennelly *et al.*, 2005).

The two-step coupled enzyme assay scheme:

$$LSU^{U1915} + RluD \rightarrow LSU^{\Psi 1915} + SSU + RlmH + SAM \rightarrow 70S^{m3\Psi 1915}$$

In the first step, LSU subunits of the *rluD/rlmH* double deletion strain were incubated with the purified RluD protein for various time intervals. In the second step, the pre-incubated mix consisting of SSU, purified native RlmH protein (at saturating concentration), and [H^3]-SAM was added to the first mix and incubated for 15 seconds. Control experiments demonstrated that in the presence of SSU, there is no detectable RluD dependent pseudouridylation during the 15 second incubation step (data not shown). Incorporation of [H^3]-methyl groups was determined by TCA precipitation and liquid scintillation counting.

The initial rates of U1915 pseudouridylation were obtained at a fixed concentration of RluD and varying concentrations of LSU (Ref. II Figure 4). Apparent K_M and k_{cat} values were calculated in a similar manner to RlmH and were based on the assumption that the Ψ1915 methylation reaction by RlmH follows the first-order kinetics. The apparent K_M value of ~1 μM for LSU and a k_{cat} value of ~2 min^{-1} for U1915 isomerization by RluD were obtained (Ref. II Table 2).

While the exact mechanism of substrate nucleotide (U1911, U1915, and U1917 in H69) recognition and pseudouridine synthesis by RluD is not known, formation of Ψs at positions 1911 and 1917 was shown to be autonomous of each other and independent of the Ψ1915 formation (Leppik *et al.*, 2007). U1911 and U1917 in H69 are likely isomerized at similar rates as U1915 given that their synthesis is stochastic and does not seem to happen in any specific order. This is based on the observation that all three H69 Ψs appear concurrently over time upon RluD treatment of the LSU (Ref. II Figure 5) as revealed by the chemical modification of 23S rRNA followed by reverse transcriptase directed primer extension analysis and separation of cDNAs by Urea PAGE (Bakin & Ofengand, 1993; Ofengand *et al.*, 2001a). Thus, as all H69 Ψs are made concurrently, the kinetic parameters determined for the synthesis of Ψ1915 can in all likelihood be extrapolated for the synthesis of Ψ1911 and Ψ1917 as well.

RluD is the first rRNA PS to be kinetically characterized and, according to current knowledge (Siibak & Remme, 2010), the only PS acting during the late stages of ribosome assembly. The rate of RluD dependent pseudouridylation *in vitro* is sufficiently fast for this modification to take place during the time frame of final LSU maturation in cells further confirming that LSU is the physiological substrate of RluD.

4. SUBSTRATE RECOGNITION AND CATALYTIC MECHANISM OF RlmH (REF. I AND III)

To shed light onto the mechanism by which the RlmH protein recognizes first its substrate base, Ψ1915 of H69, within the 70S ribosome; then its substrate atom, N3 nitrogen, within the aromatic ring of Ψ1915; and finally catalyses the transfer of a methyl group from co-factor SAM to the aforementioned nitrogen; we performed a mutagenesis study on both of the players in this process.

4.1 Ribosome mutagenesis

As already discussed, docking of the crystal structure of the RlmH dimer into the crystal structure of the 70S ribosome suggested that RlmH makes extensive contacts with the SSU and interacts with the LSU mostly in the H69 loop region (Purta *et al.*, 2008). To elucidate the substrate recognition mechanism of RlmH, the significance of individual bases in H69 loop region were assessed by monitoring the formation of $m^3\Psi1915$ *in vitro* on ribosomes with alterations in this region of 23S rRNA.

The following single base alterations in H69 of 23S rRNA were analyzed: A1912U, C1914U, A1916U, A1916C, A1919U, and A1919G (outlined in Ref. III Figure 1). Mutation A1960G located in the 23S rRNA stem-loop 71 (H71) that forms a part of the inter-subunit bridge B3 (Yusupov *et al.*, 2001), was also analyzed. Mutations A1912U, A1919U, and A1916G in H69 have previously been shown to severely impair ribosome functioning *in vitro* (Liiv *et al.*, 2005; Kipper *et al.*, 2009). A1912 and A1919 of H69 are involved in the formation of the inter-subunit bridge B2a (discussed in chapter 1.1). In current study, we were not able to analyze ribosomes containing A1916G and A1918U variants of 23S rRNA, probably due to assembly defects or instability of the mutant ribosomes in the *E. coli rluD/rlmH* double deletion strain. The *rluD/rlmH* strain was used for the expression and purification of the variant LSU so that the MN in H69 would be absent. To avoid possible defects due to incomplete assembly, the variant LSU were isolated from 70S ribosomes using streptavidin sepharose affinity chromatography (23S rRNA variants contained a streptavidin-binding aptamer in helix 25) (Leonov *et al.*, 2003). Since the absence of the RluD enzyme strongly impairs cellular growth (Gutgsell *et al.*, 2005) and therefore reduces the yield of the variant ribosomes, the *rluD/rlmH* double deletion strain was constructed using a so-called "pseudorevertant" *rluD* deletion strain. Due to compensatory mutations elsewhere in the genome, the pseudorevertant strain has a WT-like growth phenotype despite lacking a functional RluD enzyme and the Ψs in H69 (discussed in chapter 3.4).

The activity of RlmH protein on ribosomes with base alterations in 23S rRNA was tested *in vitro*. Variant LSU were pre-incubated with 10-fold molar excess of RluD protein for 10 minutes in order to introduce the H69 Ψs (foremost the prerequisite Ψ1915). Also, SSU were pre-incubated with varying concentrations (up to 6-fold molar excess compared to LSU) of native RlmH

protein and [^{3}H]-SAM (100 μM). The pre-incubation reactions were mixed together and incubated for 1 minute. It should be noted that the RlmH dependent modification of ribosomes with native H69 sequence occurs at significantly lower RlmH concentrations within seconds (k_{cat} ~5 min^{-1}) (Ref. II Table 2). Therefore, this experiment was designed to reveal major effects of the mutations in H69 on the activity of RlmH. The [^{3}H]-methyl group incorporation into TCA insoluble material was monitored by scintillation counting.

The efficiency of RlmH dependent methylation of ribosomes with native H69 sequence was ~80% and was not affected by the presence of the affinity tag used for LSU purification (data not shown). When the RlmH-dependent methylation was analyzed on the H69 variant ribosomes, all of the alterations introduced into the H69 had an inhibitory effect on the RlmH activity *in vitro* (Ref. III Figure 4A-C). The strongest negative effects were observed with replacements at position 1919 and 1912. Somewhat less severe inhibition of RlmH activity was seen with replacements at positions 1914 and 1916. Whether the RlmH substrate Ψ1915 was synthesized *in vivo* or *in vitro* did not affect the methylation efficiency, as *rlmH* deletion strain ribosomes behaved similarly to *rluD/rlmH* double deletion ribosomes treated with RluD *in vitro* (data not shown).

Since RlmH methylates H69 only in the 70S ribosomes and requires interactions with the SSU for its activity (Ref. I and II), any alteration that affects the ability of ribosomal subunits to associate would also be likely to affect the activity of the RlmH protein. In order to estimate to what extent the impairment in methylation of variant LSU was caused by defects in subunit association as opposed to the effects of the mutations on the structure of the H69 *per se* required for RlmH dependent modification, the ability of the variant LSU to form 70S ribosomes was analyzed. One A_{260} (absorbance at 260 nm) unit of both LSU and SSU, treated the same way as in methylation assay, were loaded onto sucrose density gradient, subjected to ultracentrifugation, and the ribosome profiles were analyzed with continuous monitoring of absorbance at 254 nm. As revealed by ribosome profiles, the re-association efficiency was decreased with most of the LSU variants that exhibited significant inhibitory effects on RlmH activity (Ref. III Figure 4E). The strongest defect in 70S formation was observed with the A1919U variant LSU that was also one of the poorest substrates for RlmH in methylation assay (Ref. III Figure 4). The extent of the subunit association defect of A1919U was comparable to the one caused by the A1960G mutation in H71 (located away from the H69 and forming a part of the intersubunit bridge B3), which was used as a control for a mutation known to cause an association defect (Liiv & O'Connor, 2006). However, the inhibitory effect of the A1919U mutation on the RlmH activity was significantly stronger than the one caused by the A1960G mutation (Ref. III Figure 4). The lack of correlation between the effects of the mutations in H69 on 70S formation and on methyltransferase activity of RlmH is further underscored by the A1912U replacement that significantly inhibited RlmH activity but only moderately affected 70S formation (Ref. III Figure 4). Taken together, these findings argue against the subunit association defect being the primary cause for the impair-

ment in the RlmH activity on H69 variant ribosomes. Instead, the impairment in RlmH dependent methylation could be attributed to the altered conformation of the H69 within the variant 70S ribosomes.

As the co-crystal structure of the RlmH protein in complex with the 70S is not available, the structural basis of our results can be discussed only tentatively. Evidently, a number of H69 nucleotides are important for RlmH activity *in vitro* (Ref. III Figure 4). While the adenines at positions 1912 and 1919 of H69 seem to be almost a prerequisite for the action of RlmH, mutations at positions 1914 and 1916 also exhibited a significant effect on RlmH activity. Interestingly, the most important nucleotides for the activity of RlmH, A1912 and A1919, are more distant to the actual modification site at position 1915 (Ref. III Figure 1 and Figure 5). It seems that RlmH is able to methylate Ψ1915 at least to some extent *in vivo* on ribosomes with single base alterations Ψ1911C, A1912C, A1913U, A1913G, C1914A, C1914G, A1916C, Ψ1917C, and A1918G in H69 as revealed coincidentally by the chemical modification analyses of Ψs in the H69 region (Hirabayashi *et al.*, 2006; Leppik *et al.*, 2007; Monshupanee *et al.*, 2008), however, no conclusions about the effect of these mutations on the efficiency of the methylation reaction can be drawn from these experiments.

One possible explanation for the observed effects is to assume that RlmH makes extensive and specific contacts with most of the bases in the loop region of the H69. However, this is in apparent conflict with the docking model of Purta and co-workers, which predicted contacts between RlmH and the tip of H69, not including the nucleotides A1912 and A1919 (Purta *et al.*, 2008). Molecular dynamics simulations have shown that mutations at positions 1912 and 1919 affect the conformation of the H69 (Kipper *et al.*, 2009). This conformational change in H69 may displace the Ψ1915, explaining the severe effect of the mutations at 1912 and 1919 positions on the RlmH dependent methylation despite their distance from the actual modification site. Nucleotides C1914 and A1916 (right next to the substrate Ψ1915), whose replacement exhibited less severe defects, can either interact with the RlmH protein directly or, alternatively, be important for the presentation of Ψ1915 in the proper conformation. Taken together, the fact that replacements at positions 1914 and 1916 had a smaller impact on the RlmH activity than the replacements at the more distant positions, 1912 and 1919, indicates that rather than interacting directly with the nucleosides neighboring its substrate Ψ1915, RlmH is sensitive to the conformation of the H69 within the 70S ribosome.

Interestingly, unlike RlmH, the RluD protein that modifies not just the same region of the ribosome but even the same position, namely the U1915, is relatively insensitive to single base alterations at neighboring positions in H69 as revealed by chemical modification analysis of Ψ formation in the H69 region of 23S rRNA. Both the *in vitro* (Ref. III Figure 2B) and the *in vivo* (Leppik *et al.*, 2007) analyses of RluD activity suggest that A1916 serves as an important specificity determinant for RluD.

As discussed in chapter 2.3.3, rRNA ME most likely rely on substrate dynamics, the ability of RNA to refold into an “unusual” conformation, as a

method of substrate recognition. The ability of RNA to adapt certain conformations, however, depends on its sequence. As a result, substrate recognition by ME can occur without a large number of direct contacts to conserved bases in rRNA (Byrne *et al.*, 2009). Results of the H69 mutagenesis analysis are in agreement with RlmH and RluD proteins employing the "indirect readout" mechanism of substrate recognition described previously for rRNA ME RlmD and RluA (Lee *et al.*, 2005; Hoang *et al.*, 2006). However, it is also possible that the main specificity determinants of rRNA ME lie outside the immediate vicinity of the site of modification. In case of RluD, the N-terminal S4-like domain is probably also involved in substrate recognition and binding (Vaidyanathan *et al.*, 2007) (chapter 2.3.1 and Table 2).

In addition to interacting with the H69 region of the LSU, RlmH also interacts with the SSU (Ref. I and II; Purta *et al.,* 2008). However, it is not known which regions of the SSU and to what extent the RlmH protein interacts with. The binding efficiency of the RlmH to 70S ribosomes was tested *in vitro*. Due to similar migration of RlmH and several r-proteins on the sodium dodecyl sulfate (SDS) PAGE, verifying the binding of RlmH to ribosomes required the purification of a ^{35S}Met/^{35S}Cys-labeled RlmH protein (data not shown). 70S ribosomes isolated from the WT, *rluD* deletion, and *rluD/rlmH* double deletion strains were incubated with 5-fold molar excess of the labeled RlmH in the presence of sinefungin (SAM analog, acts as a MT specific inhibitor). One part of the reaction mixture was TCA precipitated, analyzed on SDS-PAGE, and the radioactivity was visualized by a Typhoon Phosphoimager; the other part of the reaction mixture was TCA precipitated and analyzed by liquid scintillation counting. RlmH protein was shown to bind to both *rluD* and *rluD/rlmH* deletion strain, but not to *E. coli* WT strain ribosomes (data not shown). While the binding was most efficient with the *rluD/rlmH* ribosomes, it was still less than 25%. In accordance, hydroxyl-radical footprinting experiments on the *rluD/rlmH* 70S/RlmH/sinefungin complex showed only a modest protection in the H69 region and yielded no conclusive results about the interactions between the RlmH and SSU (data not shown). Furthermore, a high-resolution cryo-electron microscopy (cryo-EM) image processing and three-dimensional structure reconstitution (performed by the laboratory of Prof. D. Wilson) of the *rluD/rlmH* 70S ribosome/RlmH/sinefungin complex showed no extra density for the RlmH in the inter-subunit space. Consequently, due to the low binding efficiency of RlmH, the exact ribosomal elements beyond the H69 region recognized by RlmH have proven to be difficult to pinpoint.

In conclusion, while the exact contribution of the SSU in determining the RlmH substrate specificity remains unknown, a proper conformation of the H69 or rather the ability of H69 to obtain a certain conformation, in addition to subunit association, is likely necessary for an efficient methylation of Ψ1915 by RlmH. The modeled complex with the 70S ribosomes suggests that only a minor change in the H69 conformation is required for swinging the target Ψ into the active site of RlmH (Purta *et al.*, 2008).

4.2 RlmH mutagenesis

The crystal structure of *E. coli* RlmH dimer (Figure 7) was determined almost 10 years ago (named YbeA at the time) and was disclosed to contain an unusual topological knot (Benach *et al.,* 2003;). The extensive structural and evolutionary bioinformatics study done by Bujnicki and co-workers revealed that YbeA belonged to the SPOUT superfamily of MT (Tkaczuk *et al.*, 2007) (chapter 2.3.2). Based on sequence and structure comparison to previously characterized SPOUT MT (tRNA 2'O-MT TrmH and TrmJ, tRNA m^1G-MT TrmD, 23S rRNA 2'O-MT RlmB, and 16S rRNA m^3U-MT RsmE), Bunnicki and co-workers implicated YbeA in post-transcriptional RNA modification and predicted the location of the co-factor (at the bottom of a deep cleft) and RNA substrate binding sites at the interface of monomers (Figure 7). Conserved amino acids from both monomers were anticipated to be necessary for the active site formation (Tkaczuk *et al.*, 2007). YbeA was, indeed, confirmed to be involved in 23S rRNA $m^3\Psi$1915 formation and renamed RlmH, accordingly (Ref. I). Since there is no precedent for $m^3\Psi$ modification, the Ψ1915 recognition and methyl group transfer mechanism to N3 nitrogen of Ψ mediated by RlmH is unique. Recognition of the target atom within the Ψ is likely to be inextricably linked to the mechanism of the specific modification reaction. Binding and orientation of the Ψ will likely dictate which atom will be suitably positioned for the attack on the methyl group of SAM.

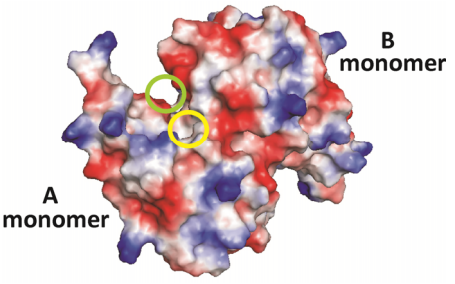

Figure 7. Structure of *E. coli* RlmH (YbeA) homodimer. Surface representation colored according to the distribution of electrostatic potential from red (-10 kT) to blue (+10 kT). Predicted co-factor and substrate binding sites between the monomers are indicated by yellow and green circles, respectively. Adapted from Benach *et al.,* 2003 (PDB ID: Ins5).

In order to shed light onto the substrate base recognition and the catalysis of methyl transfer reaction, we monitored the formation of $m^3\Psi$1915 *in vivo* in *rlmH* deletion strains expressing mutant RlmH proteins. 18 amino acids of RlmH were selected for mutagenesis study based on their conservation and/or structural proximity to the predicted co-factor binding and catalytic site (Ref. I Figure 5) (Tkaczuk *et al.*, 2007). Altogether, ~12% of the total amino acids of RlmH were analyzed. In most cases, alanine was chosen as a substitution to

eliminate side chain functionality of amino acids while avoiding potential problems due to the conformational flexibility of glycine. The low level of transcription from the plasmid encoded *ara*BAD promoter in the absence of arabinose (Lee *et al.*, 1987) was employed to achieve low amounts of mutant RlmH proteins in cells in order to reveal subtle changes in RlmH activity. A moderate level of over-expression (data not shown) from the *ara*BAD in the presence of arabinose (final concentration 0.002%) was used to reveal major changes in RlmH activity. 70S ribosomes were isolated from cells in varying growth phases (OD_{600} 0.3-2.4) and the H69 region was monitored for the presence of $m^3\Psi$1915 by primer extension analysis.

Surprisingly, all of the mutations introduced into RlmH had a negative effect on its activity (Figure 8). As expected, the defects were more evident when RlmH expression level was low. The most noticeable defects were seen with mutations of Y23, E56, E106, G107, E138, R142, and Y152. Only the Y152A mutation resulted in the total loss of the enzymatic activity. G107, E138, R142, and Y152 are universally conserved (Ref. I Figure 5 and Supplementary Figure 1). Our results support the bioinformatical predictions that L72, D73, G103, G107, and L122 are involved in SAM binding; and that E106, T126, and R142 are involved in catalysis (Tkaczuk *et al.*, 2007). However, the functional importance of E138 and Y152 was not predicted by bioinformatical analysis.

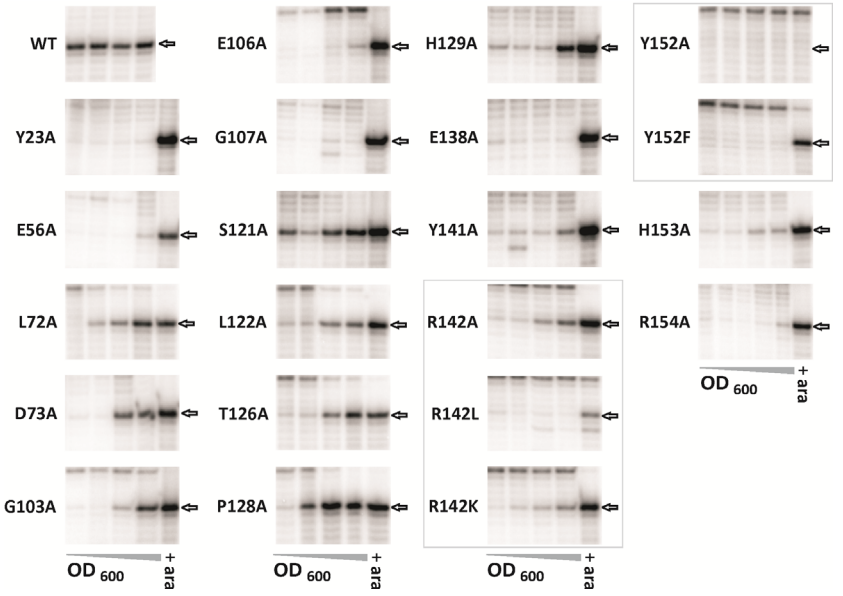

Figure 8. Effect of mutations on RlmH activity *in vivo*. Formation of $m^3\Psi$1915 in *E. coli rlmH* deletion strain expressing low or moderate amounts (+ ara) of native (WT) and mutant RlmH proteins was monitored. rRNA was extracted from ribosomes of cells collected from different growth phases (OD_{600} 0.3-2.4). Reverse transcriptase dependent

primer extension analysis followed by separation of cDNAs on Urea-PAGE was used to detect $m^3\Psi$1915. Stop signal corresponding to $m^3\Psi$1915 is indicated by an arrow.

Fortunately, the closest paralog of RlmH, the tRNA m^1G MT TrmD, has been crystallized in complex with co-factor (Ahn *et al.*, 2003). TrmD methylates N1 nitrogen of guanosine that is in a structural context that is chemically similar to the N3 nitrogen of Ψ (both nitrogens are fairly nucleophilic). Most importantly, TrmD has been relatively well characterized by site-directed mutagenesis studies (Elkins *et al.*, 2003). All in all, TrmD can provide valuable insight into the roles of amino acids located at the active site of RlmH.

The structures of the SAM binding pockets of TrmD and RlmH are nearly identical (Ahn *et al.,* 2003; Benach *et al.,* 2003; Tkaczuk *et al.,* 2007). Based on the similarity to TrmD, RlmH L72 and S121 are involved in binding of the adenosine ring of SAM, while G103 and G107 are involved in binding of the ribose moiety of SAM. In addition, D73 and L122 are likely to have a function in SAM binding. Most hydrogen bonds to SAM are presumably formed by the peptide backbone.

Based on RlmH structure, R142 lays in the putative catalytic site near the SAM binding site. TrmD equivalent of RlmH R142 appears to play a role in target base binding. While the R142A equivalent mutation completely inactivated TrmD (Elkins *et al.*, 2003), RlmH retained some enzymatic activity (Figure 8). Hydrogen bond formation between the side chain of R142 and the base of Ψ is unlikely since R142K and R142A mutations had a similar effect on RlmH activity, while the R142L mutation almost completely inactivated the enzyme. The role of R142 in substrate base binding appears to be mainly structural. Y152 probably stacks upon Ψ and is likely essential for its stabilization in the active site as RlmH was completely inactivated by substituting this tyrosine with alanine while substitution with another aromatic acid, phenylalanine, resulted in major but not complete loss of enzymatic activity at least when mutanr RlmH was present in excess in cells (Figure 8). Like its TrmD equivalent, the R154 of RlmH could be involved in interactions with RNA backbone.

TrmD D169 acts as a general base catalyst and withdraws a proton from the N1 group of guanosine allowing it to nucleophilically attack the reactive methyl group of SAM, thereby triggering the methyl transfer reaction. Similar general base catalysis mechanisms are widespread among the tRNA and rRNA MT (Schubert *et al.*, 2003; Tkaczuk *et al.*, 2007). Surprisingly, there is no obvious equivalent to TrmD D169 in the vicinity of RlmH active site to accept the proton from N3 of Ψ. As conformational rearrangements in RlmH upon substrate binding could position a more distant negatively charged residue into the vicinity of the active site, conserved aspartic (D73) and glutamic (E56, E106, and E138) acid residues located within conceivable distance from the active site were analyzed by site-directed mutagenesis. While the substitution of all of these amino acid residues had a negative effect on RlmH activity, none abolished it completely, hence, ruling out the general base candidates (Figure 8). However, the absence of a clearly recognizable catalytic general base is not unprecedented among rRNA MT.

In case of Nep1, the 18S rRNA $m^1\Psi$ MT in yeast, even determining the crystal structure in complex with the cognate substrate and co-factor did not reveal the candidate for a catalytic general base (Thomas *et al.*, 2010). Similarly, in AviRb, an avilamycin resistance 23S rRNA 2'O MT in *Streptomyces viridochromogenes*, no catalytic general base could be unambiguously identified despite structural and mutagenesis analyses (Mosbacher *et al.*, 2005). Curiously, Nep1 and AviRb also belong to the SPOUT super-family of MT (Tkaczuk *et al.*, 2007). In case of AviRb, the catalytic base was hypothesized to be water (Mosbacher *et al.*, 2005).

There are also other obvious differences between RlmH and TrmD that likely reflect their respective substrate specificities. For instance, the vicinity of the putative active site of RlmH comprises conserved residues not present in TrmD: Y23, T126, P128, H129, Y141, and H153. Substituting any of these residues with alanine had a negative effect on RlmH activity (Figure 8). Specificity for the Ψ N3 nitrogen methylation and ability to discriminate between Ψ and U suggests that H-bonding with the N1 position of Ψ is probably an important determinant for RlmH. Peculiarly, none of the 21 mutations in RlmH caused a decreased specificity for Ψ1915, as revealed by the fact that m^3U1915 formation in the *rlmH/rluD* double deletion strain was not increased compared to native RlmH (data not shown).

In conclusion, RlmH is exquisitely sensitive to mutations around the putative catalytic site. Several factors could contribute to dramatic effects of mutation on the catalytic activity of RlmH. First, residues in the active site may interact with the co-factor and the substrate and be therefore required for enzymatic activity. Second, active site residues may be involved in hydrophobic or ionic interactions across the monomer-monomer interface and could affect the catalytic activity through destabilization of the dimeric structure. Third, as the topological knot is important for SAM binding and catalytic activity, mutations could affect the enzymatic activity by perturbing the knot formation or even by perturbing the overall structure of RlmH. Determining the kinetic parameters of mutant RlmH proteins for substrate and co-factor, as well as determining the structure of RlmH in complex with its substrate and co-factor, would go a long way in helping to further elucidate the substrate recognition and catalysis mechanisms.

CONCLUSIONS

Ribosome biogenesis is a complex process involving an ordered series of events including: transcription and processing of rRNAs; binding of r-proteins; and modification of both rRNAs and r-proteins. Improperly assembled ribosomes have been implicated in several human diseases (Freed *et al.*, 2010) and insights into ribosome biogenesis may lead to new therapeutic agents. Identification and characterization of the rRNA modification enzymes will lead to a more complete understanding of the mechanisms that govern the ribosome biogenesis process as well as will help to elucidate the cellular significance of the modifications themselves. The main focus of the current study was the modification of the functionally important stem-loop 69 (H69) of the *Escherichia coli* ribosome. The following conclusions can be drawn:

I. The gene product of *ybeA* is responsible for the post-transcriptional methylation of Ψ1915 to m^3Ψ1915 in H69 of *E. coli* ribosomes. YbeA protein was renamed to RlmH according to uniform nomenclature of rRNA methyltransferases and shown to be conserved in bacteria. RlmH was the first pseudouridine methyltransferase identified and is likely to be the only one existing in bacteria.

II. RlmH has unique substrate specificity among rRNA modification enzymes; it requires prior conversion of U1915 to Ψ1915 and for the latter to be presented within the 70S ribosome. As such, RlmH is one of the latest if not the ultimate rRNA modification enzyme acting during ribosome biogenesis. RlmH dependent methylation likely coincides with translation initiation. Specificity of RlmH demonstrates that the ribosome large subunit maturation depends on the presence of ribosome small subunits.

III. RlmH is a relatively fast enzyme compared to other RNA modification enzymes. Knowledge of the substrate specificity and the kinetic parameters of RlmH made it possible to determine the kinetic parameters for RluD as well. RluD was the first pseudouridine synthase acting on rRNA to be kinetically characterized. The determined rates of H69 pseudouridylation by RluD and methylation by RlmH are compatible with the timeline of ribosome biogenesis.

IV. Substrate recognition and modification catalysis mechanisms of RlmH are sensitive to single point mutations in both the substrate H69 and in the RlmH protein itself.

REFERENCES

Adilakshmi T., Bellur D. L. & Woodson S. A. (2008). Concurrent nucleation of 16S folding and induced fit in 30S ribosome assembly. *Nature* 455, 1268–1272.

Agalarov S. C., Zheleznyakova E. N., Selivanova O. M., Zheleznaya L. A., Matvienko N. I., Vasiliev V. D. & Spirin A. S. (1998). In vitro assembly of a ribonucleoprotein particle corresponding to the platform domain of the 30S ribosomal subunit. *PNAS* 95, 999–1003.

Agarwalla S., Kealey J. T., Santi D. V. & Stroud R. M. (2002). Characterization of the 23 S ribosomal RNA m5U1939 methyltransferase from Escherichia coli. *The Journal of Biological Chemistry* 277, 8835–8840.

Agrawal R. K., Sharma M. R., Kiel M. C., Hirokawa G., Booth T. M., Spahn C. M., Grassucci R. A., Kaji A. & Frank J. (2004). Visualization of ribosome-recycling factor on the Escherichia coli 70S ribosome: functional implications. *PNAS* 101, 8900–8905.

Agrawal R. K., Spahn C. M., Penczek P., Grassucci R. A., Nierhaus K. H. & Frank J. (2000). Visualization of tRNA movements on the Escherichia coli 70S ribosome during the elongation cycle. *The Journal of Cell Biology* 150, 447–460.

Agris P. F. (1996). The importance of being modified: roles of modified nucleosides and Mg2+ in RNA structure and function. *Progress in Nucleic Acid Research and Molecular Biology* 53, 79–129.

Ahn H. J., Kim H. W., Yoon H. J., Lee B. I., Suh S. W. & Yang J. K. (2003). Crystal structure of tRNA(m1G37)methyltransferase: insights into tRNA recognition. *The EMBO Journal* 22, 2593–2603.

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

Alian A., DeGiovanni A., Griner S. L., Finer-Moore J. S. & Stroud R. M. (2009). Crystal structure of an RluF-RNA complex: a base-pair rearrangement is the key to selectivity of RluF for U2604 of the ribosome. *Journal of Molecular Biology* 388, 785–800.

Alix J. H., Hayes D. & Nierhaus K. H. (1979). Properties of ribosomes and RNA synthesized by Escherichia coli grown in the presence of ethionine. V. Methylation dependence on the assembly of E. coli 50 S ribosomal subunits. *Journal of Molecular Biology* 127, 375–395.

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

Anantharaman V., Koonin E. V. & Aravind L. (2002). Comparative genomics and evolution of proteins involved in RNA metabolism. *Nucleic Acids Research* 30, 1427–1464.

Andersen N. M. & Douthwaite S. (2006). YebU is a m5C methyltransferase specific for 16 S rRNA nucleotide 1407. *Journal of Molecular Biology* 359, 777–786.

Antoun A., Pavlov M. Y., Tenson T. & Ehrenberg M. M. (2004). Ribosome formation from subunits studied by stopped-flow and Rayleigh light scattering. *Biological Procedures Online* 6, 35–54.

Aravind L. & Koonin E. V. (1999). Novel predicted RNA-binding domains associated with the translation machinery. *Journal of Molecular Evolution* 48, 291–302.

Arnez J. G. & Steitz T. A. (1994). Crystal structure of unmodified tRNA(Gln) complexed with glutaminyl-tRNA synthetase and ATP suggests a possible role for pseudo-uridines in stabilization of RNA structure. *Biochemistry* 33, 7560–7567.

Baba T., Ara T., Hasegawa M., Takai Y., Okumura Y., Baba M., Datsenko K. A., Tomita M., Wanner B. L. & Mori H. (2006). Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Molecular Systems Biology* 2.

Bachellerie J. P. & Cavaille J. (1997). Guiding ribose methylation of rRNA. *Trends in Biochemical Sciences* 22, 257–261.

Bakin A. & Ofengand J. (1993). Four newly located pseudouridylate residues in Escherichia coli 23S ribosomal RNA are all at the peptidyltransferase center: analysis by the application of a new sequencing technique. *Biochemistry* 32, 9754–9762.

Ban N., Nissen P., Hansen J., Moore P. B. & Steitz T. A. (2000). The complete atomic structure of the large ribosomal subunit at 2.4 A resolution. *Science* 289, 905–920.

Bashan A. & Yonath A. (2008). Correlating ribosome function with high-resolution structures. *Trends in Microbiology* 16, 326–335.

Bashan A., Agmon I., Zarivach R., Schluenzen F., Harms J., Berisio R., Bartels H., Franceschi F., Auerbach T., Hansen H. A., Kossoy E., Kessler M. & Yonath A. (2003). Structural basis of the ribosomal machinery for peptide bond formation, translocation, and nascent chain progression. *Molecular Cell* 11, 91–102.

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

Benach J., Shen J., Rost B., Xiao R., Acton T., Montelione G. & Hunt J.F. (2003). Structure of ybea from e. coli. PDB 1ns5.

Ben-Shem A., Garreau de Loubresse N., Melnikov S., Jenner L., Yusupova G. & Yusupov M. (2011). The structure of the eukaryotic ribosome at 3.0 A resolution. *Science* 334, 1524–1529.

Ben-Shem A., Jenner L., Yusupova G. & Yusupov M. (2010). Crystal structure of the eukaryotic ribosome. *Science* 330, 1203-1209.

Besancon W. & Wagner R. (1999). Characterization of transient RNA-RNA interactions important for the facilitated structure formation of bacterial ribosomal 16S RNA. *Nucleic Acids Research* 27, 4353–4362.

Birenbaum M., Schlessinger D. & Hashimoto S. (1978). RNase III cleavage of Escherichia coli rRNA precursors: fragment release and dependence on salt concentration. *Biochemistry* 17, 298–307.

Blaha G., Burkhardt N. & Nierhaus K. H. (2002). Formation of 70S ribosomes: large activation energy is required for the adaptation of exclusively the small ribosomal subunit. *Biophysical Chemistry* 96, 153–161.

Blanchard S. C. & Puglisi J. D. (2001). Solution structure of the A loop of 23S ribosomal RNA. *PNAS* 98, 3720–3725.

Boehringer D., O'Farrell H. C., Rife J. P. & Ban N. (2012). Structural insights into methyltransferase KsgA function in 30S ribosomal subunit biogenesis. *The Journal of Biological Chemistry*.

Borovinskaya M. A., Pai R. D., Zhang W., Schuwirth B. S., Holton J. M., Hirokawa G., Kaji H., Kaji A. & Cate J. H. (2007). Structural basis for aminoglycoside inhibition of bacterial ribosome recycling. *Nature Structural & Molecular Biology* 14, 727–732.

Bram R. J., Young R. A. & Steitz J. A. (1980). The ribonuclease III site flanking 23S sequences in the 30S ribosomal precursor RNA of E. coli. *Cell* 19, 393–401.

Brimacombe R., Mitchell P., Osswald M., Stade K. & Bochkariov D. (1993). Clustering of modified nucleotides at the functional center of bacterial ribosomal RNA. *Faseb J* 7, 161–167.

Brodersen D. E., Clemons W. M., Jr., Carter A. P., Wimberly B. T. & Ramakrishnan V. (2002). Crystal structure of the 30 S ribosomal subunit from Thermus thermophilus: structure of the proteins and their interactions with 16 S RNA. *Journal of Molecular Biology* 316, 725–768.

Bubunenko M., Korepanov A., Court D. L., Jagannathan I., Dickinson D., Chaudhuri B. R., Garber M. B. & Culver G. M. (2006). 30S ribosomal subunits can be assembled in vivo without primary binding ribosomal protein S15. *RNA* 12, 1229–1239.

Bugl H., Fauman E. B., Staker B. L., Zheng F., Kushner S. R., Saper M. A., Bardwell J. C. & Jakob U. (2000). RNA methylation under heat shock control. *Molecular Cell* 6, 349–360.

Bunner A. E., Beck A. H. & Williamson J. R. (2010). Kinetic cooperativity in Escherichia coli 30S ribosomal subunit reconstitution reveals additional complexity in the assembly landscape. *PNAS* 107, 5417–5422.

Byrne R. T., Waterman D. G. & Antson A. A. (2009). Enzyme-RNA Substrate Recognition in RNA-Modifying Enzymes. *DNA and RNA Modification Enzymes: Structure, Mechanism, Function and Evolution* edited by Henri Grosjean.

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

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

Cannone J. J., Subramanian S., Schnare M. N., Collett J. R., D'Souza L. M., Du Y., Feng B., Lin N., Madabusi L. V., Muller K. M., Pande N., Shang Z., Yu N. & Gutell R. R. (2002). The comparative RNA web (CRW) site: an online database of comparative sequence and structure information for ribosomal, intron, and other RNAs. *BMC Bioinformatics* 3, 2.

Cantara W. A., Crain P. F., Rozenski J., McCloskey J. A., Harris K. A., Zhang X., Vendeix F. A., Fabris D. & Agris P. F. (2011). The RNA Modification Database, RNAMDB: 2011 update. *Nucleic Acids Research* 39, D195–201.

Capecchi M. R. (1967). Polypeptide chain termination in vitro: isolation of a release factor. *PNAS* 58, 1144–1151.

Ceccarelli A., Dotto G. P., Altruda F., Perlo C., Silengo L., Turco E. & Mangiarotti G. (1978). Immature 50 S subunits in Escherichia coli polyribosomes. *FEBS Letters* 93, 348–350.

Cech T. R. (2000). Structural biology. The ribosome is a ribozyme. *Science* 289, 878–879.

Chan Y. L., Dresios J. & Wool I. G. (2006). A pathway for the transmission of allosteric signals in the ribosome through a network of RNA tertiary interactions. *Journal of Molecular Biology* 355, 1014–1025.

Charette M. & Gray M. W. (2000). Pseudouridine in RNA: what, where, how, and why. *IUBMB Life* 49, 341–351.

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

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

Chatterjee K., Blaby I. K., Thiaville P. C., Majumder M., Grosjean H., Yuan Y. A., Gupta R. & de Crecy-Lagard V. (2012). The archaeal COG1901/DUF358 SPOUT-methyltransferase members, together with pseudouridine synthase Pus10, catalyze the formation of 1-methylpseudouridine at position 54 of tRNA. *RNA* 18, 421–433.

Chui H. M., Meroueh M., Scaringe S. A. & Chow C. S. (2002). Synthesis of a 3-methyluridine phosphoramidite to investigate the role of methylation in a ribosomal RNA hairpin. *Bioorganic & Medicinal Chemistry* 10, 325–332.

Cochella L. & Green R. (2005). An active role for tRNA in decoding beyond codon: anticodon pairing. *Science* 308, 1178–1180.

Cohn W. E. (1960). Pseudouridine, a carbon-carbon linked ribonucleoside in ribonucleic acids: isolation, structure, and chemical characteristics. *The Journal of Biological Chemistry* 235, 1488–1498.

Cole J. R., Chai B., Marsh T. L., Farris R. J., Wang Q., Kulam S. A., Chandra S., McGarrell D. M., Schmidt T. M., Garrity G. M. & Tiedje J. M. (2003). The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Research* 31, 442–443.

Condon C., Squires C. & Squires C. L. (1995). Control of rRNA transcription in Escherichia coli. *Microbiological Reviews* 59, 623–645.

Connolly K. & Culver G. (2009). Deconstructing ribosome construction. *Trends in Biochemical Sciences* 34, 256–263.

Connolly K., Rife J. P. & Culver G. (2008). Mechanistic insight into the ribosome biogenesis functions of the ancient protein KsgA. *Molecular Microbiology* 70, 1062–1075.

Conrad J., Niu L., Rudd K., Lane B. G. & Ofengand J. (1999). 16S ribosomal RNA pseudouridine synthase RsuA of Escherichia coli: deletion, mutation of the conserved Asp102 residue, and sequence comparison among all other pseudouridine synthases. *RNA* 5, 751–763.

Cunningham P. R., Richard R. B., Weitzmann C. J., Nurse K. & Ofengand J. (1991). The absence of modified nucleotides affects both in vitro assembly and in vitro function of the 30S ribosomal subunit of Escherichia coli. *Biochimie* 73, 789–796.

Cunningham P. R., Weitzmann C. J., Nurse K., Masurel R., Van Knippenberg P. H. & Ofengand J. (1990). Site-specific mutation of the conserved m6(2)A m6(2)A residues of E. coli 16S ribosomal RNA. Effects on ribosome function and activity of the ksgA methyltransferase. *Biochimica et Biophysica Acta* 1050, 18–26.

Czerwoniec A., Dunin-Horkawicz S., Purta E., Kaminska K. H., Kasprzak J. M., Bujnicki J. M., Grosjean H. & Rother K. (2009). MODOMICS: a database of RNA modification pathways. 2008 update. *Nucleic Acids Research* 37, D118–121.

Dallas A. & Noller H. F. (2001). Interaction of translation initiation factor 3 with the 30S ribosomal subunit. *Molecular Cell* 8, 855–864.

Dalluge J. J., Hashizume T., Sopchik A. E., McCloskey J. A. & Davis D. R. (1996). Conformational flexibility in RNA: the role of dihydrouridine. *Nucleic Acids Research* 24, 1073–1079.

Das G., Thotala D. K., Kapoor S., Karunanithi S., Thakur S. S., Singh N. S. & Varshney U. (2008). Role of 16S ribosomal RNA methylations in translation initiation in Escherichia coli. *The EMBO Journal* 27, 840-851.

Davis D. R. (1995). Stabilization of RNA stacking by pseudouridine. *Nucleic Acids Research* 23, 5020–5026.

Davis F. F. & Allen F. W. (1957). Ribonucleic acids from yeast which contain a fifth nucleotide. *The Journal of Biological Chemistry* 227, 907–915.
de Narvaez C. C. & Schaup H. W. (1979). In vivo transcriptionally coupled assembly of Escherichia coli ribosomal subunits. *Journal of Molecular Biology* 134, 1–22.
Decatur W. A. & Fournier M. J. (2002). rRNA modifications and ribosome function. *Trends in Biochemical Sciences* 27, 344–351.
Del Campo M., Kaya Y. & Ofengand J. (2001). Identification and site of action of the remaining four putative pseudouridine synthases in Escherichia coli. *RNA* 7, 1603–1615.
Del Campo M., Recinos C., Yanez G., Pomerantz S. C., Guymon R., Crain P. F., McCloskey J. A. & Ofengand J. (2005). Number, position, and significance of the pseudouridines in the large subunit ribosomal RNA of Haloarcula marismortui and Deinococcus radiodurans. *RNA* 11, 210–219.
Desai P. M. & Rife J. P. (2006). The adenosine dimethyltransferase KsgA recognizes a specific conformational state of the 30S ribosomal subunit. *Archives of Biochemistry and Biophysics* 449, 57–63.
Desaulniers J. P., Chang Y. C., Aduri R., Abeysirigunawardena S. C., SantaLucia J., Jr. & Chow C. S. (2008). Pseudouridines in rRNA helix 69 play a role in loop stacking interactions. *Organic & Biomolecular Chemistry* 6, 3892–3895.
Deutscher M. P. (2009). Maturation and degradation of ribosomal RNA in bacteria. *Progress in Molecular Biology and Translational Science* 85, 369–391.
Dohme F. & Nierhaus K. H. (1976). Total reconstitution and assembly of 50 S subunits from Escherichia coli Ribosomes in vitro. *Journal of Molecular Biology* 107, 585–599.
Dunkle J. A., Wang L., Feldman M. B., Pulk A., Chen V. B., Kapral G. J., Noeske J., Richardson J. S., Blanchard S. C. & Cate J. H. (2011). Structures of the bacterial ribosome in classical and hybrid states of tRNA binding. *Science* 332, 981–984.
Dunn J. J. & Studier F. W. (1973). T7 early RNAs and Escherichia coli ribosomal RNAs are cut from large precursor RNAs in vivo by ribonuclease 3. *PNAS* 70, 3296–3300.
Ejby M., Sorensen M. A. & Pedersen S. (2007). Pseudouridylation of helix 69 of 23S rRNA is necessary for an effective translation termination. *PNAS* 104, 19410–19415.
Elkins P. A., Watts J. M., Zalacain M., van Thiel A., Vitazka P. R., Redlak M., Andraos-Selim C., Rastinejad F. & Holmes W. M. (2003). Insights into catalysis by a knotted TrmD tRNA methyltransferase. *Journal of Molecular Biology* 333, 931–949.
Ellis J. J. & Jones S. (2008). Evaluating conformational changes in protein structures binding RNA. *Proteins* 70, 1518–1526.
Ferre-D'Amare A. R. (2003). RNA-modifying enzymes. *Current Opinion in Structural Biology* 13, 49–55.
Feunteun J., Jordan B. R. & Monier R. (1972). Study of the maturation of 5 s RNA precursors in Escherichia coli. *Journal of Molecular Biology* 70, 465–474.
Franceschi F. J. & Nierhaus K. H. (1988). Ribosomal protein L20 can replace the assembly-initiator protein L24 at low temperatures. *Biochemistry* 27, 7056–7059.
Frank J. & Agrawal R. K. (2000). A ratchet-like inter-subunit reorganization of the ribosome during translocation. *Nature* 406, 318–322.
Frank J., Sengupta J., Gao H., Li W., Valle M., Zavialov A. & Ehrenberg M. (2005). The role of tRNA as a molecular spring in decoding, accommodation, and peptidyl transfer. *FEBS Letters* 579, 959–962.

Frank J., Verschoor A., Li Y., Zhu J., Lata R. K., Radermacher M., Penczek P., Grassucci R., Agrawal R. K. & Srivastava S. (1995). A model of the translational apparatus based on a three-dimensional reconstruction of the Escherichia coli ribosome. *Biochemistry and Cell Biology* 73, 757–765.

Freed E. F., Bleichert F., Dutca L. M. & Baserga S. J. (2010). When ribosomes go bad: diseases of ribosome biogenesis. *Molecular BioSystems* 6, 481–493.

Garcia G. A. & Goodenough-Lashua D. E. M. (1998). Mechanism of RNA-modifying and editing enzymes. *Modification and Editing of RNA*. Edited by Henri Grosjean and Rob Benne.

Gausing K. (1977). Regulation of ribosome production in Escherichia coli: synthesis and stability of ribosomal RNA and of ribosomal protein messenger RNA at different growth rates. *Journal of Molecular Biology* 115, 335–354.

Gegenheimer P. & Apirion D. (1975). Escherichia coli ribosomal ribonucleic acids are not cut from an intact precursor molecule. *The Journal of Biological Chemistry* 250, 2407–2409.

Gegenheimer P., Watson N. & Apirion D. (1977). Multiple pathways for primary processing of ribosomal RNA in Escherichia coli. *The Journal of Biological Chemistry* 252, 3064–3073.

Gehrke C. W. & Kuo K. C. (1989). Ribonucleoside analysis by reversed-phase high-performance liquid chromatography. *Journal of Chromatography* 471, 3–36.

Ginsburg D. & Steitz J. A. (1975). The 30 S ribosomal precursor RNA from Escherichia coli. A primary transcript containing 23 S, 16 S, and 5 S sequences. *The Journal of Biological Chemistry* 250, 5647–5654.

Green R. & Noller H. F. (1996). In vitro complementation analysis localizes 23S rRNA posttranscriptional modifications that are required for Escherichia coli 50S ribosomal subunit assembly and function. *RNA* 2, 1011–1021.

Green R. & Noller H. F. (1999). Reconstitution of functional 50S ribosomes from in vitro transcripts of Bacillus stearothermophilus 23S rRNA. *Biochemistry* 38, 1772–1779.

Green R., Samaha R. R. & Noller H. F. (1997). Mutations at nucleotides G2251 and U2585 of 23 S rRNA perturb the peptidyl transferase center of the ribosome. *Journal of Molecular Biology* 266, 40–50.

Guillier M., Allemand F., Graffe M., Raibaud S., Dardel F., Springer M. & Chiaruttini C. (2005). The N-terminal extension of Escherichia coli ribosomal protein L20 is important for ribosome assembly, but dispensable for translational feedback control. *RNA* 11, 728–738.

Gustafsson C. & Persson B. C. (1998). Identification of the rrmA gene encoding the 23S rRNA m1G745 methyltransferase in Escherichia coli and characterization of an m1G745-deficient mutant. *Journal of Bacteriology* 180, 359–365.

Gustafsson C., Reid R., Greene P. J. & Santi D. V. (1996). Identification of new RNA modifying enzymes by iterative genome search using known modifying enzymes as probes. *Nucleic Acids Research* 24, 3756–3762.

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

Hage A. E. & Tollervey D. (2004). A surfeit of factors: why is ribosome assembly so much more complicated in eukaryotes than bacteria? *RNA Biology* 1, 10–15.
Hager J., Staker B. L. & Jakob U. (2004). Substrate binding analysis of the 23S rRNA methyltransferase RrmJ. *Journal of Bacteriology* 186, 6634–6642.
Hager J., Staker B. L., Bugl H. & Jakob U. (2002). Active site in RrmJ, a heat shock-induced methyltransferase. *The Journal of Biological Chemistry* 277, 41978–41986.
Hamma T. & Ferre-D'Amare A. R. (2006). Pseudouridine synthases. *Chemistry & Biology* 13, 1125–1135.
Hardy S. J. (1975). The stoichiometry of the ribosomal proteins of Escherichia coli. *Mol Gen Genet* 140, 253–274.
Harms J., Schluenzen F., Zarivach R., Bashan A., Gat S., Agmon I., Bartels H., Franceschi F. & Yonath A. (2001). High resolution structure of the large ribosomal subunit from a mesophilic eubacterium. *Cell* 107, 679–688.
Hayes F., Vasseur M., Nikolaev N., Schlessinger D., Sri Widada J., Krol A. & Branlant C. (1975). Structure of a 30 S pre-ribosomal RNA of E. coli. *FEBS Letters* 56, 85–91.
Heinrich T., Condon C., Pfeiffer T. & Hartmann R. K. (1995). Point mutations in the leader boxA of a plasmid-encoded Escherichia coli rrnB operon cause defective antitermination in vivo. *Journal of Bacteriology* 177, 3793–3800.
Held W. A., Ballou B., Mizushima S. & Nomura M. (1974). Assembly mapping of 30 S ribosomal proteins from Escherichia coli. Further studies. *The Journal of Biological Chemistry* 249, 3103–3111.
Held W. A., Mizushima S. & Nomura M. (1973). Reconstitution of Escherichia coli 30 S ribosomal subunits from purified molecular components. *The Journal of Biological Chemistry* 248, 5720–5730.
Helser T. L., Davies J. E. & Dahlberg J. E. (1972). Mechanism of kasugamycin resistance in Escherichia coli. *Nature: New biology* 235, 6–9.
Hennelly S. P., Antoun A., Ehrenberg M., Gualerzi C. O., Knight W., Lodmell J. S. & Hill W. E. (2005). A time-resolved investigation of ribosomal subunit association. *Journal of Molecular Biology* 346, 1243–1258.
Herold M. & Nierhaus K. H. (1987). Incorporation of six additional proteins to complete the assembly map of the 50 S subunit from Escherichia coli ribosomes. *The Journal of Biological Chemistry* 262, 8826–8833.
Hirabayashi N., Sato N. S. & Suzuki T. (2006). Conserved loop sequence of helix 69 in Escherichia coli 23 S rRNA is involved in A-site tRNA binding and translational fidelity. *The Journal of Biological Chemistry* 281, 17203–17211.
Hirokawa G., Nijman R. M., Raj V. S., Kaji H., Igarashi K. & Kaji A. (2005). The role of ribosome recycling factor in dissociation of 70S ribosomes into subunits. *RNA* 11, 1317–1328.
Hoang C., Chen J., Vizthum C. A., Kandel J. M., Hamilton C. S., Mueller E. G. & Ferre-D'Amare A. R. (2006). Crystal structure of pseudouridine synthase RluA: indirect sequence readout through protein-induced RNA structure. *Molecular Cell* 24, 535–545.
Holbrook S. R. (2008). Structural principles from large RNAs. *Annual Review of Biophysics* 37, 445–464.
Hou Y. M. & Perona J. J. (2010). Stereochemical mechanisms of tRNA methyltransferases. *FEBS Letters* 584, 278–286.

Huang L., Ku J., Pookanjanatavip M., Gu X., Wang D., Greene P. J. & Santi D. V. (1998). Identification of two Escherichia coli pseudouridine synthases that show multisite specificity for 23S RNA. *Biochemistry* 37, 15951–15957.

Jenner L., Demeshkina N., Yusupova G. & Yusupov M. (2010). Structural rearrangements of the ribosome at the tRNA proofreading step. *Nature Structural & Molecular Biology* 17, 1072–1078.

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

Kaya Y. & Ofengand J. (2003). A novel unanticipated type of pseudouridine synthase with homologs in bacteria, archaea, and eukarya. *RNA* 9, 711–721.

Kaya Y., Del Campo M., Ofengand J. & Malhotra A. (2004). Crystal structure of TruD, a novel pseudouridine synthase with a new protein fold. *The Journal of Biological Chemistry* 279, 18107–18110.

Keener J. & Nomura M. (1996). Regulation of ribosome synthesis. *Escherichia coli and Salmonella* Cellular and Molecular Biology. ASM Press, Washington DC, 1417–1431.

Kehrenberg C., Schwarz S., Jacobsen L., Hansen L. H. & Vester B. (2005). A new mechanism for chloramphenicol, florfenicol and clindamycin resistance: methylation of 23S ribosomal RNA at A2503. *Molecular Microbiology* 57, 1064–1073.

Khaitovich P., Tenson T., Kloss P. & Mankin A. S. (1999). Reconstitution of functionally active Thermus aquaticus large ribosomal subunits with in vitro-transcribed rRNA. *Biochemistry* 38, 1780–1788.

Kimura S. & Suzuki T. (2010). Fine-tuning of the ribosomal decoding center by conserved methyl-modifications in the Escherichia coli 16S rRNA. *Nucleic Acids Research* 38, 1341–1352.

King T. C., Sirdeshmukh R. & Schlessinger D. (1984). RNase III cleavage is obligate for maturation but not for function of Escherichia coli pre-23S rRNA. *PNAS* 81, 185–188.

King T. C., Sirdeskmukh R. & Schlessinger D. (1986). Nucleolytic processing of ribonucleic acid transcripts in procaryotes. *Microbiological Reviews* 50, 428–451.

Kipper K., Hetenyi C., Sild S., Remme J. & Liiv A. (2009). Ribosomal intersubunit bridge B2a is involved in factor-dependent translation initiation and translational processivity. *Journal of Molecular Biology* 385, 405–422.

Kipper K., Sild S., Hetenyi C., Remme J. & Liiv A. (2011). Pseudouridylation of 23S rRNA helix 69 promotes peptide release by release factor RF2 but not by release factor RF1. *Biochimie* 93, 834–844.

Kiss T. (2001). Small nucleolar RNA-guided post-transcriptional modification of cellular RNAs. *The EMBO Journal* 20, 3617–3622.

Kiss-Laszlo Z., Henry Y., Bachellerie J. P., Caizergues-Ferrer M. & Kiss T. (1996). Site-specific ribose methylation of preribosomal RNA: a novel function for small nucleolar RNAs. *Cell* 85, 1077–1088.

Kitahara K. & Suzuki T. (2009). The ordered transcription of RNA domains is not essential for ribosome biogenesis in Escherichia coli. *Molecular Cell* 34, 760–766.

Klaholz B. P., Myasnikov A. G. & Van Heel M. (2004). Visualization of release factor 3 on the ribosome during termination of protein synthesis. *Nature* 427, 862–865.

Klein D. J., Moore P. B. & Steitz T. A. (2004a). The contribution of metal ions to the structural stability of the large ribosomal subunit. *RNA* 10, 1366–1379.

Klein D. J., Moore P. B. & Steitz T. A. (2004b). The roles of ribosomal proteins in the structure assembly, and evolution of the large ribosomal subunit. *Journal of Molecular Biology* 340, 141–177.

Klinge S., Voigts-Hoffmann F., Leibundgut M. & Ban N. (2012). Atomic structures of the eukaryotic ribosome. *Trends in Biochemical Sciences* 37, 189–198.

Koonin E. V. (1996). Pseudouridine synthases: four families of enzymes containing a putative uridine-binding motif also conserved in dUTPases and dCTP deaminases. *Nucleic Acids Research* 24, 2411–2415.

Korostelev A., Asahara H., Lancaster L., Laurberg M., Hirschi A., Zhu J., Trakhanov S., Scott W. G. & Noller H. F. (2008). Crystal structure of a translation termination complex formed with release factor RF2. *PNAS* 105, 19684–19689.

Korostelev A., Trakhanov S., Laurberg M. & Noller H. F. (2006). Crystal structure of a 70S ribosome-tRNA complex reveals functional interactions and rearrangements. *Cell* 126, 1065–1077.

Korostelev A., Zhu J., Asahara H. & Noller H. F. (2010). Recognition of the amber UAG stop codon by release factor RF1. *The EMBO Journal* 29, 2577–2585.

Kovalic D., Giannattasio R. B., Jin H. J. & Weisblum B. (1994). 23S rRNA domain V, a fragment that can be specifically methylated in vitro by the ErmSF (TlrA) methyltransferase. *Journal of Bacteriology* 176, 6992–6998.

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

Kowalak J. A., Dalluge J. J., McCloskey J. A. & Stetter K. O. (1994). The role of post-transcriptional modification in stabilization of transfer RNA from hyperthermophiles. *Biochemistry* 33, 7869–7876.

Krzyzosiak W., Denman R., Nurse K., Hellmann W., Boublik M., Gehrke C. W., Agris P. F. & Ofengand J. (1987). In vitro synthesis of 16S ribosomal RNA containing single base changes and assembly into a functional 30S ribosome. *Biochemistry* 26, 2353–2364.

Lafontaine D., Delcour J., Glasser A. L., Desgres J. & Vandenhaute J. (1994). The DIM1 gene responsible for the conserved m6(2)Am6(2)A dimethylation in the 3'-terminal loop of 18 S rRNA is essential in yeast. *Journal of Molecular Biology* 241, 492–497.

Lane B. G., Ofengand J. & Gray M. W. (1995). Pseudouridine and O2'-methylated nucleosides. Significance of their selective occurrence in rRNA domains that function in ribosome-catalyzed synthesis of the peptide bonds in proteins. *Biochimie* 77, 7–15.

Laurberg M., Asahara H., Korostelev A., Zhu J., Trakhanov S. & Noller H. F. (2008). Structural basis for translation termination on the 70S ribosome. *Nature* 454, 852–857.

Lee N., Francklyn C. & Hamilton E. P. (1987). Arabinose-induced binding of AraC protein to araI2 activates the araBAD operon promoter. *PNAS* 84, 8814–-8818.

Lee T. T., Agarwalla S. & Stroud R. M. (2005). A unique RNA Fold in the RumA-RNA-cofactor ternary complex contributes to substrate selectivity and enzymatic function. *Cell* 120, 599–611.

Leonov A. A., Sergiev P. V., Bogdanov A. A., Brimacombe R. & Dontsova O. A. (2003). Affinity purification of ribosomes with a lethal G2655C mutation in 23 S

rRNA that affects the translocation. *The Journal of Biological Chemistry* 278, 25664–25670.
Leppik M., Peil L., Kipper K., Liiv A. & Remme J. (2007). Substrate specificity of the pseudouridine synthase RluD in Escherichia coli. *The FEBS Journal* 274, 5759–5766.
Leulliot N., Bohnsack M. T., Graille M., Tollervey D. & Van Tilbeurgh H. (2008). The yeast ribosome synthesis factor Emg1 is a novel member of the superfamily of alpha/beta knot fold methyltransferases. *Nucleic Acids Research* 36, 629–639.
Lewicki B. T., Margus T., Remme J. & Nierhaus K. H. (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. *Journal of Molecular Biology* 231, 581–593.
Lhoest J. & Colson C. (1981). Cold-sensitive ribosome assembly in an Escherichia coli mutant lacking a single methyl group in ribosomal protein L3. *European Journal of Biochemistry* 121, 33–37.
Li Z. & Deutscher M. P. (1995). The tRNA processing enzyme RNase T is essential for maturation of 5S RNA. *PNAS* 92, 6883–6886.
Li Z., Pandit S. & Deutscher M. P. (1999a). Maturation of 23S ribosomal RNA requires the exoribonuclease RNase T. *RNA* 5, 139–146.
Li Z., Pandit S. & Deutscher M. P. (1999b). RNase G (CafA protein) and RNase E are both required for the 5' maturation of 16S ribosomal RNA. *The EMBO Journal* 18, 2878–2885.
Liiv A. & O'Connor M. (2006). Mutations in the intersubunit bridge regions of 23 S rRNA. *The Journal of Biological Chemistry* 281, 29850–29862.
Liiv A., Karitkina D., Maiväli U. & Remme J. (2005). Analysis of the function of E. coli 23S rRNA helix-loop 69 by mutagenesis. *BMC Molecular Biology* 6, 18.
Lindahl L. (1973). Two new ribosomal precursor particles in E. coli. *Nature: New biology* 243, 170–172.
Lindahl L. (1975). Intermediates and time kinetics of the in vivo assembly of Escherichia coli ribosomes. *Journal of Molecular Biology* 92, 15–37.
Liu M., Novotny G. W. & Douthwaite S. (2004). Methylation of 23S rRNA nucleotide G745 is a secondary function of the RlmAI methyltransferase. *RNA* 10, 1713–1720.
Lovgren J. M. & Wikstrom P. M. (2001). The rlmB gene is essential for formation of Gm2251 in 23S rRNA but not for ribosome maturation in Escherichia coli. *Journal of Bacteriology* 183, 6957–6960.
Macmaster R., Zelinskaya N., Savic M., Rankin C. R. & Conn G. L. (2010). Structural insights into the function of aminoglycoside-resistance A1408 16S rRNA methyltransferases from antibiotic-producing and human pathogenic bacteria. *Nucleic Acids Research* 38, 7791–7799.
Maden B. E. (1990). The numerous modified nucleotides in eukaryotic ribosomal RNA. *Progress in Nucleic Acid Research and Molecular Biology* 39, 241–303.
Maiväli U. & Remme J. (2004). Definition of bases in 23S rRNA essential for ribosomal subunit association. *RNA* 10, 600–604.
Mears J. A., Cannone J. J., Stagg S. M., Gutell R. R., Agrawal R. K. & Harvey S. C. (2002). Modeling a minimal ribosome based on comparative sequence analysis. *Journal of Molecular Biology* 321, 215–234.
Meroueh M., Grohar P. J., Qiu J., SantaLucia J., Jr., Scaringe S. A. & Chow C. S. (2000). Unique structural and stabilizing roles for the individual pseudouridine

residues in the 1920 region of Escherichia coli 23S rRNA. *Nucleic Acids Research* 28, 2075–2083.
Michel G., Sauve V., Larocque R., Li Y., Matte A. & Cygler M. (2002). The structure of the RlmB 23S rRNA methyltransferase reveals a new methyltransferase fold with a unique knot. *Structure* 10, 1303–1315.
Misra T. K. & Apirion D. (1979). RNase E, an RNA processing enzyme from Escherichia coli. *The Journal of Biological Chemistry* 254, 11154–11159.
Mitchell P., Osswald M. & Brimacombe R. (1992). Identification of intermolecular RNA cross-links at the subunit interface of the Escherichia coli ribosome. *Biochemistry* 31, 3004–3011.
Mizushima S. & Nomura M. (1970). Assembly mapping of 30S ribosomal proteins from E. coli. *Nature* 226, 1214.
Monshupanee T., Gregory S. T., Douthwaite S., Chungjatupornchai W. & Dahlberg A. E. (2008). Mutations in conserved helix 69 of 23S rRNA of Thermus thermophilus that affect capreomycin resistance but not posttranscriptional modifications. *Journal of Bacteriology* 190, 7754–7761.
Moore P. B. & Steitz T. A. (2010). The roles of RNA in the synthesis of protein. *Cold Spring Harbor Perspectives in Biology* 3, a003780.
Mora L., Heurgue-Hamard V., de Zamaroczy M., Kervestin S. & Buckingham R. H. (2007). Methylation of bacterial release factors RF1 and RF2 is required for normal translation termination in vivo. *The Journal of Biological Chemistry* 282, 35638–35645.
Mosbacher T. G., Bechthold A. & Schulz G. E. (2005). Structure and function of the antibiotic resistance-mediating methyltransferase AviRb from Streptomyces viridochromogenes. *Journal of Molecular Biology* 345, 535–545.
Mueller E. G. (2002). Chips off the old block. *Nature Structural Biology* 9, 320–322.
Mueller E.G. & Ferre-D'Amare A. R. (2009). Pseudouridine formation, the most common transglycosylation in RNA. *DNA and RNA Modification Enzymes: Structure, Mechanism, Function and Evolution* edited by Henri Grosjean.
Mulder A. M., Yoshioka C., Beck A. H., Bunner A. E., Milligan R. A., Potter C. S., Carragher B. & Williamson J. R. (2010). Visualizing ribosome biogenesis: parallel assembly pathways for the 30S subunit. *Science* 330, 673–677.
Newby M. I. & Greenbaum N. L. (2001). A conserved pseudouridine modification in eukaryotic U2 snRNA induces a change in branch-site architecture. *RNA* 7, 833–845.
Ni J., Tien A. L. & Fournier M. J. (1997). Small nucleolar RNAs direct site-specific synthesis of pseudouridine in ribosomal RNA. *Cell* 89, 565–573.
Nichols R. J., Sen S., Choo Y. J., Beltrao P., Zietek M., Chaba R., Lee S., Kazmierczak K. M., Lee K. J., Wong A., Shales M., Lovett S., Winkler M. E., Krogan N. J., Typas A. & Gross C. A. (2011). Phenotypic landscape of a bacterial cell. *Cell* 144, 143–156.
Nierhaus K. H. & Dohme F. (1974). Total reconstitution of functionally active 50S ribosomal subunits from Escherichia coli. *PNAS* 71, 4713–4717.
Nikolaev N., Schlessinger D. & Wellauer P. K. (1974). 30 S pre-ribosomal RNA of Escherichia coli and products of cleavage by ribonuclease III: length and molecular weight. *Journal of Molecular Biology* 86, 741–747.
Nissen P., Hansen J., Ban N., Moore P. B. & Steitz T. A. (2000). The structural basis of ribosome activity in peptide bond synthesis. *Science* 289, 920–930.
Noller H. F. & Woese C. R. (1981). Secondary structure of 16S ribosomal RNA. *Science* 212, 403–411.

Noller H. F. (2005). RNA structure: reading the ribosome. *Science* 309, 1508–1514.
Noller H. F., Kop J., Wheaton V., Brosius J., Gutell R. R., Kopylov A. M., Dohme F., Herr W., Stahl D. A., Gupta R. & Waese C. R. (1981). Secondary structure model for 23S ribosomal RNA. *Nucleic Acids Research* 9, 6167–6189.
Nomura M. & Morgan E. A. (1977). Genetics of bacterial ribosomes. *Annual Review of Genetics* 11, 297–-347.
O'Connor M. & Dahlberg A. E. (1995). The involvement of two distinct regions of 23 S ribosomal RNA in tRNA selection. *Journal of Molecular Biology* 254, 838–847.
O'Connor M. & Gregory S. T. (2011). Inactivation of the RluD pseudouridine synthase has minimal effects on growth and ribosome function in wild-type Escherichia coli and Salmonella enterica. *Journal of Bacteriology* 193, 154–162.
O'Connor M. (2007). Interaction between the ribosomal subunits: 16S rRNA suppressors of the lethal DeltaA1916 mutation in the 23S rRNA of Escherichia coli. *Mol Genet Genomics* 278, 307–315.
O'Connor M. (2009). Helix 69 in 23S rRNA modulates decoding by wild type and suppressor tRNAs. *Mol Genet Genomics* 282, 371–380.
O'Farrell H. C., Scarsdale J. N. & Rife J. P. (2004). Crystal structure of KsgA, a universally conserved rRNA adenine dimethyltransferase in Escherichia coli. *Journal of Molecular Biology* 339, 337–353.
Ofengand J. & Bakin A. (1997). Mapping to nucleotide resolution of pseudouridine residues in large subunit ribosomal RNAs from representative eukaryotes, prokaryotes, archaebacteria, mitochondria and chloroplasts. *Journal of Molecular Biology* 266, 246–268.
Ofengand J. & Del Campo M. (2004a). Modified Nucleosides of Escherichia coli Ribosomal RNA *EcoSal—Escherichia coli and Salmonella: Cellular and molecular biology*. Chap. 4.6.1. ASM Press; Washington, DC
Ofengand J. & Del Campo M. (2004b). Modified nucleosides of Escherichia coli ribosomal RNA. *Escherichia coli and Salmonella: cellular and molecular biology*.
Ofengand J. (2002). Ribosomal RNA pseudouridines and pseudouridine synthases. *FEBS Letters* 514, 17–25.
Ofengand J., Bakin A., Wrzesinski J., Nurse K. & Lane B. G. (1995). The pseudouridine residues of ribosomal RNA. *Biochemistry and cell biology* 73, 915–924.
Ofengand J., Del Campo M. & Kaya Y. (2001a). Mapping pseudouridines in RNA molecules. *Methods* 25, 365–373.
Ofengand J., Malhotra A., Remme J., Gutgsell N. S., Del Campo M., Jean-Charles S., Peil L. & Kaya Y. (2001b). Pseudouridines and pseudouridine synthases of the ribosome. *Cold Spring Harbor Symposia on Quantitative Biology* 66, 147–159.
Ogle J. M., Brodersen D. E., Clemons W. M., Jr., Tarry M. J., Carter A. P. & Ramakrishnan V. (2001). Recognition of cognate transfer RNA by the 30S ribosomal subunit. *Science* 292, 897–902.
Ortiz-Meoz R. F. & Green R. (2011). Helix 69 is key for uniformity during substrate selection on the ribosome. *The Journal of Biological Chemistry* 286, 25604–25610.
Pai R. D., Zhang W., Schuwirth B. S., Hirokawa G., Kaji H., Kaji A. & Cate J. H. (2008). Structural Insights into ribosome recycling factor interactions with the 70S ribosome. *Journal of Molecular Biology* 376, 1334–1347.
Peil L., Virumae K. & Remme J. (2008). Ribosome assembly in Escherichia coli strains lacking the RNA helicase DeaD/CsdA or DbpA. *The FEBSJjournal* 275, 3772–3782.

Pfeiffer T. & Hartmann R. K. (1997). Role of the spacer boxA of Escherichia coli ribosomal RNA operons in efficient 23 S rRNA synthesis in vivo. *Journal of Molecular Biology* 265, 385–393.

Piekna-Przybylska D., Decatur W. A. & Fournier M. J. (2008). The 3D rRNA modification maps database: with interactive tools for ribosome analysis. *Nucleic Acids Research* 36, D178–183.

Poehlsgaard J. & Douthwaite S. (2005). The bacterial ribosome as a target for antibiotics. *Nat Rev Microbiol* 3, 870–881.

Poldermans B., Roza L. & Van Knippenberg P. H. (1979a). Studies on the function of two adjacent N6,N6-dimethyladenosines near the 3' end of 16 S ribosomal RNA of Escherichia coli. III. Purification and properties of the methylating enzyme and methylase-30 S interactions. *The Journal of Biological Chemistry* 254, 9094–9100.

Poldermans B., Van Buul C. P. & Van Knippenberg P. H. (1979b). Studies on the function of two adjacent N6,N6-dimethyladenosines near the 3' end of 16 S ribosomal RNA of Escherichia coli. II. The effect of the absence of the methyl groups on initiation of protein biosynthesis. *The Journal of Biological Chemistry* 254, 9090–9093.

Polevoda B. & Sherman F. (2007). Methylation of proteins involved in translation. *Molecular Microbiology* 65, 590–606.

Powers T., Daubresse G. & Noller H. F. (1993). Dynamics of in vitro assembly of 16 S rRNA into 30 S ribosomal subunits. *Journal of Molecular Biology* 232, 362–374.

Pruesse E., Quast C., Knittel K., Fuchs B. M., Ludwig W., Peplies J. & Glockner F. O. (2007). SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Research* 35, 7188–7196.

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

Ramagopal S. & Subramanian A. R. (1975). Growth-dependent regulation in production and utilization of acetylated ribosomal protein L7. *Journal of Molecular Biology* 94, 633–641.

Ramakrishnan V. (2002). Ribosome structure and the mechanism of translation. *Cell* 108, 557–572.

Ramamurthy V., Swann S. L., Paulson J. L., Spedaliere C. J. & Mueller E. G. (1999a). Critical aspartic acid residues in pseudouridine synthases. *The Journal of Biological Chemistry* 274, 22225–22230.

Ramamurthy V., Swann S. L., Spedaliere C. J. & Mueller E. G. (1999b). Role of cysteine residues in pseudouridine synthases of different families. *Biochemistry* 38, 13106–13111.

Raychaudhuri S., Conrad J., Hall B. G. & 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.

Raychaudhuri S., Niu L., Conrad J., Lane B. G. & Ofengand J. (1999). Functional effect of deletion and mutation of the Escherichia coli ribosomal RNA and tRNA pseudouridine synthase RluA. *The Journal of Biological Chemistry* 274, 18880–18886.

Rene O. & Alix J. H. (2011). Late steps of ribosome assembly in E. coli are sensitive to a severe heat stress but are assisted by the HSP70 chaperone machine. *Nucleic Acids Research* 39, 1855–1867.

Robertson H. D., Webster R. E. & Zinder N. D. (1968). Purification and properties of ribonuclease III from Escherichia coli. *The Journal of Biological Chemistry* 243, 82–91.

Rodnina M. V., Daviter T., Gromadski K. & Wintermeyer W. (2002). Structural dynamics of ribosomal RNA during decoding on the ribosome. *Biochimie* 84, 745–754.

Rohl R. & Nierhaus K. H. (1982). Assembly map of the large subunit (50S) of Escherichia coli ribosomes. *PNAS* 79, 729–733.

Roy M. K., Singh B., Ray B. K. & Apirion D. (1983). Maturation of 5-S rRNA: ribonuclease E cleavages and their dependence on precursor sequences. *European Journal of Biochemistry* 131, 119–127.

Roy-Chaudhuri B., Kirthi N. & Culver G. M. (2010). Appropriate maturation and folding of 16S rRNA during 30S subunit biogenesis are critical for translational fidelity. *PNAS* 107, 4567–4572.

Sakakibara Y. & Chow C. S. (2012). Role of Pseudouridine in Structural Rearrangements of Helix 69 During Bacterial Ribosome Assembly. *ACS Chemical Biology*.

Samaha R. R., O'Brien B., O'Brien T. W. & Noller H. F. (1994). Independent in vitro assembly of a ribonucleoprotein particle containing the 3' domain of 16S rRNA. *PNAS* 91, 7884–7888.

Saraiya A. A., Lamichhane T. N., Chow C. S., SantaLucia J., Jr. & Cunningham P. R. (2008). Identification and role of functionally important motifs in the 970 loop of Escherichia coli 16S ribosomal RNA. *Journal of Molecular Biology* 376, 645–657.

Schaub R. E. & Hayes C. S. (2011). Deletion of the RluD pseudouridine synthase promotes SsrA peptide tagging of ribosomal protein S7. *Molecular Microbiology* 79, 331–341.

Schluenzen F., Tocilj A., Zarivach R., Harms J., Gluehmann M., Janell D., Bashan A., Bartels H., Agmon I., Franceschi F. & Yonath A. (2000). Structure of functionally activated small ribosomal subunit at 3.3 angstroms resolution. *Cell* 102, 615–623.

Schmeing T. M. & Ramakrishnan V. (2009). What recent ribosome structures have revealed about the mechanism of translation. *Nature* 461, 1234–1242.

Schmeing T. M., Voorhees R. M., Kelley A. C., Gao Y. G., Murphy F. V. t., Weir J. R. & Ramakrishnan V. (2009). The crystal structure of the ribosome bound to EF-Tu and aminoacyl-tRNA. *Science* 326, 688–694.

Schroeder R., Barta A. & Semrad K. (2004). Strategies for RNA folding and assembly. *Nature reviews* 5, 908–919.

Schubert H. L., Blumenthal R. M. & Cheng X. (2003). Many paths to methyltransfer: a chronicle of convergence. *Trends in Biochemical Sciences* 28, 329–335.

Schuette J. C., Murphy F. V. t., Kelley A. C., Weir J. R., Giesebrecht J., Connell S. R., Loerke J., Mielke T., Zhang W., Penczek P. A., Ramakrishnan V. & Spahn C. M. (2009). GTPase activation of elongation factor EF-Tu by the ribosome during decoding. *The EMBO Journal* 28, 755–765.

Schuwirth B. S., Borovinskaya M. A., Hau C. W., Zhang W., Vila-Sanjurjo A., Holton J. M. & Cate J. H. (2005). Structures of the bacterial ribosome at 3.5 A resolution. *Science* 310, 827–834.

Scolnick E., Tompkins R., Caskey T. & Nirenberg M. (1968). Release factors differing in specificity for terminator codons. *PNAS* 61, 768–774.

Selmer M., Dunham C. M., Murphy F. V. t., Weixlbaumer A., Petry S., Kelley A. C., Weir J. R. & Ramakrishnan V. (2006). Structure of the 70S ribosome complexed with mRNA and tRNA. *Science* 313, 1935–1942.

Semrad K. & Green R. (2002). Osmolytes stimulate the reconstitution of functional 50S ribosomes from in vitro transcripts of Escherichia coli 23S rRNA. *RNA* 8, 401-411.

Semrad K., Green R. & Schroeder R. (2004). RNA chaperone activity of large ribosomal subunit proteins from Escherichia coli. *RNA* 10, 1855–1860.

Sergiev P. V., Golovina A. Y., Sergeeva O. V., Osterman I. A., Nesterchuk M. V., Bogdanov A. A. & Dontsova O. A. (2012). How much can we learn about the function of bacterial rRNA modification by mining large-scale experimental datasets? *Nucleic Acids Research*.

Seshadri A., Dubey B., Weber M. H. & Varshney U. (2009). Impact of rRNA methylations on ribosome recycling and fidelity of initiation in Escherichia coli. *Molecular Microbiology* 72, 795–808.

Shajani Z., Sykes M. T. & Williamson J. R. (2011). Assembly of bacterial ribosomes. *Annual Review of Biochemistry* 80, 501–526.

Sharpe Elles L. M., Sykes M. T., Williamson J. R. & Uhlenbeck O. C. (2009). A dominant negative mutant of the E. coli RNA helicase DbpA blocks assembly of the 50S ribosomal subunit. *Nucleic Acids Research* 37, 6503–6514.

Siibak T. & Remme J. (2010). Subribosomal particle analysis reveals the stages of bacterial ribosome assembly at which rRNA nucleotides are modified. *RNA* 16, 2023–2032.

Sirdeshmukh R. & Schlessinger D. (1985). Why is processing of 23 S ribosomal RNA in Escherichia coli not obligate for its function? *Journal of Molecular Biology* 186, 669–672.

Srivastava A. K. & Schlessinger D. (1988). Coregulation of processing and translation: mature 5' termini of Escherichia coli 23S ribosomal RNA form in polysomes. *PNAS* 85, 7144–7148.

Srivastava A. K. & Schlessinger D. (1990). Mechanism and regulation of bacterial ribosomal RNA processing. *Annual Review of Microbiology* 44, 105–129.

Steitz T. A. & Moore P. B. (2003). RNA, the first macromolecular catalyst: the ribosome is a ribozyme. *Trends in Biochemical Sciences* 28, 411–418.

Steitz T. A. (2008). A structural understanding of the dynamic ribosome machine. *Nature Reviews* 9, 242–253.

Sykes M. T., Shajani Z., Sperling E., Beck A. H. & Williamson J. R. (2010). Quantitative proteomic analysis of ribosome assembly and turnover in vivo. *Journal of Molecular Biology* 403, 331–345.

Talkington M. W., Siuzdak G. & Williamson J. R. (2005). An assembly landscape for the 30S ribosomal subunit. *Nature* 438, 628–632.

Tan J., Jakob U. & Bardwell J. C. (2002). Overexpression of two different GTPases rescues a null mutation in a heat-induced rRNA methyltransferase. *Journal of Bacteriology* 184, 2692–2698.

Taylor A. B., Meyer B., Leal B. Z., Kotter P., Schirf V., Demeler B., Hart P. J., Entian K. D. & Wohnert J. (2008). The crystal structure of Nep1 reveals an extended SPOUT-class methyltransferase fold and a pre-organized SAM-binding site. *Nucleic Acids Research* 36, 1542–1554.

Thomas S. R., Keller C. A., Szyk A., Cannon J. R. & Laronde-Leblanc N. A. (2010). Structural insight into the functional mechanism of Nep1/Emg1 N1-specific

pseudouridine methyltransferase in ribosome biogenesis. *Nucleic Acids Research* 39, 2445–2457.
Tissieres A. & Watson J. D. (1958). Ribonucleoprotein particles from Escherichia coli. *Nature* 182, 778–780.
Tkaczuk K. L., Dunin-Horkawicz S., Purta E. & Bujnicki J. M. (2007). Structural and evolutionary bioinformatics of the SPOUT superfamily of methyltransferases. *BMC Bioinformatics* 8, 73.
Toh S. M. & Mankin A. S. (2008). An indigenous posttranscriptional modification in the ribosomal peptidyl transferase center confers resistance to an array of protein synthesis inhibitors. *Journal of Molecular Biology* 380, 593-597.
Toh S. M., Xiong L., Bae T. & Mankin A. S. (2008). The methyltransferase YfgB/ RlmN is responsible for modification of adenosine 2503 in 23S rRNA. *RNA* 14, 98–106.
Tollervey D. & Kiss T. (1997). Function and synthesis of small nucleolar RNAs. *Current Opinion in Cell Biology* 9, 337–342.
Traub P. & Nomura M. (1968). Structure and function of E. coli ribosomes. V. Reconstitution of functionally active 30S ribosomal particles from RNA and proteins. *PNAS* 59, 777–784.
Vaidyanathan P. P., Deutscher M. P. & Malhotra A. (2007). RluD, a highly conserved pseudouridine synthase, modifies 50S subunits more specifically and efficiently than free 23S rRNA. *RNA* 13, 1868–1876.
Valle M., Zavialov A., Li W., Stagg S. M., Sengupta J., Nielsen R. C., Nissen P., Harvey S. C., Ehrenberg M. & Frank J. (2003). Incorporation of aminoacyl-tRNA into the ribosome as seen by cryo-electron microscopy. *Nature Structural Biology* 10, 899–906.
Vazquez-Laslop N., Ramu H., Klepacki D., Kannan K. & Mankin A. S. (2010). The key function of a conserved and modified rRNA residue in the ribosomal response to the nascent peptide. *The EMBO Journal* 29, 3108–3117.
Vester B. & Douthwaite S. (1994). Domain V of 23S rRNA contains all the structural elements necessary for recognition by the ErmE methyltransferase. *Journal of Bacteriology* 176, 6999–7004.
Voorhees R. M., Weixlbaumer A., Loakes D., Kelley A. C. & Ramakrishnan V. (2009). Insights into substrate stabilization from snapshots of the peptidyl transferase center of the intact 70S ribosome. *Nature Structural & Molecular Biology* 16, 528–533.
Wang K. T., Desmolaize B., Nan J., Zhang X. W., Li L. F., Douthwaite S. & Su X. D. (2012). Structure of the bifunctional methyltransferase YcbY (RlmKL) that adds the m7G2069 and m2G2445 modifications in Escherichia coli 23S rRNA. *Nucleic Acids Research*.
Weeks K. M. (1997). Protein-facilitated RNA folding. *Current Opinion in Structural Biology* 7, 336–342.
Weitzmann C. J., Cunningham P. R., Nurse K. & Ofengand J. (1993). Chemical evidence for domain assembly of the Escherichia coli 30S ribosome. *Faseb J* 7, 177–180.
Weitzmann C., Tumminia S. J., Boublik M. & Ofengand J. (1991). A paradigm for local conformational control of function in the ribosome: binding of ribosomal protein S19 to Escherichia coli 16S rRNA in the presence of S7 is required for methylation of m2G966 and blocks methylation of m5C967 by their respective methyltransferases. *Nucleic Acids Research* 19, 7089–7095.

Weixlbaumer A., Jin H., Neubauer C., Voorhees R. M., Petry S., Kelley A. C. & Ramakrishnan V. (2008). Insights into translational termination from the structure of RF2 bound to the ribosome. *Science* 322, 953–956.

Weixlbaumer A., Petry S., Dunham C. M., Selmer M., Kelley A. C. & Ramakrishnan V. (2007). Crystal structure of the ribosome recycling factor bound to the ribosome. *Nature Structural & Molecular Biology* 14, 733–737.

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

Wilson D. N. & Nierhaus K. H. (2005). Ribosomal proteins in the spotlight. *Critical Reviews in Biochemistry and Molecular Biology* 40, 243–267.

Wilson D. N. & Nierhaus K. H. (2007). The weird and wonderful world of bacterial ribosome regulation. *Critical Reviews in Biochemistry and Molecular Biology* 42, 187–219.

Wilson D. N., Schluenzen F., Harms J. M., Yoshida T., Ohkubo T., Albrecht R., Buerger J., Kobayashi Y. & Fucini P. (2005). X-ray crystallography study on ribosome recycling: the mechanism of binding and action of RRF on the 50S ribosomal subunit. *The EMBO Journal* 24, 251–260.

Wimberly B. T., Brodersen D. E., Clemons W. M., Jr., Morgan-Warren R. J., Carter A. P., Vonrhein C., Hartsch T. & Ramakrishnan V. (2000). Structure of the 30S ribosomal subunit. *Nature* 407, 327–339.

Winkler M. E. (1998). Genetics and Regulation of Base Modifications in the tRNA and rRNA of Prokaryotes and Eukaryotes. *Modification and Editing of RNA*. Edited by Henri Grosjean and Rob Benne.

Wireman J. W. & Sypherd P. S. (1974). In vitro assembly of 30S ribosomal particles from precursor 16S RNA of Escherichia coli. *Nature* 247, 552–554.

Woodson S. A. (2011). RNA folding pathways and the self-assembly of ribosomes. *Accounts of Chemical Research* 44, 1312–1319.

Wrzesinski J., Nurse K., Bakin A., Lane B. G. & Ofengand J. (1995). A dual-specificity pseudouridine synthase: an Escherichia coli synthase purified and cloned on the basis of its specificity for psi 746 in 23S RNA is also specific for psi 32 in tRNA(phe). *RNA* 1, 437–448.

Wurm J. P., Griese M., Bahr U., Held M., Heckel A., Karas M., Soppa J. & Wohnert J. (2012). Identification of the enzyme responsible for N1-methylation of pseudouridine 54 in archaeal tRNAs. *RNA* 18, 412–420.

Wuyts J., Perriere G. & Van De Peer Y. (2004). The European ribosomal RNA database. *Nucleic Acids Research* 32, D101-103.

Xu Z., O'Farrell H. C., Rife J. P. & Culver G. M. (2008). A conserved rRNA methyltransferase regulates ribosome biogenesis. *Nature Structural & Molecular Biology* 15, 534–536.

Yan F., LaMarre J. M., Rohrich R., Wiesner J., Jomaa H., Mankin A. S. & Fujimori D. G. (2010). RlmN and Cfr are radical SAM enzymes involved in methylation of ribosomal RNA. *Journal of the American Chemical Society* 132, 3953–3964.

Yoshida H., Yamamoto H., Uchiumi T. & Wada A. (2004). RMF inactivates ribosomes by covering the peptidyl transferase centre and entrance of peptide exit tunnel. *Genes Cells* 9, 271–278.

Young R. A. & Steitz J. A. (1978). Complementary sequences 1700 nucleotides apart form a ribonuclease III cleavage site in Escherichia coli ribosomal precursor RNA. *PNAS* 75, 3593–3597.

Yusupov M. M., Yusupova G. Z., Baucom A., Lieberman K., Earnest T. N., Cate J. H. & Noller H. F. (2001). Crystal structure of the ribosome at 5.5 A resolution. *Science* 292, 883–896.

Zengel J. M. & Lindahl L. (1994). Diverse mechanisms for regulating ribosomal protein synthesis in Escherichia coli. *Progress in Nucleic Acid Research and Molecular Biology* 47, 331–370.

Zhang W., Dunkle J. A. & Cate J. H. (2009). Structures of the ribosome in intermediate states of ratcheting. *Science* 325, 1014–1017.

Zimmermann R. A., Muto A., Fellner P., Ehresmann C. & Branlant C. (1972). Location of ribosomal protein binding sites on 16S ribosomal RNA. *PNAS* 69, 1282–1286.

SUMMARY IN ESTONIAN

Bakteriaalne ribosoom modifitseeritud nukleosiidide vaatevinklist

Ribosoomid vastutavad valgusünteesi läbiviimise eest kõikides elusrakkudes. Bakteriaalse mudelorganismi *Escherichia coli* (soolekepike) ribosoomid koosnevad kolmest ribosomaalse RNA (rRNA) molekulist ning 54 ribosomaalsest valgust (r-valgud), kusjuures RNA domineerib nii ribosoomi struktuuri kui funktsiooni seisukohast. Kuigi eukarüootide ribosoomid on suuremad ning koosnevad enamatest komponentidest, on ribosoomide struktuursed ning funktsionaalsed põhielemendid konserveerunud terve elusilooduse ulatuses. Arvestades ribosoomide suurust ja keerukust, ei ole vast üllatav, et ribosoomide süntees rakkudes on ülimalt kompleksne ning rangelt reguleeritud protsess. Ribosooomi komponentide kokkupakkimine algab samaaegselt rRNA sünteesiga ning hõlmab lisaks rRNA protsessimisele nii rRNA kui ka r-valkude modifitseerimist. Mitmed haruldased geneetilised haigused on põhjustatud vigadest ribosoomide sünteesil.

E. coli rRNAd sisaldavad 36 modifitseeritud nukleosiidi (MN), millest valdav enamus on pseudouridiinid (Ψ) ja erinevad metülatsioonid. Vaatamata sellele, et MN on enamjaolt konserveerunud ning paiknevad ribosoomi funktsionaalselt olulistes piirkondades, on nende tähtsus suures osas selguseta. Bakterites sünteesivad rRNA MN koht-spetsiifilised ensüümid, mis on *E. coli*s valdavalt küll tuvastatud, ent iseloomustatud vaid üksikutel juhtudel. Ribosoomi funktsiooneerimise seisukohast väga oluline regioon, "juuksenõel 69" (H69), sisaldab kolme konserveerunud Ψ, millest üks (positsioonis 1915) on lisaks veel ka metüleeritud ($m^3\Psi$). Kõik kolm Ψ on sünteesitud pseudouridiini süntaasi RluD poolt, kuid H69 metüleeriv ensüüm oli seni teadmata. Käesoleva töö eesmärgiks oligi tuvastada H69 pseudouridiini metüültransferaas ning iseloomustada H69 modifikatsioone ning modifikatsiooniensüüme ribosoomide sünteesi ning funktsioneerimise seisukohast.

Peamised tulemused:

1. *E. coli ybeA* geen kodeerib H69 $m^3\Psi$ metüültransferaasi. YbeA valk on bakterites konserveerunud ning nimetati ümber RlmH valguks, mis on kooskõlas kokkuleppelise rRNA metüültransferaaside nomenklatuuriga. RlmH valgu näol on tegu esimese identifitseeritud pseudouridiini metüültransferaasiga ning suure tõenäosusega ainukesega bakterites, sest peale H69 ei ole $m^3\Psi$ bakterites mujal kirjeldatud.
2. RlmH substraadispetsiifika on unikaalne rRNA modifikatsiooniensüümide seas. Esiteks, RlmH metüleerib eelistatult Ψ, ning teiseks, teeb seda 70S ribosoomil mitte aga vabadel subühikutel või rRNA-l. Sellest lähtuvalt on RlmH üks viimaseid (kui mitte kõige viimane) rRNA modifikatsiooniensüüm, mis ribosoomi sünteesil osaleb. RlmH poolne metüleerimine toimub suure tõenäosusega samaaegselt translatsiooni esimeste etappidega,

mis viitab asjaolule, et ribosoomi suure subühiku lõplik valmimine sõltub väiksest subühikust.

3. RlmH on kiire ensüüm võrreldes enamike RNA modifikatsiooniensüümidega. RlmH substraadispetsiifika ja ensüümikineetika tundmaõppimine võimaldas määrata kineetilised parameetrid ka ensüümile RluD. RluD on esimene rRNA pseudouridiini süntaas, mille ensüümikineetikat on iseloomustatud. RluD ja RlmH kineetilised konstandid on kooskõlas ribosoomide sünteesi kineetikaga rakus.
4. Üksikpunktmutatsioonid nii substraat rRNAs kui RlmH valgus endas mõjutavad oluliselt nii substraadi äratundmist kui katalüüsi kiirust, mis eristab RlmH ensüümi enamikest RNA modifikatsiooniensüümidest.

ACKNOWLEDGEMENTS

I truly feel I lucked out with my supervisors Prof. Jaanus Remme and Dr. Aivar Liiv, as well as with all my co-workers and friends. First of all, I would like to thank Jaanus for being very supportive and positive, ALWAYS. Also, for being so patient and putting up with all of my shenanigans over the years. Aivar really is the closest thing to God I am willing to believe in. All joking aside, I really appreciate everything he has taught me or has been trying to teach me, as the case may have been. I also want to thank Lauri for being a reference person for me in many aspects; and Ülo for keeping me grounded and a little bit skeptical just about everything. All the past (Arto and Smaddy) and present (Kai, Kalle, Kerli, Pavel and Triin) co-workers and students (Hanna, Maireet, and Ermo) of the molecular biology lab, as well as people from the biochemistry lab, I want to thank for hardly ever having a dull moment whether in- or outside of the lab. Tiiu and Lagle have always found the time and the energy to help with administrative issues. Prof. Douthwaite generously accepted me as a guest in his lab at the University of Southern Denmark.

My special thanks go to Anu, Katrin, Margus, and Triinu for being my course mates, co-workers, friends, and just really wonderful people. I can't imagine not having you around for the last decade or so.

I really hope I didn't forget anyone!

Lõpetuseks, suured tänud minu perekonnale, sealhulgas vanaemadele, onule ja Ailenile, igakülgse ja tingimusteta toetuse eest. Aitäh teile kõigile!

CURRICULUM VITAE (English)

I. General

1. Name: Rya Ero
2. Time and place of birth: 26.11.1981, Rakvere, Estonia
3. Citizenship: Estonian
4. Marital status: single
5. Address, telephone, e-mail: University of Tartu, Faculty of Science and Technology, Institute of Molecular and Cell Biology
Riia 23, 51010 Tartu, Estonia
tel: +372 5527374
e-mail: ryaero@ut.ee

6. Current position: University of Tartu, Institute of Molecular and Cell Biology, specialist

7. Education:

1988-1997	Rakvere Science Gymnasium
1997-2000	Tallinn Secondary Science School
2000-2004	University of Tartu, BSc, gene technology
2004-2006	University of Tartu, MSc, molecular biology
2006-...	University of Tartu, doctorate studies, molecular biology

8. Language skills: Estonian, English, German

9. Research experience:

2006-2007	University of Tokyo, Department of Biophysics and Biochemistry, Prof. S. Yokoyama's group
2008	University of Southern Denmark, Department of Biochemistry and Molecular Biology, Prof. S. Douthwaite's group (two months)
2010-...	University of Tartu, Institute of Molecular and Cell Biology, Prof. J. Remme's group, researcher

II. Scientific and research activity

1. Main research interests: Ribosome biosynthesis in *Escherichia coli*, tRNA and rRNA modifications, and RNA modification enzymes.

2. List of publications:

Ero, R., Peil, L., Liiv, A., Remme, J. 2008. Identification of pseudouridine methyltransferase in *Escherichia coli*. RNA. 14(10):2223-33.
Ero, R., Leppik, M., Liiv, A., Remme, J. 2010. RNA. Specificity and kinetics of 23S rRNA modification enzymes RlmH and RluD. 16(11):2075-84.
Leppik, M., Ero, R., Liiv, A., Kipper, K., Remme, J. 2012. Different sensitivity of H69 modification enzymes RluD and RlmH to mutations in *Escherichia coli* 23S rRNA. Biochimie. 94: 1080-89.

3. Conferences:
2008 13-th Annual Meeting of the RNA Society, Berlin, poster presentation
2009 NordForsk RNA Network Meeting, Reykjavik, oral presentation
2010 Ribosomes 2010, Orvieto, poster presentation
2012 11-th course: Frontieres of Biophysics and Structural Biology, Erice, oral presentation

4. Teaching: Practical Course in Chemistry of Nucleic Acids
Protein Biosynthesis, 2 lectures

5. Supervising: 3 BSc students, high school student (II place in Archimedes National Youth Science Awards)

CURRICULUM VITAE (Estonian)

I. Üldandmed

1. Ees- ja perekonnanimi:		Rya Ero
2. Sünniaeg ja koht:		26.11.1981, Rakvere, Eesti
3. Kodakondsus:		Eesti
4. Perekonnaseis:		vallaline
5. Aadress, telefon, e-post:		Tartu Ülikool, Loodus- ja tehnoloogiateaduskond, Molekulaar- ja rakubioloogia instituut Riia 23, 51010 Tartu, Eesti tel: +372 5527374 e-post: ryaero@ut.ee
6. Praegune töökoht, amet:		Tartu Ülikool, Molekulaar- ja rakubioloogia instituut, spetsialist
7. Haridus:	1988-1997	Rakvere Reaalgümnaasium
	1997-2000	Tallinna Reaalkool
	2000-2004	Tartu Ülikool, BSc, geenitehnoloogia erialal
	2004-2006	Tartu Ülikool, MSc, molekulaarbioloogia erialal
	2006-...	Tartu Ülikool, doktoriõpe molekulaarbioloogia erialal
8. Keelteoskus:		eesti, inglise, saksa

9. Teadusliku töö kogemus:

2006–2007	Tokio Ülikool, Biofüüsika ja biokeemia õppetool, Prof. S. Yokoyama töögrupp
2008	Lõuna-Taani Ülikool, Biokeemia ja molekulaarbioloogia õppetool, Prof. S. Douthwaite'i töögrupp (kaks kuud)
2010-...	Tartu Ülikool, Molekulaar- ja rakubioloogia instituut, Prof. J. Remme töögrupp, spetsialist

II. Teaduslik ja arendustegevus

1. Peamised uurimisvaldkonnad: Ribosoomide biosüntees bakteris *Escherichia coli*, tRNA ja rRNA modifikatsioonid ning RNA modifikatsiooniensüümid

2. Publikatsioonide loetelu:

Ero, R., Peil, L., Liiv, A., Remme, J. 2008. Identification of pseudouridine methyltransferase in *Escherichia coli*. RNA. 14(10):2223-33.
Ero, R., Leppik, M., Liiv, A., Remme, J. 2010. RNA. Specificity and kinetics of 23S rRNA modification enzymes RlmH and RluD. 16(11):2075-84.

Leppik, M., Ero, R., Liiv, A., Kipper, K., Remme, J. 2012. Different sensitivity of H69 modification enzymes RluD and RlmH to mutations in *Escherichia coli* 23S rRNA. Biochimie. 94: 1080-89.

3. Konverentsid:

2008 13-th Annual Meeting of the RNA Society, Berliin, Saksamaa, poster-ettekanne

2009 NordForsk RNA Network Meeting, Reykjavik, Island, suuline ettekanne

2010 Ribosomes 2010, Orvieto, Itaalia, posterettekanne

2012 11-th course: Frontieres of Biophysics and Structural Biology, Erice, Sitsiilia, suuline ettekanne

4. Õppetöö: Nukleiinhapete keemia praktikumi juhendamine
Valgu biosünteesi kursuse raames 2 loengut

5. Juhendamine: 3 bakalaureuseõppes oleva üliõpilase ning ühe keskkooli õpilase (Archimedese õpilaste teadustööde konkursi II preemia) juhendamine

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. 112 p.
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.
167. **Lauri Peil.** Ribosome assembly factors in *Escherichia coli.* Tartu, 2009, 147 p.
168. **Lea Hallik.** Generality and specificity in light harvesting, carbon gain capacity and shade tolerance among plant functional groups. Tartu, 2009, 99 p.
169. **Mariliis Tark.** Mutagenic potential of DNA damage repair and tolerance mechanisms under starvation stress. Tartu, 2009, 191 p.
170. **Riinu Rannap.** Impacts of habitat loss and restoration on amphibian populations. Tartu, 2009, 117 p.
171. **Maarja Adojaan.** Molecular variation of HIV-1 and the use of this knowledge in vaccine development. Tartu, 2009, 95 p.
172. **Signe Altmäe.** Genomics and transcriptomics of human induced ovarian folliculogenesis. Tartu, 2010, 179 p.
173. **Triin Suvi.** Mycorrhizal fungi of native and introduced trees in the Seychelles Islands. Tartu, 2010, 107 p.
174. **Velda Lauringson.** Role of suspension feeding in a brackish-water coastal sea. Tartu, 2010, 123 p.
175. **Eero Talts.** Photosynthetic cyclic electron transport – measurement and variably proton-coupled mechanism. Tartu, 2010, 121 p.
176. **Mari Nelis.** Genetic structure of the Estonian population and genetic distance from other populations of European descent. Tartu, 2010, 97 p.
177. **Kaarel Krjutškov.** Arrayed Primer Extension-2 as a multiplex PCR-based method for nucleic acid variation analysis: method and applications. Tartu, 2010, 129 p.
178. **Egle Köster.** Morphological and genetical variation within species complexes: *Anthyllis vulneraria* s. l. and *Alchemilla vulgaris* (coll.). Tartu, 2010, 101 p.
179. **Erki Õunap.** Systematic studies on the subfamily Sterrhinae (Lepidoptera: Geometridae). Tartu, 2010, 111 p.
180. **Merike Jõesaar.** Diversity of key catabolic genes at degradation of phenol and *p*-cresol in pseudomonads. Tartu, 2010, 125 p.
181. **Kristjan Herkül.** Effects of physical disturbance and habitat-modifying species on sediment properties and benthic communities in the northern Baltic Sea. Tartu, 2010, 123 p.

182. **Arto Pulk.** Studies on bacterial ribosomes by chemical modification approaches. Tartu, 2010, 161 p.
183. **Maria Põllupüü.** Ecological relations of cladocerans in a brackish-water ecosystem. Tartu, 2010, 126 p.
184. **Toomas Silla.** Study of the segregation mechanism of the Bovine Papillomavirus Type 1. Tartu, 2010, 188 p.
185. **Gyaneshwer Chaubey.** The demographic history of India: A perspective based on genetic evidence. Tartu, 2010, 184 p.
186. **Katrin Kepp.** Genes involved in cardiovascular traits: detection of genetic variation in Estonian and Czech populations. Tartu, 2010, 164 p.
187. **Virve Sõber.** The role of biotic interactions in plant reproductive performance. Tartu, 2010, 92 p.
188. **Kersti Kangro.** The response of phytoplankton community to the changes in nutrient loading. Tartu, 2010, 144 p.
189. **Joachim M. Gerhold.** Replication and Recombination of mitochondrial DNA in Yeast. Tartu, 2010, 120 p.
190. **Helen Tammert.** Ecological role of physiological and phylogenetic diversity in aquatic bacterial communities. Tartu, 2010, 140 p.
191. **Elle Rajandu.** Factors determining plant and lichen species diversity and composition in Estonian *Calamagrostis* and *Hepatica* site type forests. Tartu, 2010, 123 p.
192. **Paula Ann Kivistik.** ColR-ColS signalling system and transposition of Tn*4652* in the adaptation of *Pseudomonas putida.* Tartu, 2010, 118 p.
193. **Siim Sõber.** Blood pressure genetics: from candidate genes to genome-wide association studies. Tartu, 2011, 120 p.
194. **Kalle Kipper.** Studies on the role of helix 69 of 23S rRNA in the factor-dependent stages of translation initiation, elongation, and termination. Tartu, 2011, 178 p.
195. **Triinu Siibak.** Effect of antibiotics on ribosome assembly is indirect. Tartu, 2011, 134 p.
196. **Tambet Tõnissoo.** Identification and molecular analysis of the role of guanine nucleotide exchange factor RIC-8 in mouse development and neural function. Tartu, 2011, 110 p.
197. **Helin Räägel.** Multiple faces of cell-penetrating peptides – their intracellular trafficking, stability and endosomal escape during protein transduction. Tartu, 2011, 161 p.
198. **Andres Jaanus.** Phytoplankton in Estonian coastal waters – variability, trends and response to environmental pressures. Tartu, 2011, 157 p.
199. **Tiit Nikopensius.** Genetic predisposition to nonsyndromic orofacial clefts. Tartu, 2011, 152 p.
200. **Signe Värv.** Studies on the mechanisms of RNA polymerase II-dependent transcription elongation. Tartu, 2011, 108 p.
201. **Kristjan Välk.** Gene expression profiling and genome-wide association studies of non-small cell lung cancer. Tartu, 2011, 98 p.

202. **Arno Põllumäe.** Spatio-temporal patterns of native and invasive zooplankton species under changing climate and eutrophication conditions. Tartu, 2011, 153 p.
203. **Egle Tammeleht.** Brown bear (*Ursus arctos*) population structure, demographic processes and variations in diet in northern Eurasia. Tartu, 2011, 143 p.
205. **Teele Jairus.** Species composition and host preference among ectomycorrhizal fungi in Australian and African ecosystems. Tartu, 2011, 106 p.
206. **Kessy Abarenkov.** PlutoF – cloud database and computing services supporting biological research. Tartu, 2011, 125 p.
207. **Marina Grigorova.** Fine-scale genetic variation of follicle-stimulating hormone beta-subunit coding gene (*FSHB*) and its association with reproductive health. Tartu, 2011, 184 p.
208. **Anu Tiitsaar.** The effects of predation risk and habitat history on butterfly communities. Tartu, 2011, 97 p.
209. **Elin Sild.** Oxidative defences in immunoecological context: validation and application of assays for nitric oxide production and oxidative burst in a wild passerine. Tartu, 2011, 105 p.
210. **Irja Saar**. The taxonomy and phylogeny of the genera *Cystoderma* and *Cystodermella* (Agaricales, Fungi). Tartu, 2012, 167 p.
211. **Pauli Saag.** Natural variation in plumage bacterial assemblages in two wild breeding passerines. Tartu, 2012, 113 p.
212. **Aleksei Lulla.** Alphaviral nonstructural protease and its polyprotein substrate: arrangements for the perfect marriage. Tartu, 2012, 143 p.
213. **Mari Järve.** Different genetic perspectives on human history in Europe and the Caucasus: the stories told by uniparental and autosomal markers. Tartu, 2012, 119 p.
214. **Ott Scheler**. The application of tmRNA as a marker molecule in bacterial diagnostics using microarray and biosensor technology. Tartu, 2012, 93 p.
215. **Anna Balikova**. Studies on the functions of tumor-associated mucin-like leukosialin (CD43) in human cancer cells. Tartu 2012, 129 p.
216. **Triinu Kõressaar.** Improvement of PCR primer design for detection of prokaryotic species. Tartu 2012, 83 p.
217. **Tuul Sepp.** Hematological health state indices of greenfinches: sources of individual variation and responses to immune system manipulation. Tartu 2012, 117 p.