Accessory factors for ribosomal assembly

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2 ABSTRACT

The assembly of ribosomal RNA (rRNA) and ribosomal proteins (r-proteins) into ribosomal subunits (30S and 50S) is a complex process. Transcription of rRNA requires antitermination proteins and the primary transcripts are processed by ribonucleases. R-proteins and rRNAs are chemically modified, the r-proteins bind to the rRNAs and the formed RNA-protein complexes are folded into mature ribosomal subunits. All these processes are well-coordinated and overlapping. Non-ribosomal factors are required for proper assembly and maturation of the ribosomal subunits. Two of these factors are the RimM and RbfA proteins, which bind to 30S subunits and are important for efficient processing of 16S rRNA. Lack of either RimM or RbfA results in a reduced amount of polysomes and a lower growth rate. An increased amount of RbfA can partially compensate for deficiencies shown by a RimM lacking mutant.

Here, mutations that alter phylogenetically conserved amino acids in RimM have been constructed. One of these (rimM120), which resulted in the replacement of two adjacent tyrosines by alanines, reduced the growth rate three-fold and also decreased the processing efficiency of 16S rRNA. The RimM120 mutant protein showed a much reduced binding to the 30S subunits. Suppression of the rimM120 mutant was achieved by increased amount of the RimM120 protein, by overexpression of rbfA, or by mutations that changed r-protein S19 or 16S rRNA. A variant of r-protein S13, which was previously isolated as a suppressor to a deletion of rimM (∆rimM), suppressed also the rimM120 mutation. The wild-type RimM protein, but not the RimM120 protein, was shown to bind r-protein S19 in the 30S subunits. The changes in S13, S19 and 16S rRNA that compensated for the deficiencies shown by the rimM mutants are all located within a small region of the head of the 30S subunit, suggesting that this region is the likely target for the RimM action.

To isolate RbfA variants that show reduced association with the 30S subunits, phylogenetically conserved, surface exposed amino acid residues of RbfA were changed to alanines or, in some instances, to amino acids of the opposite charge to that in the wild-type protein. Alterations of F5, R31, D46 and D100 had the largest effect on growth.

Mutations in the metY-nusA-infB operon, isolated as suppressors to the ∆rimM mutant, were shown to increase the amounts of RbfA. In a ∆rimM mutant, all RbfA protein was found associated with the 30S subunits and no free RbfA was detected.

The RlmB protein was shown to be the methyltransferase responsible for the formation of Gm2251 in 23S rRNA in Escherichia coli. Unlike a Saccharomyces cerevisiae mutant that lacks the orthologue to RlmB, Pet56p, which methylates mitochondrial rRNA, a ∆rlmB mutant did not show any defects in ribosomal assembly.
3 PAPERS IN THIS THESIS

This thesis is based upon the following publications, which are referred to in the text by their roman numerals (I-IV).


4 INTRODUCTION

4.1 STRUCTURE AND FUNCTION OF RIBOSOMES.

In the ribosomes, mRNAs are translated into new peptides by polymerization of amino acids in an order determined by the codon sequences of the mRNAs. The most studied organism, when it comes to basic cellular functions, is the bacterium *Escherichia coli*. This is the organism this thesis will deal with if nothing else is noted.

**Components of the ribosomes.** The ribosome consists of two subunits named 30S and 50S based on their sedimentation coefficients. The 30S subunit consists of 16S rRNA, 1542 nucleotides in length, and 21 ribosomal proteins (r-proteins) (S1-S21), while the 50S subunit consists of 5S and 23S rRNA, 120 (or 121) and 2904 nucleotides in length, respectively, as well as 33 r-proteins (L1-L6, L7/L12, L9-L11, L13-L25, L27-L36) (Zengel and Lindahl, 1994). The 16S and 23S rRNAs and several r-proteins are chemically modified by special enzymes (see section 4.6). All the ribosomal components have to assemble in a precise manner to form functional ribosomes (see section 4.4).

**The three-dimensional structure of the ribosome.** Several methods to elucidate the structure of the ribosomes have been employed. Cryo electron microscopy and x-ray crystallography have been used to reveal the structure of ribosomal components, whole subunits or complete 70S ribosomes. The highest resolution has been achieved by x-ray crystallography but these studies have been hampered by the difficulties to produce crystals of sufficient quality. High-resolution structures of the 30S subunit from *Thermus thermophilus* (Wimberly, *et al.*, 2000), the 50S from *Haloarcula marismortui* (Ban, *et al.*, 2000) and from *Deinococcus radiodurans* (Harms, *et al.*, 2001) have been produced (Figure 1). In addition, structures of ribosomes and ribosomal subunits in complex with translation factors and antibiotics have been resolved (Smith, *et al.*, 1992; McCutcheon, *et al.*, 1999; Frank and Agrawal, 2000; Carter, *et al.*, 2001; Klaholz, *et al.*, 2004; Yonath and Bashan, 2004). Furthermore, interactions between the different components have been studied by using footprinting and crosslinking techniques (Mueller and Brimacombe, 1997).
Figure 1. Crystal structures of the ribosomal subunits. The structures are viewed both from the subunit interface (left) and solvent side (right). A: 30S subunit from *Thermus thermophilus* (Wimberly, *et al.*, 2000). The 16S rRNA is in gray; the identity of the differently colored proteins is indicated. *T. thermophilus* does not have the S21 protein but has the Thx protein not present in *E. coli*. B: 50S subunit from *Deinococcus radiodurans* (Harms, *et al.*, 2001). The 23S rRNA is in light gray, 5S rRNA in dark gray; the identity of the differently colored proteins is indicated. Protein names in red mark the approximate positions of proteins that did not resolve well in the crystal. L34 is not marked because it is positioned behind L2 and is not visible. *D. radiodurans* lacks the L25 protein but a different protein, CTC, is present and is proposed to replace L25. The coordinates for the structures were retrieved from the Protein Data Bank, PDB IDs: 1FJF (30S) and 1NKW (50S).
The translation process. The translation process can be divided into three parts: initiation, elongation and termination. After termination, the ribosomes are recycled and can take part in a new round of translation.

**Initiation.** During initiation of translation, the 30S subunit, initiator tRNA and mRNA form a complex with the help of initiation factors (IF) 1, 2 and 3. The binding of the 30S subunit to the mRNA is usually mediated by r-protein S1 and the anti-Shine-Dalgarno sequence in 16S rRNA, which basepairs to the Shine-Dalgarno (SD) sequence in the mRNA (Shine and Dalgarno, 1974; Boni, et al., 1991). The formylmethionine charged initiator tRNA binds to the peptidyl (P) site of the 30S subunit and, via codon-anticodon basepairing, to the start codon of the mRNA. IF2 facilitates the binding of initiator tRNA to the P site and blocks the aminoacyl (A) site (Carter, et al., 2001). IF3 keeps the two ribosomal subunits apart and also prohibits initiation with non-initiator tRNAs or initiation at wrong codons (Hartz, et al., 1990). The function of IF1 is less known but it improves the function of the other IFs. After this primary initiation complex is formed, the 50S subunit binds to it, the IFs are released and elongation can proceed (reviewed by Gualerzi and Pon, 1990).

**Elongation.** During elongation of translation, ternary complexes consisting of elongation factor Tu (EF-Tu), aminoacylated tRNA, and GTP, bind to a translating ribosome so that the anticodon of the tRNA can interact with the mRNA in the A-site. If the tRNA cannot form a proper codon-anticodon interaction, it will be rejected. On the other hand, if the right tRNA enters the A-site, the codon-anticodon interaction will be correct, resulting in a conformational change in the 30S subunit, leading to GTP hydrolysis and release of EF-Tu. After this, a proofreading step occurs where there is a second chance for improper tRNAs to be rejected. If the tRNA is not rejected it is accommodated so that the aminoacyl group ends up in the peptidyl transferase center of the 50S subunit. Next, the peptide on the P-site tRNA is transferred to the aminoacyl group on the A-site tRNA and is thereby lengthened by one amino acid. The A-site tRNA will end up in a hybrid state, in the A-site of the 30S subunit and in the P-site of the 50S subunit (the A/P state) and the P-site tRNA ends up in the P-site of the 30S subunit and in the exit (E) site of the 50S subunit (the P/E state) (Moazed and Noller, 1989). After peptidyl transfer, the elongation factor G (EF-G) binds to, and induces a conformational change of the ribosome (Frank and Agrawal, 2000). EF-G hydrolyzes a GTP and translocation takes place; the deacylated tRNA in the P/E site shifts to the E-site and the peptidyl-tRNA in the A/P site shifts to the P-site (Rodnina, et al., 1997). During the translocation, the mRNA is shifted one codon, while maintaining the interaction with the peptidyl-tRNA. The
next codon of the mRNA will after the translocation reside in the A-site, and be available for interaction with a new tRNA.

Termination and recycling. Termination of translation occurs when the ribosome encounters a stop codon, which is recognized by release factor 1 (RF1) or RF2 (RF1 recognizes UAA and UAG stop codons, whereas RF2 recognizes UAA and UGA stop codons), after which the peptide is hydrolyzed from the P-site tRNA. Then RF3 binds to the ribosome inducing the release of RF1 or RF2, after which RF3 hydrolyzes GTP to release itself (reviewed by Kisselev and Buckingham, 2000). The ribosome recycling factor (RRF), together with EF-G and IF3, dissociate the 30S and 50S subunits and remove the remaining tRNA (and mRNA) from the post translation complex to free the ribosomal subunits for a new round of translation (Karimi, et al., 1999; Hirokawa, et al., 2002).

Secondary functions of the ribosome. Although protein synthesis is the main function of the ribosome, there are several secondary functions connected to translation. The ribosome is involved in all translational regulation, which is important for optimization of protein synthesis rates.

Transcription-translation coupling. The transcription and translation processes are coupled in the sense that ribosomes initiate translation on nascent mRNAs while the mRNAs are being synthesized, and follow behind the RNA polymerase (RNAP). The coupling between RNAP and ribosomes is important for proper transcription and prohibits premature Rho dependent termination of transcription, since the ribosomes prevent Rho-mRNA interactions (Adhya and Gottesman, 1978). The termination of non-coupled transcripts might serve to avoid production of untranslatable mRNAs.

Stringent response. The ribosomes are also involved in eliciting the stringent response, that is the rapid shut-down of stable RNA synthesis upon different kinds of nutritional restrictions. Upon amino acid starvation there is an increase of unaminoacylated tRNAs and when they enter the A-site of the ribosome, the stringent response effector guanidine-3’-pyrophosphate-5’-pyrophosphate (ppGpp) is produced by the RelA protein bound to the ribosomes (Haseltine and Block, 1973; Ramagopal and Davis, 1974). The effector molecule, ppGpp, binds to the RNAP and decreases transcription preferably from stable RNA promoters (see also 4.3).

Folding and export. The folding and export of the newly synthesized peptides are influenced by the ribosomes. The ribosome itself has folding assisting capabilities (Das, et al., 1992), but can also bind the trigger factor chaperone that assists folding of newly produced peptides (Stoller, et al., 1995). The ribosome is also able to bind the signal recognition particle that promotes secretion and membrane integration of several proteins (Rinke-Appel, et al., 2002). The association of both chaperones and
the protein export machinery with ribosomes directly links translation to folding and export. This ensures that the affected peptides are properly folded and/or exported immediately upon production.  

**Trans-translation.** If a ribosome translates a truncated mRNA lacking a stop codon, the ribosome will stall at the end of the mRNA. A specialized RNA, the transfer-messenger RNA (tmRNA), can release such stalled ribosomes by a process called trans-translation (Keiler, et al., 1996). The tmRNA, which has a tRNA like part that can be charged with alanine, enters the stalled ribosome in a complex with EF-Tu, much like a tRNA but without recognizing any codon. The peptide on the P-site tRNA is then transferred to the alanine on the tmRNA and the ribosome translocates. Translation then shifts to use the tmRNA as an mRNA. The tmRNA has a short open reading frame that ends with a stop codon so that the translation can terminate in the regular way. In addition, this reading frame codes for a peptide that is a recognition signal for several proteases, resulting in degradation of what would most likely be an aberrant protein (Keiler, et al., 1996). In addition, the truncated mRNA seems to be destabilized by the trans-translation process (Yamamoto, et al., 2003).

**Thermo sensor.** The ribosome has also been proposed to act as a thermo sensor and mediates both cold shock and heat shock responses by an unknown mechanism (VanBogelen and Neidhardt, 1990).

### 4.2 **IN VITRO ASSEMBLY OF RIBOSOMES.**

**30S.** Active 30S subunits can be reconstituted *in vitro* by using 16S rRNA and small subunit proteins purified from ribosomes (Traub and Nomura, 1968) or *in vitro* transcribed 16S rRNA and recombinant proteins (Krzyzosiak, et al., 1987; Culver and Noller, 1999). In the first assembly step, 16S rRNA and 15 of the 30S proteins assemble into a reconstitution intermediate (RI₃₀), which sediments at about 21S (Traub and Nomura, 1969; Held and Nomura, 1973). Next, RI₃₀ is transformed into an activated intermediate (RI₃₀*); this step is rate limiting and requires heat (40°C), magnesium ions (10mM) and generally high ionic conditions (Traub and Nomura, 1969; Held and Nomura, 1973). Even though no proteins are added at this step, the sedimentation is changed to about 26S. During the last step, the remaining proteins are added and RI₃₀* is converted to mature 30S subunits (Traub and Nomura, 1969; Held and Nomura, 1973). Large conformational changes occur during the conversion of RI₃₀ to RI₃₀*, whereas when RI₃₀* is further matured to 30S only minor changes take place (Holmes and Culver, 2004). The assembly can be initiated by two proteins,
S4 and S7. S4 nucleates assembly in the 5’ and central domains of 16S rRNA, that fold into the body of the 30S subunit, while S7 nucleates assembly in the 3’ major domain of 16S rRNA that folds into the head of the 30S subunit (Nowotny and Nierhaus, 1988). The assembly process starts in the 5’ part of the 16S rRNA and progresses generally towards the 3’ part (Powers, et al., 1993). This is correlated with the fact that S7 binds relatively late even though it is an assembly initiator. The r-proteins bind to the forming particle in an ordered fashion; while some proteins can bind to naked rRNA, others require certain other proteins to bind first. The interdependence of the ribosomal protein binding has been determined and can be visualized in an assembly map (Held, et al., 1974; Nierhaus, 1991; Grondek and Culver, 2004).

50S. Production of 50S subunits in vitro resembles the process for 30S (Nierhaus and Dohme, 1974; Dohme and Nierhaus, 1976). First, the two rRNAs (5S and 23S) and about two thirds of the proteins form a first intermediate, RI_{50}(1), sedimenting at 33S. Thereafter, an activation step at 44°C and 4 mM Mg^{2+} is required to produce a product, RI_{50}*(1), which sediments at 41S. The remaining proteins are added and the particle is converted to RI_{50}(2) sedimenting at 48S. The final transformation of RI_{50}(2) to mature 50S particles requires the conditions, 50°C and 20 mM Mg^{2+} (Nierhaus and Dohme, 1974; Dohme and Nierhaus, 1976). Unlike 30S subunits, active 50S subunits cannot be made from in vitro transcribed rRNA because of a requirement of one or more modifications between positions 2445 and 2523 in 23S rRNA (Green and Noller, 1996). Yet, as for 30S, the assembly of 50S can be initiated by two proteins, L24, which usually binds first, and L3, which binds later (Nowotny and Nierhaus, 1982). The assembly map of the large subunit proteins has also been determined (Herold and Nierhaus, 1987).

4.3 REGULATION OF RIBOSOMAL SYNTHESIS.

The synthesis of ribosomal components is tightly regulated, since enough ribosomes must be produced for optimal growth in various conditions, while avoiding excess production due to the high energy cost.

**Regulation of rRNA synthesis.** The rRNAs are transcribed from seven operons (rrn, all containing 16S, 23S and 5S rRNA, as well as one or more tRNAs (Figure 2). The rRNA promoters. The main regulatory step in ribosomal biosynthesis is the transcription initiation of the rRNA operons, which exhibits stringent regulation, growth rate regulation and homeostatic regulation. The ribosomal RNA operons are
transcribed from two promoters, P1 and P2 (Figure 2). Both the P1 and P2 core promoters have consensus or near consensus -10 and -35 boxes but with a spacing of 16 basepairs, which is one shorter than in the consensus promoter. Upstream of the P1 and P2 core promoters lies UP elements, which are AT rich sequences that increase transcription by specifically binding the C terminal domain of the RNAP alpha subunit (Ross, et al., 1993). The enhancing effect of the UP element on initiation is about 20-fold at P1 promoters (except for rrnD P1, for which the effect is 50-fold) and four-fold at P2 promoters (Hirvonen, et al., 2001; Murray, et al., 2003a). Further upstream of the P1 promoters are binding sites for the transcription factor FIS (Ross, et al., 1990). Binding of FIS to these sites leads to a further increase in transcription initiation by six to eight times (except for rrnD P1, for which the increase is less than three-fold) (Hirvonen, et al., 2001). The nucleoid associated protein H-NS can down-regulate rRNA transcription, in part due to competition with FIS for DNA binding at the promoters (Afflerbach, et al., 1998), but also due to trapping of the transcriptional initiation complex after a trinucleotide has been formed (Schröder and Wagner, 2000).

**Figure 2.** Schematic view of an rRNA operon with a blow-up of the promoter region. Shown is a representative of four of the seven operons, where there is one tRNA gene between the genes for 16S and 23S rRNA. The other three operons contain two tRNA genes at this location (not shown). Downstream of the 5S rRNA, there is one tRNA present in two of the operons, one of which also contains a second gene for 5S rRNA (not shown), whereas there are two tRNAs in another operon (not shown) and none in the remaining four operons. The number of FIS sites in the different P1 promoter regions varies from three to five.
Regulation of transcription initiation. Proper regulation of the \textit{rrn} promoters requires a GC rich discriminator region between the -10 box and the transcriptional start point (Travers, 1980). The \textit{rrn} promoter architecture, including the discriminator region, only permits the RNAP to form unusually unstable open complexes at these promoters (Gourse, 1988). This in turn leads to a requirement for a high level of the initiating nucleotide triphosphate, iNTP (Gaal, \textit{et al.}, 1997). The stringent regulation is mediated by the concentration of ppGpp, which increases drastically during amino acid starvation (Cashel, 1969). The stringent effector ppGpp reduces the transcriptional initiation by further destabilizing the open complexes or by competition with iNTP for RNAP binding (Barker, \textit{et al.}, 2001; Jöres and Wagner, 2003). The DksA protein is involved in regulation by both iNTP and ppGpp, because it makes the open initiation complexes even more unstable and potentiates the regulation by these molecules (Paul, \textit{et al.}, 2004a). DksA has been proposed to coordinate and stabilize the binding of ppGpp to the RNAP (Perederina, \textit{et al.}, 2004). The iNTP levels are responsible for the homeostatic regulation (Gaal, \textit{et al.}, 1997). Since mRNA translation is the process in the cell that consumes most NTPs, the NTP levels can give an indication of the translational capacity compared to the amount of available energy. The iNTP and ppGpp levels contribute differently to the regulation of rRNA transcription during the different growth phases (Murray, \textit{et al.}, 2003b). During stationary phase and out-growth from stationary phase, the iNTP levels regulate the transcription. On the other hand, at nutritional up-shifts and down-shifts during exponential growth, ppGpp is the main regulator. When the cells enter stationary phase, both iNTP and ppGpp levels are responsible for down-regulation of the transcriptional initiation. The P1 promoter is the strongest during exponential growth in rich medium while the downstream P2 promoter dominates at low growth rates and in stationary phase (Murray and Gourse, 2004). When the P1 promoter is highly active, the transcription from this promoter will block the transcription of the P2 promoter (Gafny, \textit{et al.}, 1994). How the growth rate regulation is accomplished is not known (Paul, \textit{et al.}, 2004b). FIS, H-NS and ppGpp might take part in this regulation but none of them is solely responsible (Afflerbach, \textit{et al.}, 1998; Bartlett, \textit{et al.}, 2000). FIS is up-regulated in exponential growth and both H-NS and ppGpp levels are inversely correlated to the growth rate (Lazzarini, \textit{et al.}, 1971; Nilsson, \textit{et al.}, 1992; Afflerbach, \textit{et al.}, 1998).

Antitermination of rRNA transcription. The rRNA genes contain sites that can act as Rho dependent terminators (Kingston and Chamberlin, 1981). To avoid premature termination of rRNA transcription, which could be a problem especially since Rho can bind to untranslated transcripts (Adhya and Gottesman, 1978), an antitermination
system acts during rRNA transcription. Antitermination sequences located directly downstream of the P2 promoters and before the 23S rRNA genes will promote interactions between antitermination factors and the RNAP as it passes through these sequences (Li, et al., 1984; Szymkowiak and Wagner, 1987; Squires, et al., 1993). These associations allow the RNAP to pass through Rho dependent terminators (Li, et al., 1984; Albrechtsen, et al., 1990). The antitermination factors identified so far are NusA, NusB and NusG, as well as r-proteins S4, S10 (NusE), L3 and L13 (Nodwell and Greenblatt, 1993; Vogel and Jensen, 1997; Torres, et al., 2001; Torres, et al., 2004). NusA also promotes rapid transcription of the rRNA operons even in the presence of ppGpp, which is known to slow down transcriptional elongation (Vogel and Jensen, 1997).

Regulation of ribosomal protein synthesis. The synthesis of r-proteins is coupled to rRNA synthesis to ensure that all ribosomal components are made in stoichiometric amounts (Reviewed by Zengel and Lindahl, 1994). For most r-protein encoding operons, one of the encoded r-proteins has a regulatory role. If this protein is produced in excess over the amount of rRNA, it can bind to its own mRNA and inhibit translation of one of the early genes in that operon. Due to translational coupling, downstream genes are also translated less efficiently and the stability of the mRNA will be reduced. For one operon (S10), the regulation is both at the transcriptional and translational level, and is in both cases mediated by r-protein L4. The ribosomal proteins involved in antitermination (see above) might also help coordinate rRNA and r-protein synthesis (Torres, et al., 2001). If there is an excess of rRNA, the r-proteins involved in antitermination might be sequestered by the rRNA and there will be a shortage of these r-proteins available for antitermination, resulting in less efficient rRNA transcription.

4.4 PRODUCTION OF RIBOSOMES IN VIVO.

Although there is considerable in vitro data concerning ribosomal synthesis, the production of ribosomes in vivo is less understood. Both similarities and differences between the in vivo and the in vitro assembly process exist. The process in vitro is slow and requires very specific conditions; this is obviously not the case in vivo. The in vivo process is quite complex where rRNA transcription, processing and modification (see sections 4.5 and 4.6) as well as r-protein binding, and folding of the whole complex have to be well coordinated. A number of non-ribosomal proteins that seem to assist in the subunit formation have been identified (see section 4.7).
Intermediates in ribosome assembly. Precursor particles that are intermediates in 30S and 50S ribosomal subunit production have been found (Mangiarotti, et al., 1968; Hayes and Hayes, 1971). These particles are similar but not identical to the in vitro assembly intermediates. Formation of 30S proceeds via a 21S particle to an immature 30S particle and eventually into the mature 30S subunit (Lindahl, 1975). Similarly, formation of 50S proceeds via a 32S particle to a 43S particle, then to an immature 50S particle and finally into the mature 50S subunit (Lindahl, 1975). Both the 30S and the 50S intermediates are short lived and the most abundant of the immature subunits are those that sediment as the mature ones (Lindahl, 1975). The 21S precursor particle contains nine r-proteins (S1, S4, S5, S8, S13, S15, S16, S17, and S20) (Nierhaus, et al., 1973). The 32S particle contains about half of the large subunit proteins (L1, L4, L9, L10, L13, L17, L18, L20, L21, L22, L24, L25, L27, L29 and L30) and the 43S particle has an additional eight proteins (L3, L7, L11, L23, L14, L15 L33 and L19) (Nierhaus, et al., 1973). The precursors sedimenting like mature particles probably contain all r-proteins present in the mature subunits.

Transcription and assembly. The transcription of the rRNAs and the assembly of the ribosomal subunits are tightly linked. Initial cleavages of the rRNA transcript to produce the rRNA precursors (see section 4.5) occur already during transcription (Hofmann and Miller, 1977). Binding of ribosomal proteins to the rRNA starts before the transcription has been completed (de Narvaez and Schaup, 1979). Ribosomal RNA that is transcribed by the T7 polymerase assembles into abnormal ribosomal particles with low translational activity (Lewicki, et al., 1993). This is probably due to the high elongation rate of the T7 polymerase that leads to misfolding of the rRNAs and induces assembly defects. It is possible to imagine that the antitermination machinery also influences the assembly process, either by bringing the two ends of the rRNA together, which could facilitate folding and processing, or by bringing in the r-proteins involved in the antitermination process to a favorable position for rRNA binding (Torres, et al., 2001).

The role of precursor sequences. The leader sequence before and the internal spacers between the mature rRNA sequences are important for ribosomal assembly. Mutations in the 16S rRNA leader sequence that are present in 17S precursor rRNA (see section 4.5) but not in mature 16S rRNA affect the production of functional 30S subunits. The 30S subunits, produced with 16S rRNAs that have altered leader sequences, are largely non-functional in translation (Theißen, et al., 1993). This is probably due to altered folding of the 16S rRNA (Balzer and Wagner, 1998; Besancon and Wagner, 1999). For the production of active 50S subunits, the flanking sequences of the 23S rRNA are important and the base pairing between the 5’ and 3’
ends is crucial (Liiv and Remme, 1998). The 5’ end of pre-23S forms a secondary structure by itself before the transcription of the 3’ end has been completed and then subsequently it forms a different structure by base pairing to the 3’ end. The temporary structure at the 5’ end seems to be important for proper folding of 23S rRNA (Liiv and Remme, 2004).

**Quality control of ribosomes.** In the event that the assembly process fails and aberrant ribosomal subunits form, there is a system for their degradation. Two exo ribonucleases, polynucleotide phosphorylase (PNP) and RNaseR degrade defective rRNA (Cheng and Deutscher, 2003). In the absence of these nucleases, accumulation of rRNA fragments and defective ribosomal subunits occurs, leading to slower formation of new 70S ribosomes (Cheng and Deutscher, 2003). If an imbalance between the subunits arises, there might be a mechanism to degrade the subunit in excess. If so, PNP and RNaseR are likely candidates for this action.

**Coupling between the assembly of 50S and 30S.** The synthesis of the two subunits seems to be connected. For example, an L22 mutant that is defective in the production of 50S subunits is also affected in the synthesis of the 30S subunits, even though L22 does not seem to influence the assembly of 30S directly (Pardo, et al., 1979). Furthermore, a deletion of the gene for the 23S rRNA pseudouridine synthase RluD, not only results in a 50S maturation defect but also affects 30S assembly (Ofengand, et al., 2001) (see also section 4.6). The opposite can also be the case; a spectinomycin resistant mutant in which the 30S maturation is aberrant also has a defect in 50S production (Nashimoto and Nomura, 1970). An assembly defect in one subunit might lead to an excess of a free regulatory r-protein (see above) and this could in turn lead to a down regulation of certain r-proteins of both subunits. The existence of a more direct coupling that balances the production of the two subunits cannot be excluded.

### 4.5 PROCESSING OF RIBOSOMAL RNA.

**Early processing.** The ribosomal RNA operons contain the genes for 16S, 23S and 5S rRNA, as well as different tRNAs (Figure 2). All these RNA genes are transcribed into one long transcript (the 30S rRNA) which is processed by different RNases to produce the mature products. First, RNaseIII separates the precursors for 5S, 16S and 23S rRNA by cleaving at certain double stranded RNA structures (Figure 3) (Nikolaev, et al., 1973; Ginsburg and Steitz, 1975). For 16S rRNA, the cleavage at the 5’ end requires that the 3’ end has been transcribed but for 23S the 5’ end can be
cleaved before the 3’ end of the same molecule has been transcribed (King and Schlessinger, 1983). The RNaseIII cleavages are rapid, usually completed before the entire 30S rRNA has been transcribed; the RNaseIII uncleaved precursor can only be seen in an RNaseIII deficient strain (Nikolaev, et al., 1973; Hofmann and Miller, 1977).

**16S rRNA maturation.** The 16S rRNA precursor produced by RNaseIII is the 17S RNA, which has 115 nucleotides extra in the 5’ end and 33 extra in the 3’ end (Young and Steitz, 1978). Ribosomes produced in vitro that contain the 17S precursor are deficient in translation (Wireman and Sypherd, 1974). After the RNaseIII cleavage, the RNA is further processed in the 5’ end by RNaseE to a precursor, which is 66 nucleotides longer than mature 16S rRNA (Dahlberg, et al., 1978; Li, et al., 1999b). After this step, there is an immediate cleavage by RNaseG to give the mature 5’ end (Li, et al., 1999b). However, both RNaseE and RNaseG can form mature 5’ ends in absence of the other (Li, et al., 1999b). The RNase(s) involved in the 3’ processing of the RNaseIII product to form mature 16S rRNA has not been identified, however, this step is less efficient if the 5’ end has not been cleaved by RNaseE (Li, et al., 1999b). The initial cleavage by RNaseIII is not absolutely required for the further processing and mature 5’ and 3’ ends can be formed in an RNaseIII mutant (King and Schlessinger, 1983; Srivastava and Schlessinger, 1989). The cleavage by RNaseE is a late step in the assembly of 30S subunits and 17S RNA is found in particles co-sedimenting with mature 30S subunits (Lindahl, 1973). The conversion of 17S to mature 16S rRNA is dependent on proper maturation of the 30S subunits and several mutants affected in 30S assembly have increased amounts of 17S RNA (see section 4.7). In fact, it has been suggested that the conversion of 17S to 16S rRNA is dependent on the functionality of the ribosomal subunits and that this processing step occurs when the newly formed subunits initiate translation (Mangiarotti, et al., 1974).

**23S maturation.** RNaseIII cleaves the 5’ end of free 23S rRNA precursors as well as those already incorporated in 50S subunits seven and three nucleotides from the mature end, respectively (Bram, et al., 1980; Sirdeshmukh and Schlessinger, 1985; Allas, et al., 2003). The RNaseIII cleavage in the 3’ end produces a 23S rRNA precursor with eight extra nucleotides compared to mature 23S rRNA (Bram, et al., 1980). If RNaseIII is missing, mature 23S rRNA cannot be formed and instead a number of longer RNA molecules are formed (King, et al., 1984). These abnormal RNA species can still be inserted into functional 50S particles. The enzyme(s) required for the final maturation of the 5’ end is unknown, although it appears as if this step occurs in translating ribosomes (Srivastava and Schlessinger, 1988).
Figure 3. Proposed structures of rRNA precursors from the *rrnB* operon. Residues retained in the mature rRNAs are shown in bold, however, most of the mature RNAs are shown schematically as black lines. The different RNase cleavage sites are marked with arrows; question marks indicate unknown RNases. A: 16S rRNA precursor (Klein, et al., 1985), B: 23S rRNA precursor (Klein, et al., 1985) and C: 5S rRNA precursor (Roy, et al., 1983). The 16S and 23S rRNA precursors can form secondary structures beyond what is shown here.
mature 3’ ends are produced by the exonuclease RNaseT. This processing is more efficient in 23S rRNA precursors in ribosomes than in free 23S rRNA precursors (Li, et al., 1999a).

5S maturation. The RNaseIII derived 5S rRNA precursor extends from the RNaseIII site at the 3’ end of 23S rRNA (except for 5S rRNA from rrfF, which is the second 5S rRNA gene in the rrnD operon) to the end of the rRNA transcript (or possibly to the RNaseP site of the tRNA at the end of some operons) (Singh and Apirion, 1982). The RNaseIII processing is proceeded by RNaseE cleavage that leads to a 5S rRNA precursor with three extra nucleotides both at the 5’ and the 3’ end (Ghora and Apirion, 1978; Roy, et al., 1983). The three 5’ nucleotides are removed one at a time, but removal of the last one is delayed and seems to require active translation (Feunteun, et al., 1972). The enzyme(s) responsible for the final 5’ trimming has not been identified. As for 23S rRNA, the mature 3’ ends are produced by RNaseT (Li and Deutscher, 1995).

4.6 MODIFICATIONS OF RIBOSOMAL COMPONENTS.

Modified nucleotides in rRNA. The E. coli rRNAs have several modified nucleotides, 11 in 16S rRNA and 25 in 23S rRNA (summarized in Table 1), but none in 5S rRNA. The enzymes responsible for the different modifications are only known in about half of the cases. The modifications are clustered in the functionally important regions, the peptidyl transferase center in 50S and the decoding region in 30S (Brimacombe, et al., 1988; Smith, et al., 1992).

Pseudouridines. Pseudouridines are made by isomerization of uridines so that the uracil base is attached to the ribose by the C5 instead of the N1. There is one pseudouridine (Ψ) in 16S rRNA and ten Ψ in 23S rRNA (Del Campo, et al., 2001); one of the Ψ in 23S rRNA is further methylated to m^3Ψ (Kowalak, et al., 1996). All of the enzymes (and their corresponding genes) responsible for pseudouridine synthesis are known (Del Campo, et al., 2001). Null mutations of most of the corresponding genes do not give any apparent growth defects. The only null mutation that has a drastic effect is that of rluD, which gives a five-fold reduction in growth rate (Gutgsell, et al., 2001) (see also below). However, the lack of modification cannot account for this phenotype, because an RluD variant that lacks the pseudouridine synthase ability can restore the growth of the rluD null mutant (Gutgsell, et al., 2001). In addition, an rluA null mutant is out-competed by a wild-type strain in a competition experiment (Raychaudhuri, et al., 1999).
Methylations. Methyl groups can be added to nucleotides, both on the 2′-Oxygen of the ribose and on different positions of the base. In contrast to pseudouridine synthases, only the genes for some of the methyltransferases are known. The 16S rRNA methyltransferases identified so far are RsmB, which methylates C967 to m⁵C (Tscherne, et al., 1999a), RsmC, which methylates G1207 to m²G (Tscherne, et al., 1999b) and RsmA (KsgA), which methylates both A1518 and A1519 to m⁶₂A (Helser, et al., 1971; van Buul and van Knippenberg, 1985). The 23S methyltransferases identified so far are RlmA₁ (RrmA), which methylates G745 to m¹G (Gustafsson and Persson, 1998), RumB, which methylates U747 to m⁵U (Madsen, et al., 2003), RumA, which methylates U1939 to m²U (Agarwalla, et al., 2002) and RrmJ (FtsJ), which methylates the ribose of U 2552 (Caldas, et al., 2000a). Different methyltransferases have different substrate requirements. Some, like RsmB, RlmA₁ and RumA can methylate free rRNA but not rRNA in ribosomal particles (Weitzmann, et al., 1991; Hansen, et al., 2001; Agarwalla, et al., 2002), while others, like RsmC, RrmJ and the m²G966 methyltransferase, require more or less complete ribosomal particles (Weitzmann, et al., 1991; Tscherne, et al., 1999b; Caldas, et al., 2000a).

Table 1. Modifications in rRNA of E. coli.a

<table>
<thead>
<tr>
<th>16S rRNA</th>
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<th>Position</th>
<th>Gene for modifying enzyme</th>
<th>Reference for gene</th>
</tr>
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<td>516</td>
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<td>(Wrzesinski, et al., 1995a)</td>
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<tr>
<td>m⁷G</td>
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<td></td>
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<tr>
<td>m²G</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>967</td>
<td>rsmB</td>
<td>(Tscherne, et al., 1999a)</td>
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<tr>
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<td>rsmC</td>
<td>(Tscherne, et al., 1999b)</td>
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<tr>
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<td></td>
<td></td>
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<tr>
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<tr>
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<td>rsmA (ksgA)</td>
<td>(Helser, et al., 1971; van Buul and van Knippenberg, 1985)</td>
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<tr>
<td>m⁶₂A</td>
<td>1519</td>
<td>rsmA (ksgA)</td>
<td>(Helser, et al., 1971; van Buul and van Knippenberg, 1985)</td>
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</tr>
</tbody>
</table>
### Table 1. Continued.

<table>
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<th>Modified nucleotide</th>
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<th>Gene for modifying enzyme</th>
<th>Reference for gene</th>
</tr>
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<tr>
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<td>(Gustafsson and Persson, 1998)</td>
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<td>(Madsen, et al., 2003)</td>
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<tr>
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<td>$rluC$</td>
<td>(Conrad, et al., 1998; Huang, et al., 1998)</td>
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<td></td>
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<tr>
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<td></td>
</tr>
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<td>(Huang, et al., 1998; Raychaudhuri, et al., 1998)</td>
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</tr>
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<td></td>
<td></td>
</tr>
<tr>
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<td>$rluE$</td>
<td>(Del Campo, et al., 2001)</td>
</tr>
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</tr>
<tr>
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<td>$m^2A$</td>
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<td></td>
</tr>
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<td>$rluC$</td>
<td>(Conrad, et al., 1998; Huang, et al., 1998)</td>
</tr>
<tr>
<td>$Um$</td>
<td>2552</td>
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<td>(Caldas, et al., 2000a)</td>
</tr>
<tr>
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<td>$rluC$</td>
<td>(Conrad, et al., 1998; Huang, et al., 1998)</td>
</tr>
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<td>(Del Campo, et al., 2001)</td>
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<td>$Ψ$</td>
<td>2605</td>
<td>$rluB$</td>
<td>(Del Campo, et al., 2001)</td>
</tr>
</tbody>
</table>

a) From Rozenski et al. 1999, except C*2501 (Andersen, et al., 2004) and Ψ2604 (Del Campo, et al., 2001) in 23S rRNA.

b) $Ψ$, pseudouridine; $D$, dihydrouridine; $m^xN$, nucleotide N methylated at position x of the base; $Nm$, nucleotide N methylated at the 2’-O of the ribose; $C^*$, unknown modification.

c) $rluD$ is only responsible for the conversion of U1915 to $Ψ$ and not for the methylation.
Modification enzymes and ribosome assembly. The modifications are made during the assembly and maturation of the ribosomal particles, such that in mature ribosomes almost all of the positions susceptible to modification are fully modified. The modification enzymes that can use free rRNA as a substrate are likely to be involved in early steps of ribosomal subunit synthesis, while others are involved in later steps. Mutations in genes for some of the modification enzymes give rise to defects in the maturation of the ribosomal subunits. In an rluD null mutant there is less 70S ribosomes than in a wild-type strain and a 50S subunit assembly intermediate sedimenting at 39S accumulates (Ofengand, et al., 2001). Surprisingly, the maturation of the 30S subunits is also affected in the rluD null mutant and sedimentation of 30S subunits shifts to 27S (Ofengand, et al., 2001). Moreover, a deletion of rrmJ results in reduced amounts of 70S ribosomes and polysomes, and an accumulation of a 50S precursor particle sedimenting at 40S (Bügl, et al., 2000; Caldas, et al., 2000b). Furthermore, an rlmA' mutant has reduced levels of 70S ribosomes and polysomes, resulting in elevated levels of free subunits (Gustafsson and Persson, 1998). The rlmA' mutant also displays a lower growth rate and a lower translational elongation rate than a wild-type strain (Gustafsson and Persson, 1998). However, the slow growth is not due to the lack of the corresponding modification (Liu, et al., 2004). A similar situation is also evident in yeast (Saccharomyces cerevisiae) mitochondria; while the 21S Gm2270 methyltransferase Pet56p is required for the production of large ribosomal subunits (Sirum-Connolly and Mason, 1993; Sirum-Connolly, et al., 1995), the modification per se is not required for the maturation of the subunit.

Acetylations. R-proteins can also be acetylated; S5, S18, and L12 are all acetylated on the N-terminal nitrogen by the proteins RimJ, RimI, and RimL, respectively (Cumberlidge and Isono, 1979; Isono and Isono, 1980; Isono and Isono, 1981). The acetylated form of L12 is L7 and both forms are found in mature ribosomes, although their relative amounts vary with the growth phase (Ramagopal and Subramanian, 1975). The L12 to L7 conversion is accomplished before or during 50S assembly, but not in mature subunits (Ramagopal and Subramanian, 1975). Deletion of the gene for any of these three acetyltransferases has no effect on the growth (Isono and Isono, 1980; Isono and Isono, 1981; White-Ziegler, et al., 2002).

Other modifications. About half of the S11 protein molecules have an isoaspartate residue (David, et al., 1999). Ribosomal protein L16 has at least one more uncharacterized modification in addition to the N-terminal methylation (Brosius and Chen, 1976; Arnold and Reilly, 1999). R-protein S6 is modified by RimK, which adds up to four glutamate residues at the C-terminus (Kade, et al., 1980; Kang, et al., 1989). R-protein S12 has a methylthio group on the aspartate at position 88 (Kowalak and Walsh, 1996).

4.7 ACCESSORY FACTORS FOR RIBOSOMAL ASSEMBLY.

It has become apparent that the synthesis of ribosomal subunits requires non-ribosomal factors, apart from enzymes for rRNA processing and modification of rRNA and r-proteins. These factors would assist in folding and assembly of the ribosomal subunits.

DEAD-box helicases. One group of proteins associated with ribosomal subunit assembly is the DEAD-box RNA helicases and three examples, SrmB, DbpA and CsdA, have so far been identified in E. coli. Deletion of the gene for any of these three proteins gives a cold sensitive phenotype (Nashimoto, 1993; Jones, et al., 1996; Charollais, et al., 2003). Several DEAD-box proteins are also involved in ribosomal maturation in eukaryotes (Tanner and Linder, 2001). The DEAD-box helicases unwind double stranded RNA structures in an ATP dependent manner (Tanner and Linder, 2001). These proteins are believed to assist in ribosomal assembly by resolving secondary structures that otherwise would inhibit proper folding of the rRNA. This would be especially important at low temperature when RNA secondary structures are more stable.

The SrmB protein. In a cold sensitive srmB mutant, a 32S precursor of 50S accumulates and the maturation of both 16S and 23S rRNA is impaired (Nashimoto,
et al., 1985; Nashimoto, 1993). SrmB is also a high copy suppressor of a mutation in the gene for L24 that has a negative effect on the assembly of the 50S subunit (Nishi, et al., 1988). A strain in which the srmB gene has been inactivated grows as well as a wild-type strain at 37°C, but not at low temperature (Nashimoto, 1993; Charollais, et al., 2003). An srmB deletion mutant shows an abnormal polysome profile; the amount of polysomes is decreased, resulting in increased amounts of the free subunits, and a pre-50S particle that sediments at 40S accumulates (Charollais, et al., 2003). SrmB is found in the slowest sedimenting part the 50S peak after sucrose gradient centrifugation of cellular extracts, indicating that SrmB binds to a, probably immature, subset of the 50S subunits. When purified SrmB protein is mixed with an extract from a ΔsrmB strain it cosediments with the 40S particle. The 40S particle contains pre-23S rRNA and lacks some r-proteins (L13, L28, L34, L35, L36) and has lower amounts of several other r-proteins (Charollais, et al., 2003). In the 40S particle, the assembly of the subunit is probably stalled because of a misfolded rRNA secondary structure that in a wild-type strain would normally be unwound by SrmB.

The DbpA protein. DbpA was identified as a DEAD-box protein by homology to other DEAD-box family members (Iggo, et al., 1990). DbpA has an ATPase activity, which is dependent on a small part of the 23S rRNA (nucleotides 2496 to 2588) that in the mature 50S subunit is located in the peptidyl transferase center (Fuller-Pace, et al., 1993; Nicol and Fuller-Pace, 1995), and a helicase activity which is dependent on helix 92 within this region (Diges and Uhlenbeck, 2001).

The CsdA protein. CsdA (DeaD) was identified as a high copy suppressor of temperature sensitive mutations in rpsB (for r-protein S2) (Toone, et al., 1991) that restores the binding of r-proteins S1 and S2 to the 30S subunit in one of the rpsB mutants (Moll, et al., 2002). CsdA is a cold shock protein that unwinds double stranded RNA in an ATP independent manner (Jones, et al., 1996). Several functions have been suggested, all of which are consistent with an RNA helicase activity: stabilization of mRNAs (Iost and Dreyfus, 1994; Brandi, et al., 1999), degradation of mRNAs (Jones, et al., 1996; Yamanaka and Inouye, 2001), enhancement of translational initiation (Lu, et al., 1999) and ribosomal maturation. During sucrose gradient centrifugation, CsdA co-sediments with 50S subunits (most is found in the slowest sedimenting part of the 50S peak), and to a lesser extent with 30S and 70S (Jones, et al., 1996; Charollais, et al., 2004). A csdA deletion mutant grows normally at 37°C, but not at lower temperatures: at 15°C the generation time is 16 hours compared to eight hours for a wild-type strain (Jones, et al., 1996). The polysome profile of a csdA deletion mutant is abnormal at low temperature; the amounts of polysomes and 70S are lower and the amounts of 30S and 50S are higher compared
to a wild-type strain (Charollais, et al., 2004). Like the Δsrmb strain, the ΔcsdA mutant accumulates a 40S particle, which is a 50S precursor that contains pre-23S rRNA and almost completely lacks some large subunit r-proteins (L6, L16, L25, L28, L32, L33, and L34) and has reduced amounts of other r-proteins (Charollais, et al., 2004). CsdA is also found in the 40S peak of a Δsrmb mutant (Charollais, et al., 2004). The growth rate of a ΔcsdA mutant is only half that of a Δsrmb mutant at 20°C, although the polysome profile defect is not as pronounced as for the Δsrmb mutant, indicating that CsdA has important functions apart from ribosomal maturation (Charollais, et al., 2004).

**Protein chaperones.** Chaperones are also proposed to be directly involved in the assembly of ribosomal subunits since both DnaK and GroEL seem to participate in this process. In contrast to the DEAD-box proteins, DnaK and GroEL appear to be more important at higher temperatures.

**DnaK and ribosomal maturation.** A temperature sensitive dnaK point mutant has less 70S and accumulates ribosomal precursor particles at non-permissive temperature (45°C): two 50S precursors sedimenting at 45S and 32S, as well as one 30S precursor sedimenting at 21S (Alix and Guérin, 1993). These precursors are able to form mature particles and disappear when the cells are shifted back to permissive temperature (Alix and Guérin, 1993). In addition, these precursors slowly convert to mature particles also at non-permissive temperature (El Hage and Alix, 2004). The 21S precursor contains 17S rRNA but lacks S3, S10, S14 and S21, and has less of S1, S2 and S5 (El Hage and Alix, 2004). Both the 32S and the 45S contain pre-23S rRNA, however, in 45S some of the 23S rRNA is mature (El Hage and Alix, 2004). The 45S particles lack r-proteins L2, L6, L9, L16, L25, L27, L28, L30 and L32 (El Hage and Alix, 2004). A ΔdnaK strain, which also is temperature sensitive (Paek and Walker, 1987), shows similar defects as the temperature sensitive dnaK point mutant at high temperatures but is unaffected at temperatures up to about 40°C (El Hage, et al., 2001). During in vitro assembly of the 30S subunit, DnaK can substitute for the heat activation step that converts the RI₃₀ intermediate to RI₃₀⁺ (Maki, et al., 2002; Maki, et al., 2003). DnaK has strong affinity for r-proteins S4, S12, S17, and S21, as well as weaker affinity for S3, S5, S8, S16 and S19 (Maki, et al., 2002). The temperature sensitive dnaK mutation can be suppressed by S4 overproduction (Maki, et al., 2002).

**GroEL and ribosomal maturation.** Overexpression of the chaperonin GroEL can partially compensate for the lack of DnaK at 44°C (El Hage, et al., 2001). Temperature sensitive groEL mutants have a low amount of 70S and accumulate a 45S precursor of 50S at non-permissive temperatures (El Hage, et al., 2001). GroEL
has also been implicated in the RNaseE-dependent processing of pre-5S rRNA (Sohlberg, et al., 1993). Ribosomal proteins S2, L9 and L7/L12 co-immuno-precipitate with GroEL (Houry, et al., 1999). Furthermore, reduction of GroEL and GroES also reduces the number of post-translationally added glutamates on S6 (see above) (Kanemori, et al., 1994).

**Other ribosomal maturation assisting factors.** In addition to the proteins mentioned above, other proteins involved in ribosomal maturation also exist. *The Era protein.* Era is an essential GTPase (March, et al., 1988), the gene for which is cotranscribed with *rnc*, the gene for RNaseIII. The Era protein consists of two domains, an N-terminal GTPase domain and a C-terminal KH domain (Chen, et al., 1999). The KH domain of Era mediates binding to the cytoplasmic membrane (March, et al., 1988; Lin, et al., 1994; Hang and Zhao, 2003) and to 16S rRNA (Sayed, et al., 1999; Meier, et al., 2000; Hang and Zhao, 2003). Era can also be autophosphorylated and most of the protein is phosphorylated *in vivo* (Sood, et al., 1994). Several different functions have been proposed for Era, among which are roles in DNA replication or cell division (Gollop and March, 1991; Britton, et al., 1997; Britton, et al., 1998), coordination between carbon and nitrogen uptake (Powell, et al., 1995), expression of heat shock proteins (Lerner and Inouye, 1991) as well as ribosomal maturation and translation. An S100 extract from Era depleted cells does not promote *in vitro* translation by ribosomes from a wild-type strain, but the ribosomes from the Era depleted cells are functional in translation if they are provided with an S100 extract from *era*<sup>+</sup> cells (Sayed, et al., 1999). Overexpression of the rRNA methyltransferase RsmA suppresses the cold sensitive E200K alteration in Era (Lu and Inouye, 1998) and overexpression of Era suppresses an *rbfA* (see below) null mutation (Inoue, et al., 2003). The amount of r-protein S6 increases if cells are depleted for Era (Lerner and Inouye, 1991). In addition to its ability to bind 16S rRNA, Era can also bind to 30S subunits (Sayed, et al., 1999). Moreover, the GTPase activity of Era is stimulated by 16S rRNA (Meier, et al., 1999; Meier, et al., 2000). Some mutations in *era* increase the amount of 17S rRNA (Nashimoto, et al., 1985; Inoue, et al., 2003). Depletion of Era results in an increased amount of 70S (Sayed, et al., 1999), but in the E200K *era* mutant, the amount of 70S decreases and free ribosomal subunits become more abundant (Inoue, et al., 2003).

*The RbfA protein.* The *rbfA* gene was isolated as a high copy suppressor of a cold sensitive mutation (C23U) in 16S rRNA (Dammel and Noller, 1995). The C23U mutation weakens the base pairing of this base to G11 and destabilizes the 5’ terminal helix of mature 16S rRNA (Dammel and Noller, 1993). RbfA has a structure that consists mainly of a KH domain, but has a AxG motif instead of the usual GxxG.
motif (Huang, et al., 2003). The growth rate of an *rbfA* null mutant is about two fold lower than that of a wild-type strain at 42°C and the difference is even more pronounced at lower temperatures (almost three-fold at 26°C) (Dammel and Noller, 1995). The *rbfA* mutation is lethal together with mutations that destabilize the 5’ terminal helix of 16S rRNA (Dammel and Noller, 1995). The *rbfA* null mutant has a reduced processing of 16S rRNA, leading to an accumulation of the 17S precursor of 16S rRNA (Bylund, et al., 1998) and is defective in polysome formation, resulting in increased amounts of free ribosomal subunits, (Dammel and Noller, 1995). The 16S rRNA processing defect and the inability to form polysomes become increasingly more severe with decreasing temperature (Xia, et al., 2003). The expression of RbfA increases during cold shock and in an *rbfA* null mutant, the cold shock state becomes permanent after a temperature downshift (Jones and Inouye, 1996). RbfA is bound to the 30S subunits, but not to the 50S subunits or the 70S ribosomes, after sucrose gradient centrifugation of cellular extracts (Dammel and Noller, 1995). At 37°C, about 20% of RbfA is associated with 30S, whereas at 15°C, when the total level of RbfA is higher, about 40% is associated with 30S (Xia, et al., 2003). Interestingly, if the 25 most C-terminal amino acids of RbfA are removed, the resulting protein, which does not bind to 30S subunits, can trans-complement the cold-shock and 16S processing defects but not completely restore the low growth rate of the *rbfA* null mutant (Xia, et al., 2003). Further, this C-terminally truncated RbfA cannot suppress the C23U mutation in 16S rRNA

The RimM protein. The gene for RimM is located in an operon that also contains the genes for r-proteins S16 and L19 and the tRNA^m^ethyltransferase TrmD (Byström, et al., 1983). However, the expression of RimM is only one twelfth of that of S16 or L19 due to an mRNA secondary structure that reduces the translational initiation of the *rimM* gene (Wikström and Björk, 1988; Wikström, et al., 1992). A *rimM* deletion mutant has a low growth rate, only one fifth of a wild-type strain in rich medium at 37°C and this difference is accentuated at 42°C and 21°C or in minimal media at 37°C (Persson, et al., 1995; Bylund, et al., 1997). The *rimM* deletion mutant is also deficient in translation initiation and shows a reduced translational elongation rate (Bylund, et al., 1997). This mutant accumulates the 17S precursor to 16S rRNA (Bylund, et al., 1998). The slow growth and the translation defects of the *rimM* deletion strain can be partially suppressed by alterations in the C terminal part of r-protein S13 or by overproduction of RbfA (Bylund, et al., 1997; Bylund, et al., 1998). The RimM protein binds to 30S subunits but not to 70S ribosomes and the amount of RimM is highest in the slowest sedimenting part of the 30S peak obtained after sucrose gradient centrifugation of cellular extracts, which
might indicate that RimM binds to an immature subpopulation of the 30S subunits. (Bylund, et al., 1997). The C terminal half of RimM is proposed to fold into a PRC β-barrel structure (Anantharaman and Aravind, 2002).

*The YrdC protein.* A *yrdC* mutation was isolated as a suppressor to a temperature sensitive mutation in *prfA*, the gene for RF1. The *yrdC* mutant shows increased levels of the 17S precursor to 16S rRNA, and of free ribosomal subunits, resulting in less polysomes (Kaczanowska and Rydén-Aulin, 2004). Further, the YrdC protein binds double stranded RNA *in vitro* (Teplova, et al., 2000).

*The RsgA protein.* RsgA is a GTPase that is activated by ribosomes, especially by 30S subunits but also by 70S ribosomes and to a lesser extent by 50S subunits (Daigle, et al., 2002; Daigle and Brown, 2004). This protein has an OB β-barrel fold that mediates binding to ribosomes, which is strongest in the presence of the non-hydrolyzable GTP analogue GDPNP (Daigle, et al., 2002; Daigle and Brown, 2004). The binding of RsgA to 30S and the concomitant GTPase stimulation is abolished by A-site binding aminoglycoside antibiotics (Himeno, et al., 2004). RsgA dissociates 70S in the presence of GDPNP *in vitro* (Himeno, et al., 2004). Deletion of *rsgA* results in a 2.3-fold reduction in growth rate and reduced amounts of 70S compared to 30S and 50S and also in an increased amount of 17S RNA (Himeno, et al., 2004). Interestingly, free 30S subunits from the deletion mutant cannot activate the GTPase activity of RsgA *in vitro*, but free 30S from a wild-type strain or 30S subunits from 70S ribosomes of the mutant can (Himeno, et al., 2004). These findings suggest that, in the Δ*rsgA* mutant, there is an accumulation of immature 30S subunits unable to stimulate the GTPase activity of RsgA.

*Other mutations.* There are also additional mutations that confer abnormal ribosome maturation but have not been mapped to any precise location on the chromosome. In *rimB* and *rimD* mutants, a 43S precursor to 50S accumulates and in a *rimC* mutant a 32S precursor to 50S accumulates (Bryant and Sypherd, 1974). A temperature sensitive *rimH* mutant has less ribosomal particles and rRNA and the ratio of 17S to mature 16S is increased (Johnson, et al., 1976). An erythromycin resistant *eryC* mutant has 26S and 43S particles instead of 30S and 50S and shows large amounts of precursors to 16S and 23S rRNA (Pardo and Rosset, 1977). These mutations affecting ribosomal maturation could however be in genes for ribosomal components or in the genes for some of the known proteins discussed above. Whether these mutations directly affect ribosomal synthesis, or if the observed effects are indirect, have yet to be investigated.
5 AIMS OF THIS THESIS

The aim of this thesis was to understand the function of non-ribosomal proteins involved in the assembly of ribosomes in *Escherichia coli*. The specific aims were:

- To investigate the function of the RimM protein in ribosomal maturation.
- To study the role of RbfA in ribosomal maturation and how it is connected to the function of RimM.
- To examine the function of the RlmB methyltransferase in rRNA methylation and a possible role in assembly of 50S ribosomal subunits.
6 RESULTS AND DISCUSSION

6.1 RLMB IS REQUIRED FOR rRNA METHYLATION BUT NOT FOR RIBOSOMAL ASSEMBLY.

In yeast (*Saccharomyces cerevisiae*), Pet56p methylates the 2’O of the G (resulting in Gm) in position 2270 of the mitochondrial large subunit rRNA. The Pet56 protein is required for formation of functional mitochondrial ribosomes, but this is not dependent on its methylation activity (Sirum-Connolly and Mason, 1993; Sirum-Connolly, *et al.*, 1995). There are orthologues to Pet56p in bacteria, the most similar protein in *E. coli* is RlmB (previously YjfH). In Paper III, the effects of a deletion of the *rlmB* gene are reported. The *rlmB* deleted strain was completely lacking Gm in its rRNA, confirming that RlmB is responsible for the formation of Gm2251, which is the only Gm in rRNA of *E. coli*. The *rlmB* deletion mutant grew as well as a wild-type strain and could not be out-competed by the wild type in a competition experiment. Unlike PET56 in *S. cerevisiae*, deletion of *rlmB* did not affect the maturation of the large ribosomal subunits. Ribosomal precursors were converted into mature subunits, as quickly as in the wild-type strain and polysome profiles were not affected. One explanation for this discrepancy could be that the N-terminal part of Pet56p, for which no homologous sequence is present in the RlmB protein, takes part in ribosomal maturation. In *E. coli*, this function could be executed by another protein or possibly not even be needed due to ribosomal structure differences. However, the purpose of the methylation *per se* remains elusive.

6.2 MUTAGENESIS OF RIMM AND ISOLATION OF SUPPRESSOR MUTATIONS.

Mutagenesis of selected positions in *rimM*. In order to elucidate the function of RimM in ribosomal maturation, phylogenetically conserved amino acids of RimM were replaced by alanines (Paper IV). Most of the amino acid substitutions did not confer any growth defect (G17A, K18A, G20A, G24A, R26A, G27A, E36A + D37A, E102A + E103A and K109A + D110A). One of the substitutions (D137A) resulted in a significant growth defect. However, in the mutant containing this substitution, the level of the RimM protein was lower than in a wild-type strain, suggesting that the
RimM-D137A protein was unstable. Another mutant (rimM120), in which the highly conserved tyrosines in positions 106 and 107 were changed to alanines, showed a three-fold reduced growth rate compared to the wild-type strain, even though the level of the RimM protein level was not affected. However, if only position 106 or 107 was altered, there was no, or just a small, effect on the growth rate, respectively. Thus, the two tyrosines are important for the function of RimM, however, one aromatic amino acid in either of these positions seems sufficient.

**Suppressors to the rimM120 mutation.** Several compensatory mutations to the rimM120 mutation were isolated. Two of these were similar to those that suppress deficiencies of the ΔrimM mutant by overexpressing rbfA (see section 6.4); one had a deletion of the infB terminator preceding rbfA and the other contained a frame shift mutation in nusA, identical to one of the ΔrimM suppressor mutations (Paper IV). Some suppressor mutations were located in the respective genes for r-protein S19 and 16S rRNA (Paper IV). Other suppressor mutations resulted in increased expression of the RimM120 protein (Paper I). In addition, intragenic suppressor mutations were also obtained, one of which that resulted in a valine in position 106 of the RimM protein and another (rimM131) that changed the asparagine in position 84 to lysine.

**Overexpression of rimM.** The translation of rimM is restricted by an mRNA secondary structure that blocks the translation initiation region (Wikström and Björk, 1988; Wikström, *et al.*, 1992). Several of the rimM120 suppressor mutations weakened this structure, in some cases without changing the encoded peptide sequence (see Figure 3 in Paper I), resulting in a several-fold increased expression of rimM. Thus, increased expression of the rimM120 mutant protein seems to compensate for its reduced function. Some of the mutations were in the stop codon of rpsP (for r-protein S16), changing it to sense codons. Since rpsP is directly upstream of and in the same reading frame as rimM, the stop codon mutations resulted in the synthesis of a hybrid protein between S16 and RimM (Paper I). The S16-RimM protein was found in much higher amounts in the mutants than the RimM protein in a wild-type strain, explaining the suppression of the slow growth. The hybrid protein was assembled into 70S ribosomes and polysomes, just like r-protein S16 but unlike RimM. S16 is essential for growth (Persson, *et al.*, 1995) and important for assembly but not for the activity of 30S subunits *in vitro* (Held and Nomura, 1975). Since the strain expressing the hybrid protein grew quite well and did not have any severe defect in 30S assembly it must have retained the function of S16 in ribosomal assembly.
Mutations in ribosomal components. Among the alterations that suppressed the rimM120 mutation, some were in r-protein S19 and 16S rRNA (see Figures 5 and 9 in Paper IV). Five different mutations resulting in 16S rRNA alterations (C962U, C970G, ∆A974, A975U and G1015A) and one mutation (rpsS876) resulting in an alteration in S19 (H83Y) were isolated. The 16S rRNA mutations were also able to suppress the ∆rimM mutation quite well. It has previously been shown that a ∆rimM mutation can be suppressed by mutations in rpsM, the gene for r-protein S13 (Bylund, et al., 1997). At least one of these (rpsM873) was also able to suppress the rimM120 mutation (Paper IV). The rpsS876 mutation was more efficient in suppressing the slow growth of the rimM120 than of the ∆rimM mutant, whereas the rpsM873 mutation was equally efficient in both rimM mutants. The fact that the rpsS876 mutation seemed specific for the rimM120 mutant indicates that the suppression by rpsS876 requires the RimM protein. The suppression might result from the rpsS876 mutation facilitating RimM120 function. All of the alterations in ribosomal components that suppress the defects of the rimM mutants are located in a small portion of the head of the 30S subunit (Figure 9 in Paper IV). Thus, this part of the ribosome is the likely target for the action of RimM in the maturation of the 30S subunits.

6.3 INTERACTIONS BETWEEN RIMM AND RIBOSOMAL COMPONENTS.

Ribosomal defects in rimM mutants.
Polysome profiles. Both the ΔrimM and rimM120 mutants had lower amounts of polysomes compared to the wild-type strain (Paper IV). The polysome defects were partially corrected for by the rpsM873 and rpsS876 suppressor mutations. The growth rate of suppressor-free, as well as suppressor-containing rimM mutants, correlated very well with the amount of polysomes, suggesting that the growth rate of the mutants is limited by the translational capacity.
Processing of 16S rRNA. The rimM120 mutant showed an increased amount of the 17S precursor to 16S rRNA, however, not to the same degree as the ΔrimM mutant (Paper IV). The rpsS876 suppressor partially restored the processing of 17S in the rimM120 mutant, whereas the rpsM873 suppressor mutation did not. However, neither of the suppressor mutations improved the processing in the ΔrimM mutant. The 16S rRNA processing deficiency of both rimM mutants was suppressed by the alterations in 16S rRNA, although the suppression was in most cases more efficient
in the rimM120 than in the \( \Delta \)rimM mutant. The processing of 17S rRNA was partially restored by the intragenic rimM131 suppressor mutation (Paper IV). There appears to be at least two mechanisms for suppression of the rimM mutants. One mechanism compensates for the absence of RimM, but does not restore the processing of 16S rRNA, as in the rpsM873 mutant. The other mechanism results in improved processing and is, to some extent, dependent on the presence of the RimM protein, as in the rpsS876 mutant. The distinction between these mechanisms is not clear-cut and in some suppressor strains a combination of the two is observed. However, the A975U alteration in 16S rRNA is not consistent with any of these mechanisms because it is quite efficient in restoring the 16S rRNA processing in the \( \Delta \)rimM mutant.

**Association of RimM to 30S subunits.** Wild-type RimM is found associated with 30S subunits after sucrose gradient centrifugation of cellular extracts (Bylund, *et al.*, 1997), whereas the rimM120 mutant protein shows much reduced binding to the 30S subunits (Paper I). Neither the intragenic suppressor mutation rimM131, nor the suppressor mutations altering S13, S19 or 16S rRNA could restore the interaction with the 30S subunits (Paper IV). In the suppressor strains overproducing RimM, the total amount of RimM bound to 30S was restored even though the fraction of RimM bound to the 30S subunits was the same as in the rimM120 mutant (Paper I). It is difficult to envision how the intragenic suppressor mutation rimM131 could improve the 16S rRNA processing when the binding to the 30S subunits was still deficient. One explanation might be that the weak binding of RimM to 30S subunits and the reduced processing of 16S rRNA are separate effects of the rimM120 mutation and that the weak binding of RimM would be sufficient to promote efficient processing. The rimM131 mutation might then improve the processing without affecting the binding to the 30S subunits. Another explanation could be that there are two different interactions between RimM and the 30S subunits: one strong binding, which can be seen after sucrose gradient centrifugation and one weaker interaction that is not detected in these experiments. The suppression would then be due to an improved ‘weaker’ interaction.

**RimM co-purification experiments.** A GST-RimM hybrid protein was used to pull out cellular components that interact with RimM (Paper IV). At 4°C, several proteins co-purified with the GST-RimM protein but not with a GST-RimM120 hybrid protein. These proteins were identified as r-proteins of the 30S subunit. When the purification was executed at room temperature, most of the ribosomal proteins were washed off by 0.6 M NaCl treatment. The only protein that remained bound to RimM was r-protein S19. The binding of the RimM120 protein to the 30S subunits was not
restored by the alteration in S19 caused by the rpsS876 suppressor mutation. From these results, it can be concluded that the two tyrosines in position 106 and 107, which are important for the function of RimM, are crucial for the binding of RimM to the S19 protein in the 30S subunits. The binding of RimM to S19 correlates well with the identification of alterations lying in, or close to, S19 in the 30S subunits as suppressors to the *rimM* mutations. Taken together, these results map the target for RimM to the S19 region of the 30S subunits.

### 6.4 RbfA AND SUPPRESSION OF ∆RIEIM.

**Mutations that increase the amount of RbfA.** Several mutations in the *metY-nusA-infB* operon were isolated as suppressors to the ∆*rimM* mutation; these are described in Paper II. All of the characterized mutations resulted in an increased amount of RbfA, which is encoded by the fifth gene of the *metY-nusA-infB* operon. Overexpression of RbfA is known to compensate for deficiencies in the ∆*rimM* mutant (Bylund, *et al.*, 1998). Increased amounts of RbfA were achieved in several ways: In one suppressor strain there was a duplication in the chromosome that placed a second copy of the *rbfA* gene after the *yhbM* promoter, resulting in efficient transcription of *rbfA*. In another strain, an IS2 element had been inserted in the end of the *infB* gene, creating a new promoter that resulted in strong expression of *rbfA*. Some suppressor mutations had deletions that removed the *infB* terminator, located upstream of *rbfA*, that normally terminates more than 80% of the transcription. Nine of the suppressor mutations were in the *nusA* gene. These mutations decreased the ability of NusA to stimulate transcriptional termination, which resulted in increased read-through of internal terminators of the *metY-nusA-infB* operon. Both the terminators directly after the *metY* gene and the terminator between *infB* and *rbfA* were read through more efficiently in the *nusA* mutants, resulting in higher levels of RbfA.

**Localization of RbfA in a ∆rimM mutant.** Total cell extracts from the ∆*rimM* strain MW37 (Persson, *et al.*, 1995) and the wild type strain MW100 (Wikström, *et al.*, 1988) were subjected to sucrose gradient centrifugation and selected fractions were analyzed by Western blotting with an antiserum against RbfA. In the wild-type strain, most of the RbfA protein was found associated with the 30S subunits and only a small amount of free RbfA was seen (Figure 4A). This is in contrast to the large amount of free RbfA reported earlier (Xia, *et al.*, 2003). This discrepancy might be due to differences in the buffers used for extract preparation or to strain differences as
Xia et al. used the strain MC4100, which has several mutations not present in strain MW100. In the ΔrimM mutant, all RbfA protein was bound to the 30S subunits and no free RbfA was detected (Figure 4B). Thus, it is probable that overexpression of RbfA can suppress the rimM mutations because it overcomes the limited amount of free RbfA available for the production of mature 30S subunits in the rimM mutants. The limiting amounts of free RbfA could be a consequence of RimM depletion causing an accumulation of a 30S assembly intermediate that binds RbfA.

**Figure 4.** Subcellular localization of RbfA. Cellular extracts were fractionated by sucrose gradient centrifugation and indicated fractions were analyzed with anti RbfA antibodies in Western blotting experiments. **A.** MW100 (wild type). **B.** MW37 (ΔrimM).
6.5 STRUCTURAL AND FUNCTIONAL STUDIES OF RbfA.

**Amino acid substitutions in RbfA.** In order to identify amino acid residues of RbfA that are important for its function, especially for the binding to 30S subunits, phylogenetically conserved surface exposed residues were changed, in most cases to alanines (Figure 5). The effects of these amino acid substitutions on the growth of the cells, scored as relative colony size, are summarized in Table 2. A growth reduction at 30°C and higher was only observed for the substitutions with the most severe effects on growth. In most cases, significant differences were only observed below 25°C. However, most of the alterations did not have any effect and double substitutions did not reduce the growth more than the most severe of the two single substitutions. When the arginine at position 31 was changed to a negatively charged glutamate, the effect on growth was more pronounced than when it was changed to an alanine, suggesting that the positive charge of the arginine is important in this position. In contrast, the lysine substitution for aspartate in position 100 had less effect than the alanine substitution, suggesting that the charge in this position is not crucial but that the small hydrophobic side chain of alanine should be avoided. Thus, arginine 31 is more likely than aspartate 100 to be involved in any specific interaction.

**Table 2.** Growth of *rbfA* mutants.

<table>
<thead>
<tr>
<th>RbfA alteration</th>
<th>colony size a</th>
<th>RbfA alteration</th>
<th>colony size a</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>+++</td>
<td>R80A</td>
<td>+++</td>
</tr>
<tr>
<td>F5A</td>
<td>++</td>
<td>F78A + R80A</td>
<td>++(+</td>
</tr>
<tr>
<td>R7A</td>
<td>++(+)</td>
<td>K85A</td>
<td>+++</td>
</tr>
<tr>
<td>F5A + R7A</td>
<td>++</td>
<td>R88A</td>
<td>+++</td>
</tr>
<tr>
<td>R10A</td>
<td>++(+)</td>
<td>K85A + R88A</td>
<td>+++</td>
</tr>
<tr>
<td>R25A</td>
<td>+++</td>
<td>R90A</td>
<td>+++</td>
</tr>
<tr>
<td>K28A + D29A</td>
<td>++(+)</td>
<td>R88A + R90A</td>
<td>+++</td>
</tr>
<tr>
<td>R31A</td>
<td>++(+)</td>
<td>F97A</td>
<td>+(+)</td>
</tr>
<tr>
<td>R31E</td>
<td>(+)</td>
<td>F98A</td>
<td>+++</td>
</tr>
<tr>
<td>D46A</td>
<td>++</td>
<td>D100A</td>
<td>+</td>
</tr>
<tr>
<td>K51A</td>
<td>+++</td>
<td>D100K</td>
<td>++</td>
</tr>
<tr>
<td>Y53A</td>
<td>+++</td>
<td>E121A + E122A</td>
<td>+++</td>
</tr>
<tr>
<td>F78A</td>
<td>+++</td>
<td></td>
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</tr>
</tbody>
</table>

a) Relative colony sizes on rich medium plates at room temperature (21-22 °C).
with 30S subunits. The importance of position 31 is further emphasized by the fact that the R31E substitution had the most obvious effect of all the created substitutions.

**The role of RbfA in ribosomal assembly.** It is intriguing to speculate about the function of RbfA. Evidence points to an interaction with the 5’ terminal helix of 16S rRNA (Dammel and Noller, 1995), which cannot coexist with the precursor structure shown in Figure 3A. Lack of RbfA leads to a reduced processing of the 17S to 16S rRNA (Bylund, et al., 1998) and the formation of the 5’ helix might be required for this processing. If this is the case, RbfA might be involved in the formation of the 5’ helix and as a consequence also influences the 16S rRNA processing. Alternatively, the processing might be required for the formation of the 5’ terminal helix of 16S rRNA, in which case RbfA must be involved both before and after the processing step. The depletion of free RbfA in the ΔrimM mutant suggests that RbfA binds to the 30S subunits before RimM but is not released until after RimM has bound. Such a scenario could be important for the timing of the maturation steps, in which RbfA and RimM participate.

![Figure 5. Three-dimensional structure of RbfA (Huang, et al., 2003) shown in two opposite orientations. The last 25 amino acids of RbfA are not included in the structure; the last position is arginine 108. The positions in RbfA that were altered are shown in color. The different colors indicate the different effects on the growth by the single alanine substitutions (except for K28 and D29, which were only changed simultaneously): positions in green did not affect the growth at room temperature; positions in yellow gave a slight effect (‘+++’ in Table 2); positions in light orange, dark orange and red had larger impacts on growth (‘++’, ‘+(+)’ and ‘+’, respectively, in Table 2). The coordinates for the RbfA structure were from the Protein Data Bank, PDB ID 1KKG.](image-url)
7 CONCLUSIONS

The main conclusions from the work presented in this thesis are:

- The RimM protein binds to r-protein S19 in 30S subunits. The two tyrosines in positions 106 and 107 are required for this binding.

- The target for the action of RimM in assembly is in the S19 region of the head of the 30S subunit.

- Lack of RimM leads to a depletion of free RbfA, which explains the observed suppression of \textit{rimM} mutants by overexpression of \textit{rbfA}.

- RlmB is the methyltransferase that is responsible for the formation of Gm2251 in 23S rRNA, however, it is not required for proper assembly of the 50S subunit.
8 FUTURE PERSPECTIVES

The understanding of the assembly and maturation of ribosomal subunits is far from complete and further detailed studies of the participating components are required.

To find out if the action of RimM is leading to a conformational change in the 30S precursor particle, the susceptibility of the 16S rRNA in 30S particles from the ΔrimM mutant to RNA modifying agents can be investigated, before and after addition of purified RimM protein.
It would be interesting to test if RimM can influence \textit{in vitro} assembly of 30S subunits, especially if it can facilitate the conversion of RI\textsubscript{30} to RI\textsubscript{30}* at low temperature.
If the structure of 30S subunits from the ΔrimM mutant was determined by cryo-EM, this could give clues to what goes wrong in the maturation of 30S subunits when RimM is missing.

The approaches suggested above for elucidation of the function of RimM, might also be applied to study the role of RbfA in assembly of the 30S subunits. Further, it might be possible to find interaction partners to RbfA by co-purification experiments in a similar way as was done for RimM.
If mutations could be isolated, that suppress the slow growth of \textit{rbfA} amino acid substitution mutants, they would enhance the understanding of the function of RbfA. It would also be interesting to investigate if there are any interactions between RimM and RbfA during the maturation of 30S subunits.

In addition, a synergistic lethality screen could be set up to identify mutations that result in a requirement for RlmB. This might give clues to the function of Gm in position 2251 of the 23S rRNA.
The time as a PhD student has been nice, largely due to the nice people in the department. During the years I have met a lot of people and I want to thank you all for making this a good and rewarding time.

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I am very grateful for the people that have handled different practical things, so that I have been able to concentrate on the research. Thanks to the secretaries Marita, Berit, Anitha, to the dishwashing and media personnel and to P-A and Johnny. Thank you, Marcus and Matt for reading my thesis and suggesting improvements.
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