The importance of maturation factors in 30S ribosomal subunit assembly

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“He attacked everything in life with a mix of extraordinary genius and naive incompetence, and it was often difficult to tell which was which.”

Douglas Adams (1952 - 2001)

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Abstract

The assembly of the ribosome is a complex process that needs to be highly efficient to support maximum growth. Although the individual subunits of the ribosome can be reconstituted *in vitro*, such a reaction is inefficient in comparison to the assembly rate *in vivo*. What differentiates the *in vivo* from the *in vitro* assembly is primarily the presence of ribosome assembly proteins. These are proteins that assist in the assembly of the ribosomal subunits but are not part of the mature ribosome. In bacteria, the ribosome assembly proteins include rRNA processing enzymes and rRNA/ribosomal protein (r-protein) modifying enzymes. One set of ribosome assembly proteins, the ribosome maturation factors, have been difficult to classify due to their differences in structure and their apparent lack of similarities with regard to function. As part of this thesis, the previously uncharacterized RimP (ribosome maturation) protein formerly known as P15A or YhbC, was studied. Deletion of the *rimP* gene affected the growth rate more severely at 44°C than at 37°C and 30°C. Polysome profile analysis revealed a decrease in the amount of translating ribosomes and a corresponding increase in the amount of free 50S and 30S ribosomal subunits. The disproportionate large increase in 50S relative to 30S subunits indicated a 30S assembly defect. RimP was shown to localize to the 30S ribosomal subunit, and an accumulation of 17S rRNA, a precursor to 16S rRNA, supports a role for RimP in 30S subunit maturation.

The results from *in vitro* reconstitution experiments have given valuable insights in the assembly of the 30S subunit. By using a recently developed method, the role of ribosome maturation factors Era, RimM and RimP during *in vitro* reconstitutions of the 30S subunit was investigated. Era was found to increase the incorporation rate for most of the late binding r-proteins, while RimM and RimP had more specific effects. RimM increased the incorporation rate for r-proteins S19 and S9 and inhibited the incorporation of S13 and S12, whereas RimP increased the incorporation rate primarily for S12 and S5.

A comparison of the ribosome maturation factors RimP and RbfA (ribosome binding factor A) revealed structural similarities between the N-terminal domain of RimP and the single domain of RbfA. RbfA is a 15 kDa protein that was found to high copy-suppress a dominant C23U 16S rRNA mutation giving rise to cold-sensitivity in *E. coli*. A number of chromosomal suppressor mutations that increased the growth rate of an *rbfA* null mutant were isolated. The five strongest suppressor mutations were localized to the *rpsE* gene, for r-protein S5 and resulted in amino acid substitutions in three positions: G87A, G87S, G91A, A127V and A127T. These alterations improved translation and the processing of 16S rRNA in the *rbfA* null mutant. Moreover, they also suppressed the slow growth of the C23U rRNA mutant at 30, 37 and 44°C.

Sammanfattning på svenska

Monteringen av ribosomen är en komplex process som måste vara effektiv för cellen skall kunna växa så fort som möjligt. Det är visat sedan tidigare att ribosomens två subenheter kan monteras ihop *in vitro* och sedan vara del av en ribosom som gungerar vid proteinsyntes, dock är den typen av rekonstrueringsreaktioner mycket ineffektiva i jämförelse med vad som krävs *in vivo*. Skillnaden mellan dessa två tillstånd är primärt *in vivo*-reaktionens närvaro av hjälpproteiner. Hjälpproteinerna assisterar monteringen av ribosomens subenheter men är själva inte en del av den färdiga ribosomen. Inom denna klass av proteiner återfinns proteiner som t.ex. processerar ribosomalt RNA och proteiner som modifierar ribosomalt RNA och ribosomala protein. En klass av hjälpproteiner, mognadsfaktorerna, har varit svåra att klassificera på grund av strukturella olikheter och en brist på funktionella likheter. En del i detta avhandlingsarbete var karaktäriseringen av den tidigare okända mognadsfaktorn RimP, tidigare kallad YhbC eller P15A. En deletion av *rimP* hade störst påverkan på tillväxthastigheten vid 44°C, men effekter kunde även ses vid 30°C och 37°C. En analys av den ribosomala statusen visade på en minskning av ribosomer aktiva i translation och en motsvarande ökning av fria 50S- och 30S-subenheter. Den oproportionerligt höga ökningen av fria 50S-subenheter, i relation till 30S-subenheter, indikerade att något var fel i monteringen av 30S-subenheten. RimP-proteinet återfanns lokaliserat till fria 30S-subenheter, och en ökning av omoget 16S ribosomalt RNA i en stam som saknar RimP stödjer dess roll i monteringen av 30S-subenheten.

Rekonstrueringsexperiment *In vitro* har gett många värdefulla ledtrådar till hur 30S-subenheten monteras ihop. Genom att använda en nyligen utvecklad metod kunde vi undersöka hur mognadsfaktorerna Era, RimM och RimP påverkade monteringen av ribosomens 30S subenhet *in vitro*. Era ökade inkorporeringshastigheten av många av de ribosomala proteiner som inkorporeras sent i monteringen av 30S, medans RimM och RimP uppvisade mer specifika effekter. RimM ökade inkorporeringshastigheten för de ribosomala proteinerna S19 och S9, men dessutom inhiberade RimM inkorporeringen av de ribosomala proteinerna S13 och S12. RimP uppvisade den tydligaste effekten av de undersökta proteinerna genom att kraftigt öka

inkorporeringshastigheten för det ribosomala proteinet S12, och ökade även inkorporeringshastigheten för det ribosomala proteinet S5. En jämförelse av de två mognadsfaktorerna RbfA och RimP visade på strukturella likheter mellan RimP:s N-terminala domän och den enda domänen hos RbfA. RbfA är ett 15 kDa protein som upptäcktes som en hög-kopiesupressor av en dominant C23U-mutation i 16S ribosomalt RNA som leder till köld-känslighet hos *E. coli*. Ett antal kromosomala supressormutationer som ökade tillväxthastigheten för en mutant som saknar RbfA isolerades och de fem starkaste av dessa lokaliserades till *rpsE* genen som kodar för det ribosomala proteinet S5. Mutationerna gav upphov till aminosyra utbyten i tre positioner i S5: G87A, G87S, G91A, A127T och A127V. Förändringarna i S5 förbättrade translationen och processningen av 16S ribosomalt RNA i mutantensom saknar RbfA. Dessutom förbättrade mutationerna tillväxthastigheten hos C23U-mutanten vid 30, 37 och 44°C.

Papers in this thesis

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

I **Stefan Nord, Göran O. Bylund, J. Mattias Lövgren and P. Mikael Wikström.** 2009. The RimP protein is important for maturation of the 30S ribosomal subunit. Journal of Molecular Biology 386:742-753

II **Anne E. Bunner, Stefan Nord, P. Mikael Wikström and James R. Williamson** 2010. The effect of ribosome assembly cofactors on *in vitro* 30S subunit reconstitution. Journal of Molecular biology 398:1-7

III **Stefan Nord, Hasan Tükenmez, P. Mikael Wikström** 2010. Substitutions in the C terminal domain of the *E. coli* ribosomal protein S5 suppress the lack of the ribosome maturation factor RbfA as well as the dominant cold-sensitive C23U mutation in 16S rRNA. Manuscript in preparation.

The Ribosome

The synthesis of ribosomes is highly energy-consuming using up to 40% of the energy available to the cell. During optimal growth conditions one cell of *Escherichia coli* may contain up to 70,000 ribosomes, constituting approx. 50% of the dry mass of the cell. *E. coli* must be able to cope with varying environmental conditions, ranging from the nutrient rich human intestine to the often nutrient poor environment outside of the human host. This puts an extreme pressure on the adaptive abilities of the cell and demands an efficient utilization of available nutrients so as to achieve maximum growth. The synthesis of ribosomes therefore has to be highly regulated so as not to waste energy on producing ribosomes that are not needed and at the same time also be able to adapt quickly as soon as the conditions support faster growth.

The bacterial ribosome is referred to as the 70S ribosome, where S is the Svedberg coefficient describing the rate of sedimentation through a sucrose gradient, and is made up of the large, 50S, and the small, 30S, subunits. The mature *E. coli* ribosome consists of roughly two thirds RNA and one-third protein (Schuwirth et al. 2005). The 30S subunit contains one rRNA species 1542 nucleotides in length, named 16S, and 21 r-proteins, while the 50S subunit contains two rRNA species, 23S (2904 nucleotides) and 5S (120 nucleotides), and 33 r-proteins. The solving of the crystal structure of the ribosome revealed that not only is the rRNA a major part of the ribosome, it is the core that although de-proteinized contains self-organizing properties (Ramakrishnan 1986; Ramakrishnan and Moore 2001). Further, the 23S rRNA catalyses the formation of the peptide bonds without the involvement of any protein constituents (Ban et al. 2000; Nissen et al. 2000; Yusupov et al. 2001) demonstrating that the ribosome, in fact, is a ribozyme, i.e. an RNA based enzyme.

The ribosome has two primary functions, of which one is the translation of the RNA message into amino acids and from this follows the second function, the formation of peptide bonds between the amino acids to join them into a polypeptide (protein). The decoding function of the ribosome resides in the 30S subunit while the catalysis of peptide bond formation resides in the 50S subunit.

Much research has been focused on the mechanisms of the different processes of translation, but far less effort has been made to understand the assembly of the ribosome.

The coordinated assembly of a ribosome

The single rRNA of the 30S ribosomal subunit, named 16S in its mature form, and the two rRNA molecules of the 50S ribosomal subunit, named 23S and 5S, make up the core component of the ribosome and constitute approx. 65 % of its volume. The rRNA is transcribed from a varied number of rRNA operon(s), depending on the organism; in *Escherichia coli,* for example, there are seven rRNA operons. Transcription of the rRNA operons accounts for more than half of the cell's total RNA synthesis under rapid growth conditions (Condon et al. 1993). The rRNA, apart from containing the instructions for its own folding, also contains the binding sites for many of the r-proteins. These proteins are part of the mature ribosome, in contrast to the proteins that aid in ribosome assembly without being part of the ribosome. The synthesis of the r-proteins for the individual ribosomal subunits is coordinated with the synthesis of rRNA. In *E. coli,* there are 19 r-protein operons that contain r-protein genes clustered together, or r-protein genes together with genes for other proteins involved in translation. Results from both *in vivo* and *in vitro* experiments support the idea that the addition of r-protein to rRNA is a co-transcriptional event i.e., the addition of the r-proteins to the rRNA being transcribed is achieved as soon as the respective r-protein binding site emerges from the RNA polymerase (de Narvaez and Schaup 1979; Powers et al. 1993).

Once the individual 50S and 30S subunits have matured they can join to form 70S ribosomes. This joining of the two subunits occurs at the late stages of the initiation of translation.

The process of translation

The first step of translation is initiation, which is promoted by the three initiation factors IF1-3 and involves the interaction of the Shine-Dalgarno sequence (SD), a stretch of 6-9 nucleotides (nt) upstream of the translation initiation start codon on the mRNA, with the anti-SD sequence at the 3' end of

the 16S rRNA (Shine and Dalgarno 1974). After the interaction between the 30S subunit and the mRNA, the initiation factors promote start codon accommodation in the P-site and the recruitment of the 50S subunit resulting in a mature and translationally active 70S ribosome. The second step of translation is elongation, that involves the formation of peptide bonds between the amino acids attached to the tRNAs recruited to the corresponding codons of the mRNA based on the fit between the anticodon and the codon. The last step of translation is termination, which involves the recruitment of translation release factors to the fully translated mRNA locked into the ribosome. The release factors promote the release of the peptide chain from the P-site. The two subunits can then be recycled, which resets them to a translation initiation ready state.

Ribosomal RNA (rRNA)

In *E. coli* there are seven rRNA operons (*rrnA-E*, *rrnG* and *rrnH*). All have a more or less similar organization with two tandem promoters, P1 and P2, the gene for 16S rRNA, spacer tRNA(s), the gene for 23S rRNA, the gene for 5S rRNA and distal tRNA(s) (Brosius et al. 1981). The differences in the types and numbers of tRNAs encoded aside, there are sequence differences between the operons in the genes, control and spacer regions (Brosius et al. 1978; Boros et al. 1979; Shen et al. 1982; Plaskon and Wartell 1987; Harvey et al. 1988).

Regulation of rRNA transcription

The main determinant of ribosome synthesis is the regulation of the ability of the RNA polymerase to initiate transcription initiation at the rRNA operon promoters. The rRNA synthesis is dependent on antitermination mechanisms, responds to the availability of amino acids (the so-called stringent response) and varies in relation to growth rate.

The *rrn* operon P1 and P2 promoters are among the strongest in the cell. The P1 promoter is most active in rapidly growing cells while P2 seems more important at lower growth rates and in stationary phase (Murray and Gourse 2004). A few core features of these two promoters and the regions surrounding

them create the basis for a high rate of, but at the same time tightly controlled, transcription of rRNA. The upstream UP element and the transcription factor FIS, which binds upstream of the UP element, promote up to a 200-fold increase in transcription from the P1 promoter *in vivo* (Hirvonen et al. 2001). The UP element enhances the association of the RNA polymerase to the promoter region and FIS bends and untwists the -10 promoter region to promote open complex formation (Auner et al. 2003; Barnard et al. 2004). Another protein involved in the regulation of *rrn* expression is the histone-like protein H-NS that acts as a repressor of FIS-dependent activation and traps the RNA polymerase in an open complex on the DNA (Dame et al. 2002).

Antitermination

Transcription of rRNA is positively regulated by an antitermination system. In bacteria like *E. coli*, transcription and translation of mRNA are coupled, which means that when the mRNA exits the RNA polymerase, the ribosomes adhere to the transcript and starts translating, i.e. before the RNA polymerase has reached the end of the sequence to be transcribed. A major consequence of the translation of the mRNA is that the ribosomes prevent premature termination of transcription dependent on the termination protein Rho (Adhya and Gottesman 1978; Kingston and Chamberlin 1981). Since rRNAs are not translated and therefore cannot be protected by ribosomes, there is an antitermination system that prevents the Rho protein from acting on the emerging rRNA transcript. All rRNA operons in *E. coli* contain antitermination sequences, the so-called nut-like sequence element, due to its similarity to antitermination sequences in bacteriophage lambda, residing in their leader and spacer regions. Assembly of the antitermination complex, consisting of NusA, NusB, NusE and NusG, is dependent on the existence of the nut-like sequence element. Interestingly, r-protein S10 (NusE) as well as some other r-proteins (S1, S4, L3, L4 and L13) have been implicated in the antitermination system (Torres et al. 2001).

Stringent response

The stringent response is the cellular action taken when the availability of amino acids needed for rapid growth in the cell's surrounding becomes low and

after a complex set of responses leads to a shut-down of the synthesis of rRNA and tRNA in benefit of amino acid synthesis (Paul et al. 2005). This diversification in metabolic capacity is a fundamental defensive and adaptive process that is achieved through the small nucleotides guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) collectively called (p)ppGpp. The proteins responsible for the synthesis and degradation of (p)ppGpp are RelA and SpoT. RelA is a (p)ppGpp synthase that responds exclusively to amino acid starvation by sensing deacetylated tRNA bound to the A-site of the ribosome (Cashel 1975). SpoT is a (p)ppGpp hydrolase with dual functions, it is both a (p)ppGpp synthase and a (p)ppGpp hydrolase (Xiao et al. 1991). SpoT is thought to be responsible for (p)ppGpp production in response to most stresses and nutrient limitations except amino acid starvation through an unknown mechanism (Magnusson et al. 2005). Recently, the RNA polymerase binding protein DksA was found as a regulator of (p)ppGpp induced termination of rRNA transcription by aiding the binding of ppGpp to the secondary channel of the RNA polymerase close to the active site, leading to immediate transcription cessation of rRNA and tRNA genes (Artsimovitch et al. 2004; Paul et al. 2004; Perederina et al. 2004). This model for DksA involvement in stringent response seems to be generally accepted although at least one earlier report showed a DksA independent effect of ppGpp on transcription from rRNA promoters *in vivo* (Brown et al. 2002).

Growth rate dependent regulation

Growth rate dependent regulation is the cells way of fine-tuning the rRNA transcription so as to, under static nutrient conditions, be able to supply just the amount of ribosomes needed to use the available nutrients optimally. Limited growth, as in stationary phase, will then lead to a decrease of rRNA transcription. Growth rate dependent regulation has been studied extensively for decades and many are the models that have been proposed on how this regulation is achieved. A thorough review of previous results and some additional experiments have put the nucleotide ppGpp back in the spotlight, and this far seems to be a good candidate for the signal molecule being responsible not only for the stringent response, but also for growth rate dependent regulation (Dennis et al. 2004; Bremer and Dennis 2008).

Maturation of rRNA

Processing of rRNA

The 16S, 23S and 5S rRNA, as well as, different tRNA species are transcribed as one primary transcript. The first endoribonuclease to cleave this transcript is RNase III, which separates the different precursor rRNAs and the tRNAs (Nikolaev et al. 1973; Ginsburg and Steitz 1975). RNase III cleaves the stems formed by pairing of the 5' and 3' ends of 16S and 23S, respectively, in the primary transcript. In a strain deficient of RNase III, the formation of mature 16S can occur, but mature ends of the precursor 23S rRNA will not form (King and Schlessinger 1983).

The 16S precursor 17S, generated by the RNase III cleavage contains 115 extra nucleotides at the 5' end and 33 extra nucleotides at the 3' end relative to mature 16S rRNA (Young and Steitz 1978). Subsequent processing by RNase E generates a 16S precursor with 66 extra nucleotides at the 5' end. A final cleavage by RNase G generates the mature 5' end of 16S rRNA (Li et al. 1999). However, it has been shown that RNase E and RNase G separately can form mature 5' ends (Li et al. 1999). The RNase responsible for generating the mature 3' end of 16S rRNA has yet to be identified.

The RNase III cleavage of the primary rRNA transcript generates a 23S precursor with 7 or 3 nucleotides at the 5' end and 8 extra nucleotides at the 3' end in relation to mature 23S rRNA. Maturation of the 3' end of 23S rRNA is achieved through the actions of the exoribonuclease RNase T, while the enzyme responsible for the final processing of the 5' end is unknown.

Modification of rRNA

In addition to being processed before achieving its mature form as seen in the 70S ribosome, the rRNA also receives specific modifications added at certain nucleotides. The 16S rRNA is modified in 11 positions of which one contains a pseudouridine and the others receive methyl groups. The 23S rRNA is known to contain modifications in 25 positions of which nine contain a pseudouridine, 13 receive one or two methyl groups, one contains a

pseudouridine and a methyl group while one position contains an unidentified modification. The majority of the modifications in 16S and 23S are located in the decoding center and the peptidyl transferase center, respectively. These localizations indicate a function in translation that is supported by a generally high degree of conservation, which normally indicates an important function such as translation, but experimental data in support of an involvement in translation remain to be seen. Recent discoveries have shown that modifications at position 1402 in 16S rRNA by RsmL and RsmH are important for fine-tuning of the P site and increases the decoding fidelity (Kimura and Suzuki 2010).

An alternative explanation to the presence of modifications in rRNA is that they mark the completion of certain steps in ribosome assembly and that the responsible enzymes have other roles in ribosome assembly than just modifying rRNA. This has been suggested at least for positions 1518 and 1519 in 16S rRNA, which are dimethylated by the enzyme KsgA. The deletion of the *ksgA* gene shows no discernable phenotype, which has been a conundrum since the gene is nearly universally conserved. Recent discoveries have shown that KsgA and the modifications that it is responsible for seem to be part of a check point separating the pre-30S subunits from the complete 30S subunits (Xu et al. 2008). KsgA has been shown to bind to pre-30S subunits that are compositionally complete but in a translationally inactive conformation (Desai and Rife 2006).

Folding of 16S rRNA

One of the most impressive features of rRNA is the ability to fold into a functional state during *in vitro* conditions with only the assistance of the r-proteins (Traub and Nomura 1968). Although both subunits can be assembled *in vitro*, the slow and inefficient assembly of the 50S subunit has lead to the 30S subunit becoming the model particle for ribosome assembly (Nomura 1973; Green and Noller 1999). The folding of the 16S rRNA can be divided roughly into three parts, the folding of the 5'-, central-, and 3'-domains.

The individual domains can be assembled *in vitro*, much in the same way as for the whole 30S. The body consists of the 5' rRNA (nucleotides 1-526) and r-proteins S4, S16, S17 and S20 (Weitzmann et al. 1993); the platform consists

of the central rRNA (nucleotides 547-895) and r-proteins S6, S8, S11, S15 and S18 (Agalarov et al. 1998); and the head consists of the 3' rRNA (nucleotides 923-1542) and r-proteins S2, S3, S7, S9, S10, S13, S14 and S19 (Samaha et al. 1994). Although the separately assembled domains are similar in shape and size to their counterparts in mature 30S they lack the tertiary binding r-proteins. This is most probably due to that the tertiary binding r-proteins require interactions with rRNA and/or r-proteins in the other domains. The 5' domain is unique in the sense that it can form a native 30S conformation without the help of any of the r-proteins, indicating that it is the fold of the rRNA that determines r-protein binding and not the r-proteins that determine the fold of the rRNA (Adilakshmi et al. 2005).

The r-proteins

The proteins that are part of the mature 30S subunit are denoted "S proteins" for "Small subunit" since they are only found in the 30S subunit and in the 30S that is part of the 70S. The 21 r-proteins in the 30S are S1-S21. The 33 r-proteins being part of the mature 50S subunit are denoted "L proteins" for "Large subunit". The r-proteins in the 50S are denoted L1-L6, L7/L12, L9-L11, L13-L25, L27-L36. The specific functions of the r-proteins remain under investigation, but apart from extra-ribosomal roles, such as feedback regulation of their own expression, they seem to perform a general role in stabilizing the tertiary rRNA structures.

Modification of r-proteins

Some r-proteins are post-translationally modified. Proteins S11, L3, L7/L12, L16, L33 and L11 are methylated (Terhorst et al. 1972; Brosius and Chen 1976; Chen et al. 1977; Colson et al. 1979), whereas S5, S18 and L12 are acetylated (Cumberlidge and Isono 1979; Isono and Isono 1980). R-protein S6 is modified with up to six glutamic acid residues at its C-terminal end (Kang et al. 1989). Apart from being methylated, protein S11 can also be modified by the addition of an isoaspartate (David et al. 1999). Protein S12 has a methylthio-aspartic acid (Kowalak and Walsh 1996), and L16 has one unknown modification besides its methylation (Arnold and Reilly 1999). The

modifications mentioned above are the ones known to date and there may be additional modifications that have not yet been found. There is not much known about the function of r-protein modifications and with one exception the loss of a modification gives no phenotypic change. The lack of methylation of r-protein L3 leads to slow growth, cold-sensitivity and accumulation of subunit precursors (Lhoest and Colson 1981). Why r-protein modifications in general seem to be redundant under laboratory conditions remains to be investigated.

Regulation of r-protein synthesis

R-protein synthesis needs to be as tightly controlled as the synthesis of rRNA since the utilization of r-protein is dependent on the availability of other r-proteins and rRNA. Regulation is achieved, for most of the r-proteins, through an autogenous feedback mechanism where one protein acts as a repressor, usually at the translational level, of the synthesis from the operon it is encoded by (Nomura et al. 1984). As long as there is a demand for r-proteins in ribosome assembly, the pool of non-utilized repressor r-proteins will be low. However, if the need for r-proteins decreases, due to lower amounts of free rRNA, the amount of free repressor r-proteins relative to that of assembling subunits will increase and hence the repressor proteins will bind to their own mRNAs and block translation. To date ten r-protein repressors have been identified: S1, S4, S7, S8, S15, S20, L1, L4, L10 and L20 (Kaczanowska and Ryden-Aulin 2007).

Ribosome assembly factors

The discovery that ribosomal subunits could be assembled *in vitro* using only mature rRNA and total protein from either the 30S ribosomal subunit or the 50S subunit (TP30 and TP50, respectively) indicates that the rRNAs contain much of the information needed for complete assembly (Traub and Nomura 1968; Nierhaus and Dohme 1974; Dohme and Nierhaus 1976; Krzyzosiak et al. 1987; Culver and Noller 1999). However, the *in vitro* reactions required non-physiological conditions such as increased temperature, high ionic concentrations and long incubation times, in order to produce mature subunits. These observations suggested that something was missing from the *in vitro*

reactions that are present *in vivo*. Over the past 15 years, it has become clear that there are ribosome assembly factor proteins that assist in the assembly of the ribosomal subunits but are not part of the mature subunits. These proteins belong to different classes and can have other functions apart from their function in ribosome assembly.

RNA chaperones, RNA helicases and ribosome dependent GTPases

Although the rRNA itself seems to contain many of the instructions needed for self-assembly, the efficiency is not sufficient to meet the requirements during *in vivo* conditions. It has been predicted that in a randomly generated RNA molecule about 50% of the bases will form a double helix (Lorsch 2002). Thus, the self-assisted folding would generate mis-folded structures in the rRNA of different stabilities. Interactions in these structures need to be broken for the correct structure to form and such events are probably the rate limiting steps during *in vitro* assembly (Held et al. 1974; Herold and Nierhaus 1987). Probably, this explains why *in vitro* assembly requires a heating step to be completed. *In vivo*, these kinetically trapped mis-folded structures might be resolved by RNA chaperones to facilitate the correct folding. To date, the only RNA chaperone suggested to be involved in translation in *E. coli* is the translation initiation factor IF1, although the specific target of its chaperone activity is not known (Croitoru et al. 2006).

RNA helicases that act upon secondary and tertiary structures in RNA play key roles in RNA processing, degradation, translation and ribosome assembly (Fuller-Pace 1994). Many of the RNA helicases belong to the family of proteins called DEAD-box helicases, due to their characteristic DEAD motif (Cordin et al. 2006). In *E. coli* there are three known DEAD-box helicases suggested to be involved in ribosome assembly: CsdA, DbpA and SrmB (Diges and Uhlenbeck 2001; Charollais et al. 2003; Charollais et al. 2004). Notably, none of these take part in the assembly of the 30S subunit. CsdA was found as a suppressor of an alteration in r-protein S2 and was later shown to be a cold-shock-inducible ATP-independent RNA helicase (Toone et al. 1991; Jones et al. 1996). SrmB was suggested to be a nucleic acid-dependent ATPase when it was found as a

high-copy suppressor of a temperature-sensitive mutation in the gene for r-protein L24 (Nishi et al. 1988). DbpA is a 3’ to 5’ ATP dependent RNA helicase with specificity for hairpin 92 in 23S rRNA (Diges and Uhlenbeck 2001; Henn et al. 2010). The precise role of any of these proteins is not known.

Ribosome assembly GTPases

Apart from the role GTPases play in many essential processes in the cell e.g., cell cycle regulation and/or metabolism, some are also implicated in the assembly of the ribosomal subunits. Most of the ribosome assembly GTPases are essential for growth, and in the cases where null mutants are viable their growth is severely impaired due to a reduction in 70S ribosomes (Campbell et al. 2005; Hwang and Inouye 2006; Matsuo et al. 2006; Schaefer et al. 2006; Uicker et al. 2006; Loh et al. 2007).

RbgA, ribosome biogenesis GTPase A, is a widely conserved protein but is absent from *E. coli* and other proteo-gamma bacteria (Morimoto et al. 2002). A *Bacillus subtilis* strain lacking RbgA is devoid of 50S and accumulates a 45S intermediate (Uicker et al. 2006). This intermediate lacks r-proteins with molecular weights similar to L16, L27 and L36 suggesting that these proteins are missing and that RbgA is responsible for their incorporation (Britton 2009).

Obg (SpoOB-associated GTP binding protein) is an essential conserved GTPase that has been implied in such diverse processes as ribosome assembly, chromosome segregation and replication and in *B. subtilis* and *Streptomyces* spp. it is involved in the regulation of sporulation (Okamoto and Ochi 1998; Lin et al. 1999; Wout et al. 2004; Foti et al. 2007). A role for Obg in ribosome assembly was indicated when Obg was found to co-elute with purified ribosomes and interacted with ribosomal protein L13 (Scott et al. 2000). Interestingly, over-expression of Obg can alleviate the slow growth phenotype and increases the levels of 70S ribosomes seen in a strain lacking the ribose methylation at U2552 in 23S rRNA due a deletion of *rrmJ*, without restoring the actual modification (Tan et al. 2002). Further, Obg interacts specifically with SpoT that is involved in the regulation of rRNA synthesis (see aboveWout

et al. 2004). This observation is indicative of the connection between ribosome assembly and regulation of rRNA synthesis (Wout et al. 2004).

EngA/Der (double Era-like GTPase). Members that belong to this unique family of GTP binding proteins contain two sequential G-domains located in their N-terminal part and an RNA binding KH domain in the C-terminal part (Robinson et al. 2002). The first indication that EngA is involved in ribosome assembly was the observation that over-expression of EngA could rescue the slow growth phenotype of an *rrmJ* mutant, i.e. the same effect seen as when over-expressing Obg (Tan et al. 2002). Later it was shown that EngA binds only to free 50S subunits and not to 50S in the 70S ribosomes or to the 30S subunits (Bharat et al. 2006).

Era (*E. coli* Ras-like protein) is a GTPase that is highly conserved and is essential in *E. coli* (Inoue et al. 2003). Era has affinity for both 16S rRNA and the protein component of 30S (Meier et al. 2000; Tu et al. 2009). Depletion of Era affects maturation of 16S rRNA, which is interesting since Era is in contact with the 3' domain of 16S rRNA (Tu et al. 2009). Whether or not Era is involved in the maturation of the 3' end of 16S rRNA remains to be elucidated. The binding of Era to RNA increases its GTPase activity (Meier et al. 2000) however, it is not known if this is important for the ribosome assembly function. The over-expression of Era can suppress the lack of RbfA, another ribosome assembly protein, and this suppression seems to be independent of the GTPase activity of Era, and even enhanced by the lack of it (Inoue et al. 2003).

The ribosome maturation factors.

Apart from the relatively well-characterized classes of proteins, presented above, involved in ribosome assembly of either the 50S or 30S subunits, there are ribosome maturation factors whose roles are far less understood. These proteins are not members of the same protein family based on their three-dimensional structures but contain e.g. RNA binding domains found in other proteins involved in ribosome assembly. With very few exceptions, the information we have about the function of these proteins comes from studies on the phenotypes of strains in which the corresponding genes have been deleted.

In general, the phenotypes are similar with a decrease in 70S ribosomes and polysomes and a concomitant increase in free subunits, an accumulation of immature rRNA species and either a temperature-sensitivity (Ts) or a cold sensitivity (Cs) phenotype. These similarities and the ability to suppress some of these phenotypes of a particular mutant by over-expressing one of the other ribosome assembly factors, have lead to a view of ribosome assembly proteins being functionally redundant. Recent studies may have shifted the idea from functional redundancy to a more assembly pathway oriented way of looking at the function of ribosome assembly proteins (Talkington et al. 2005; Bunner et al. 2010). Hence, assembly of the ribosomal subunits can occur through many pathways simultaneously and if the lack of one assembly protein negatively affects one pathway this may be compensated by shifting assembly over to another pathway.

RbfA (ribosome binding factor A) was found as a high copy suppressor of a C23U cold-sensitive mutation in the 5' region of 16S rRNA (Dammel and Noller 1993; Dammel and Noller 1995). The C23U mutation, or the lack of RbfA, results in cold-sensitive growth and a decrease in 70S ribosomes with a corresponding increase in 50S and 30S subunits. Further, strains that either lack RfbA or that harbor the C23U mutation accumulate 17S rRNA. The C23U mutation is thought to shift the equilibrium between two competing secondary structures in 16S rRNA to favor that found in immature 16S rRNA at the expense of the formation of helix 1 found in mature 16S rRNA, which would explain the accumulation of 17S rRNA. The phenotypic similarities between the RbfA lacking mutant and that harboring the C23U mutation lead to the suggestion that the C23U mutation disrupts the binding of RbfA to helix 1 (Dammel and Noller 1993), although this remains to be proven experimentally. The recent docking of RbfA to the 30S subunit had the potential of discerning whether RbfA binds to helix 1 or not (Datta et al. 2007). Interestingly, RbfA seems to dock close to helix 1 but does not show a direct interaction; RbfA rather seems to interact directly with parts of helix 28, 29, 44 and 45 in the 16S rRNA and also with ribosomal proteins S9 and S13 (Datta et al. 2007). It was suggested that the lack of direct interaction with helix 1 was due to the use of *Thermus thermophilus* RbfA that has a shorter C-terminal tail than in *E. coli* (Datta et al. 2007). It has been suggested that a longer C-terminal tail region of

RbfA is of importance for bacterial species living at lower temperatures as a way of stabilizing/enabling the formation of the mature form of helix 1 (Datta et al. 2007).

RimM (Ribosome maturation factor M) is a 21kDa two-domain protein (Bylund et al. 1997; Suzuki et al. 2007). Deletion of the gene coding for the RimM protein results in a decrease in mature 70S ribosomes and a corresponding increase in free 50S and 30S subunits (Lovgren et al. 2004). The *rimM* deletion mutant also has higher amounts of immature 16S rRNA indicating a defect in the maturation of the 30S subunits (Bylund et al. 1998). The association of RimM to r-protein S19 in the 30S subunits and to S19 free in solution supports the involvement of RimM in the maturation of the 30S subunit (Lovgren et al. 2004; Suzuki et al. 2007). Two adjacent tyrosines (pos 106-107) in RimM seemed crucial for the interaction with the 30S subunits (Lovgren et al. 2004). Some alterations in r-protein S13 suppress partially the deficiencies of a *rimM* deletion mutant but not those of a mutant expressing a RimM variant with alanines substituted for the two tyrosines. Conversely, mutations in helices 31 and 33b of 16S rRNA, as well as, an amino acid substitution (H83Y) in r-protein S19 suppress more efficiently the deficiencies of the *rimM* tyrosine-to-alanine substitution mutant than those of the *rimM* deletion mutant (Lovgren et al. 2004). R-protein S13 binds to helix 31 while r-protein S19 binds to helix 33b and S13 interacts with S19 during assembly of the 30S subunit. Thus, all these data links RimM to the maturation of a very specific region of the 30S subunit. Interestingly, over-expression of RbfA suppresses partially the deficiencies of the *rimM* mutants, indicating that these two proteins might have some functions in common.

RimJ (Ribosome maturation factor J) has been identified as the N-acetyltransferase of r-protein S5, but was recently found to be more directly involved in the maturation of the 30S ribosomal subunit (Cumberlidge and Isono 1979; Janda et al. 1985; Yoshikawa et al. 1987; Roy-Chaudhuri et al. 2008). RimJ was found as an extragenic suppressor of an S5 (G28D) mutation resulting in a cold-sensitivity phenotype. The suppression was independent of the acetyltransferase activity, hence RimJ might have dual functions in ribosome assembly.

In vitro assembly of the 30S ribosomal subunit

30S subunits that are active in translation can be reconstituted *in vitro* by the use of 16S rRNA and total 30S r-proteins purified from ribosomes [TP30] (Traub and Nomura 1968). Similar results are obtained when using *in vitro* transcribed 16S rRNA and recombinant proteins (Krzyzosiak et al. 1987; Culver and Noller 1999). The initial experiments on the sequential addition of r-proteins to 16S rRNA lead to the classical assembly map showing the r-protein binding order and their different interaction dependencies, RNA/protein, protein/protein or both, needed for binding of each specific r-protein and hence progression of assembly. Based on these data the r-proteins have been divided into three groups: primary-, secondary- and tertiary-binding proteins. The primary binding proteins bind directly to rRNA and initiates nucleation while the secondary and tertiary binding proteins need binding of at least one protein from the preceding group to be able to bind. Besides the binding order of r-proteins, the assembly of the 30S subunit seems to have a 5' to 3' polarity (Powers et al. 1993). *In vitro* reconstitution of the 30S ribosomal subunit at low temperature (0-15°C) displays a three-step assembly. Upon incubation of TP30 and 16S rRNA, assembly stalls after formation of a distinct 30S ribosomal intermediate (RI30). The RI30 sediments as a 21S particle and is composed of 16S rRNA and primary and secondary binding r-proteins (Held and Nomura 1973). RI_{30} is not a uniform intermediate but rather a collection of unstable particles in the process of assembling (Held and Nomura 1973; Talkington et al. 2005). The RI_{30} does not impose a complete block in assembly, but the observation that assembly at 15°C takes more than two days is a strong indication that the RI_{30} is not a good substrate for further assembly (Talkington et al. 2005). Incubating the RI_{30} at higher temperature (approx. 42°C) and high ionic concentrations gives a new intermediate referred to as an activated ribosomal intermediate (RI^*_{30}), which sediments as a 26S particle (Holmes and Culver 2004). The difference in sedimentation is due only to conformational changes since it is achieved without the incorporation of additional r-proteins (Holmes and Culver 2004). Addition of tertiary proteins to the RI^*_{30} will generate a mature 30S subunit.

In vitro assembly of the 50S ribosomal subunit

Although there are similarities with the *in vitro* assembly of the 30S ribosomal subunit, the *in vitro* assembly of the 50S subunit is much more complex. The 23S rRNA is roughly twice the size of 16S rRNA, there are nearly twice as many r-proteins and a second rRNA molecule, the 5S, that need to be incorporated to form a mature 50S subunit. In addition, there are four steps needed to complete the *in vitro* reconstitution of the 50S subunit. First the rRNA, 23S and 5S, is incubated with a subgroup of r-proteins at 4°C, forming the first 50S intermediate $RI_{50}[1]$. Then, the $RI_{50}[1]$ must be incubated at 44°C to be able to form the activated $RI^*_{50}[1]$, sedimenting at 41S. This complex is then incubated with the remaining r-proteins, forming a new intermediate, $RI_{50}[2]$ that sediments as a 48S particle. To become a mature 50S subunit this particle has to be incubated at a high Mg^{2+} concentration and at high temperature (50°C) (Herold and Nierhaus 1987).

In vivo assembly of the ribosome

The *in vitro* data on ribosomal subunit assembly have given clues to how the process proceeds *in vivo*. However, *in vivo* assembly still remains poorly understood. Ribosomal subunit intermediates have been found for both subunits, both *in vitro* and *in vivo* (Mangiarotti et al. 1968; Traub and Nomura 1968; Hayes and Hayes 1971). *In vivo*, a 21S intermediate of the 30S subunit has been found, whereas for the 50S subunit, 32S and 43S intermediates have been identified (Lindahl 1975). Apart from these distinct intermediates it is thought that more stable intermediates co-migrate with their respective 30S and 50S subunits during sucrose gradient centrifugations, which complicates the analysis of any such potential assembly intermediates (Lindahl 1975).

The transcription, and hence availability, of rRNA is the major determinant of the co-transcriptional ribosome assembly. *In vivo* experiments where T7 RNA polymerase transcribed rRNA was used as an assembly template gave rise to abnormal subunit particles with low translational activity (Lewicki et al. 1993). This was attributed to an effect of the high elongation rate of the T7 RNA polymerase, resulting in mis-folding and hence assembly defects.

The primary transcripts of the rRNA operons contain regions that need to be processed before the subunits can be part of a functional ribosome. It has been shown that the leader sequence of 16S rRNA is important for correct folding and also for assembly of the 30S ribosomal subunit. The leader sequence is only part of the 17S rRNA but not of mature 16S rRNA. Despite the absence of the leader region in mature 16S rRNA, point mutations in this region give rise to translationally inactive 30S subunits (Theissen et al. 1993). The effect of the 16S rRNA leader mutations might be due to interference with rRNA folding events during assembly (Balzer and Wagner 1998). Similarly, the maturation of the 50S ribosomal subunit is also dependent on the flanking regions of the 23S rRNA. The base-pairing between the 5' and 3' ends of the 23S rRNA is essential (Liiv and Remme 1998). Also, the base-pairing occurring within the 5' region before transcription has reached the 3' region of the 23S rRNA seems to be important for proper folding (Liiv and Remme 2004). These observations highlight some short-comings of the *in vitro* reconstitution experiments performed with mature rRNA, that although informative might give a too simplistic view of the assembly of the ribosome.

Quality control and degradation of ribosomes.

If the assembly of the ribosome fails, the cell needs a way of removing these ribosomes from translation. The degradation of ribosomes has been studied for certain physiological conditions such as starvation for carbon, nitrogen, phosphate or magnesium ions (Ben-Hamida and Schlessinger 1966; Jacobson and Gillespie 1968; Maruyama and Mizuno 1970). A recent publication proposes a simple model for the degradation of ribosomes that are not needed. The model is based on a difference in the susceptibility to endonucleolytic degradation, where the 70S ribosome is less susceptible than the 30S and 50S ribosomal subunits (Zundel et al. 2009). The identity of the endonuclease(s) responsible for the degradation remains to be discovered. Two other proteins involved in the degradation of rRNA are the exoribonucleases polynucleotide phosphorylase (PNP) and RNase R. The deletion of the genes for each of these exoribonucleases causes an accumulation of rRNA fragments that interferes with ribosomal subunit assembly, conceivably through depletion of the r-protein pool in competition with the true 16S rRNA. The depletion of

r-proteins would lead to a decrease in ribosomes that would derepress rRNA synthesis resulting in a further accumulation of rRNA fragments to such an extent that it might be toxic to the cell (Cheng and Deutscher 2003).

Coupling of 50S and 30S ribosomal subunit assembly.

There are indications that there is a link between the assembly of the 50S and 30S subunits. For example, when the assembly of one subunit is impaired, the assembly of the other subunit is also affected. Alterations in r-proteins S5 and L22 affect the maturation of their respective subunit but also that of the other subunit (Nashimoto and Nomura 1970; Nashimoto et al. 1971). A deletion of the gene *rluD*, coding for a pseudouridine synthase, which modifies positions 1911, 1915 and 1917 in 23S rRNA, does not only affect the maturation of the 50S but also that of the 30S (Gutgsell et al. 2005). Deletion of the genes for either of the 50S interacting ribosome assembly proteins SrmB and CsdA causes an accumulation of 17S rRNA (Charollais et al. 2003; Charollais et al. 2004). Similar effects have also been seen by the addition of ribosome specific drugs. The 50S binding antibiotic chloramphenicol affects also the assembly of the 30S ribosomal subunit (Siibak et al. 2009). The mechanism behind the coupling between the assembly of the 50S and 30S subunits remains to be elucidated.

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 RimP protein in ribosome maturation.

- To investigate the role of RbfA in the maturation of the 30S ribosomal subunit.

- To investigate effects of the ribosome assembly factors RimM, RimP and Era on *in vitro* reconstitution of the 30S ribosomal subunit.

- To investigate the connection between RbfA, RimP in 30S maturation and the formation of helix 1 in 16S rRNA.

Results and discussion

The RimP protein is important for maturation of the 30S ribosomal subunit.

The location of the *rimP* gene in the *nusA-infB* operon indicated a possible involvement of RimP in translation since most of the genes in this operon encode proteins that are directly or indirectly involved in translation. One of the encoded proteins, RbfA, is a ribosome maturation factor. The initial characterization of the role of RimP was carried out by using a strain, MW187, harboring an in-frame deletion of the *rimP* gene, MW187.

Growth of the *rimP* deletion mutant is most affected at high temperature.

The growth of the *rimP* deletion mutant MW187 was slower than that of the wild-type strain at all tested temperatures, but the difference was more pronounced at higher than lower temperatures (Paper I). The slow growth of the *rimP* mutant was complemented by the introduction of a plasmid carrying the *rimP* gene indicating that the growth defects were due to the lack of the RimP protein.

The *rimP* deletion mutant displays a decrease in 70S ribosomes.

To investigate if RimP had a role in translation, the translational status of the *rimP* deletion mutant MW187 was examined by looking at the distribution between translating ribosomes and free ribosomal subunits. The *rimP* deletion mutant MW187 grown at either 30, 37 or 44°C displayed increased amounts of free 30S and 50S subunits with a corresponding decrease in translating ribosomes compared to the wild-type strain as demonstrated by analysis of total cell extracts after sucrose gradient centrifugations (Paper I). The increase in the amount of 30S and 50S subunits was disproportionate as the amount of 30S subunits was substantially lower than that of the 50S subunits, indicating that the assembly of the 30S subunit was defective explaining the decrease in ribosomes active in translation. The defects were seen at all tested temperatures

with the most severe effects at 44°C in agreement with the results from growth rate measurements.

The *rimP* deletion mutant accumulates immature 16S rRNA.

The reduced amounts of translating ribosomes and disproportionately low amounts of 30S subunits in the *rimP* mutant lead us to suspect that the RimP protein was involved in the maturation of the 30S subunit. It has been suggested that one of the last steps of maturation of the 30S ribosomal subunit is the final processing of the immature 16S rRNA (17S) to its mature form 16 rRNA, hence an increase in 17S rRNA would indicate a defect in the maturation of the 30S ribosomal subunit. To analyze the presence of 17S rRNA, total RNA was extracted from the *rimP* deletion mutant MW187 grown at 30, 37 or 44°C and used in primer extension analysis of 16S rRNA. The mutant showed an accumulation of 17S rRNA at the tested temperatures with the highest amounts at 44°C (Paper I). Thus, the defects in the processing of 16S rRNA correlated with the defects in growth and translation of the *rimP* deletion mutant.

Localization of RimP to the 30S ribosomal subunit.

The defects of the *rimP* deletion mutant pointed to that the RimP proteins might be involved in the maturation of the 30S ribosomal subunit suggesting that RimP might localize to the 30S ribosomal subunits. To test this hypothesis, fractions obtained after centrifugation of a cell extract of, a wild-type strain, through a sucrose gradient was analyzed for the presence of the RimP protein by Western blotting. The RimP protein was found to co-localize with the 30S ribosomal subunits, but not with either the 50S ribosomal subunit or the 70S ribosome supporting a role for RimP in the maturation of the 30S subunit (Paper I)

The lack of RimP is not suppressed by increased amounts of other maturation factors.

It has been shown that the over-expression of some ribosomal maturation factors can compensate for the loss of another ribosome maturation factor (Lu and Inouye 1998; Bylund et al. 2001; Inoue et al. 2003). To investigate whether the growth defect of the *rimP* deletion mutant MW187 could be suppressed by

increased expression of any known ribosome maturation factor, plasmids expressing the genes for RbfA, RimM, Era, RsgA, KsgA and DnaK were introduced into the mutant. Expression of neither of the genes on the plasmids suppressed the slow growth of the *rimP* deletion mutant.

RimP might have a role that is distinct from other ribosome maturation factors.

Many mutants defective in ribosome assembly are cold-sensitive. The function ascribed to ribosome maturation factors in general is to facilitate the folding of rRNA, either by direct interaction with the rRNA or indirectly by the interaction with r-proteins. Folding of the rRNA is an energy requiring process that, if hampered, would display the most severe defects in a low energy surrounding. Hence, the removal of a ribosome maturation factor would show the most severe effects at low temperature since the low free energy state would lead to an increased need for enzymatic catalysis of rRNA conformational changes. The fact that the lack of RimP had more severe effects at high than at low temperature indicates that RimP might have a role in ribosome maturation to stabilize a structure that is unstable at high temperatures.

The effect of ribosome maturation factors on the *in vitro* reconstitution of the 30S ribosomal subunit.

The investigation of the *in vivo* functions of ribosome assembly factors is hampered by the fact that there are no methods to analyze their function when they are present in the cell. Much of what is known about this group of protein is derived from the observations of the phenotypes associated with the lack of a specific ribosome maturation factor. The same problem existed in the field of r-proteins and the studies of their addition to the maturing ribosomal subunits *in vivo*. This was partially overcome by using purified ribosomal components to study assembly of the ribosomal subunits *in vitro* (Traub and Nomura 1968; Nierhaus and Dohme 1974; Dohme and Nierhaus 1976). Those *in vitro* experiments were hallmark events in the field of ribosome assembly and gave rise to the now classic assembly maps of the 30S and 50S ribosomal subunit.

To gain insight into the function of a sub-population of the ribosome maturation factors, the proteins Era, RimM, RimP and RbfA were purified and incorporated into the *in vitro* reconstitution experiments. The individual ribosome maturation factors were pre-incubated with mature 16S rRNA, isolated from mature 30S subunits, and ^{15}N-labeled total 30S r-proteins (TP30), also isolated from mature 30S subunits. Samples were taken out after different time points and chased with unlabeled (^{14}N) r-proteins to allow for the completion of the assembly of the 30S subunit. The fully assembled 30S subunits were then analyzed by quantitative mass spectrometry for the incorporation of ^{15}N-labeled r-protein in relation to a ^{14}N-non-labeled control at the different time points yielding an incorporation rate that was compared with that for the assembly of the 30S subunit without the addition of a ribosome maturation factor (Paper II).

Era increases the incorporation rate of most late binding r-proteins.

The addition of the GTPase Era gave no increase in the incorporation of specific r-protein but rather a general effect on most late binding r-proteins (Paper II). This effect in the later stages of 30S ribosomal subunit assembly is consistent with the hypothesis that Era might be involved in the final maturation of 17S rRNA to 16S rRNA (Sharma et al. 2005). The incorporation rate of r-protein S5, S9, S11 and S12 was approx. two-fold increased by the addition of Era. Slightly increased incorporation rates were obtained for the 3' domain r-proteins S7, S10, S13, S14 and S19. Based on these observations, Era does not seem to be aiding the incorporation of specific r-proteins but rather to facilitate the folding of the rRNA resulting in a general increase in the incorporation of a sub-population of r-proteins. Since the effects of Era spans several proteins throughout the later steps of assembly it is likely that Era enters the 30S assembly in the initial part of the late stages and resides on the assembling 30S subunit for a period of time, possibly until the final maturation. During *in vitro* reconstitution reactions, r-proteins S21 and S2 bind transiently to the assembling 30S and therefore are hard to detect using PC/QMS (Talkington et al. 2005). We observed a stabilization of the incorporation of r-protein S2 (unpublished data), which further supports a function for Era in the latest steps of assembly.

RimM primarily increases the incorporation rates of r-proteins S9 and S19.

Addition of RimM showed the most prominent effects on the incorporation of S9 (2-fold) and S19 (4-fold) with more modest effects on the incorporation of S10 and S3 (both 1.5-fold increase). These results are consistent with previous work on RimM that suggested a role in the assembly of the 3' domain (head) of the 30S ribosomal subunit. Surprisingly, the incorporation of r-proteins S12 and S13 was blocked by RimM after an initial period of normal incorporation rates. The inhibition of the incorporation of S13 might result from RimM preventing S13 from binding to S19 since these two proteins are known to interact in the 30S subunit (Brodersen et al. 2002). The inhibition of S12 is more difficult to explain since it is part of the 5' domain and neither S12, nor the assembly of the 5' domain have been shown to be involved in the assembly of the 3' domain.

RimP primarily increases the incorporation of r-proteins S5, S9 and S12

The ribosome maturation factor RimP was the protein that showed the most striking effect on r-protein incorporation rates, when added to the *in vitro* reconstitution mixtures. RimP primarily increased the incorporation rates of r-proteins S12 (6-fold), S5 (2-fold) and S9 (2-fold). Based on previous *in vivo* interaction studies, where RimP was found to interact with r-protein S5 and S7 (Butland et al. 2005), our hypothesis was that RimP would increase the incorporation of either, or both, of these two proteins. It has been shown previously that the incorporation of S12 is slightly dependent on the presence of S5 (Traub and Nomura 1968), but it is unlikely that the two-fold increase in the S5 incorporation rate can explain the six-fold incorporation increase of S12 in our experiments. The two-fold increase in the incorporation of r-protein S9 might be explained by the slight increase in the incorporation of r-protein S7 since the prior binding of S7 has been shown to increase the incorporation rate of S9 26-fold (Bunner et al. 2010).

Substitutions in the C terminal domain of the *E. coli* ribosomal protein S5 suppress the lack of the ribosome maturation factor RbfA as well as the dominant cold-sensitive C23U mutation in 16S rRNA.

Overexpression of *rimP* is lethal to an *rbfA* null mutant.

Since the genes for the two ribosome maturation factors RimP and RbfA are found in the same operon and that the two proteins share structural similarities, we wanted to test if either of the two could high-copy suppress the lack of the other. The plasmid carrying the gene for *rbfA* did not suppress the growth defects of the *rimP* deletion mutant MW187. However, the introduction of the plasmid carrying the gene for *rimP* into the *rbfA* null mutant strain GOB162 arrested growth at 30, 37 and 44°C (Paper III). Further experiments demonstrated that the growth arresting effect was most pronounced at 44°C. Looking at ribosome assembly as a multitude of pathways that are interdependent suggest that the over-expression of *rimP* in the absence of *rbfA* might have increased the rate of a RimP dependent assembly pathway while that dependent on RbfA was slowed down due to the lack of RbfA. Alternatively RimP might be dependent on the actions of RbfA in order to dissociate from the assembling 30S after having performed its function. The effects of either of the hypothesis would be the same, the assembling 30S subunit would be trapped in a conformation that is incompatible with further steps of assembly, and hence the lack of 30S subunits will lead to a lack of 70S ribosomes that in turn arrests growth.

Isolation of chromosomal suppressor mutations towards an *rbfA* null Δ*rimP135* double mutant.

Previous attempts to isolate chromosomal suppressor mutations towards either of the *rbfA* null or the *rimP* deletion mutations have been unsuccessful (data not shown). We reasoned that it might be easier to obtain suppressor mutations to either of them by using a strain lacking both RbfA and RimP. After repeated sub-culturing and plating of the double mutant, faster-growing derivatives appeared (Paper III). A total of 32 faster- growing clones were obtained, of which 12, 11 and 9 were isolated at 30, 37 or 44°C, respectively.

Initial observations indicated that the strongest suppressors were those isolated at 30°C.

Localization of a subset of suppressor mutations to the *rpsE* gene, for r-protein S5.

All of the strongest suppressor mutations, except one, localized to the gene *rpsE*, encoding r-protein S5. Five nucleotide substitutions that resulted in amino acid substitutions at three different positions in the C-terminal part of r-protein S5 were identified as being G87A/S, G91A and A127V/T. Among the acquired suppressor-containing mutants, the same nucleotide substitutions were obtained in individually isolated clones, suggesting a certain degree of saturation. It is interesting to note that the amino acid substitutions occur on a triple β-sheet surface resembling an RNA recognition motif (RRM) (Davies et al. 1998), which suggest that the alterations might change how S5 interacts with the surrounding rRNA, the closest structure being Helix 1. Previously known alterations in S5 have been found as suppressors giving spectinomycin resistance and/or having effects on translational fidelity. Amino acid substitutions conferring spectinomycin resistance are found in positions 20-22, and 28 of S5, while those that display a reduced translational accuracy are located on a C-terminal surface that faces r-protein S4. The amino acid substitutions discovered here (Paper III) reside on a surface that is distinct from those containing previously identified alterations in S5. Thus, the effect of the amino acid substitutions isolated in the work presented in Paper III on the translational machinery might be novel.

The *rpsE* mutations suppress the lack of *rbfA*.

To examine if the *rpsE* mutations suppressed the simultaneous lack of RbfA and RimP or if they were directed towards one of the mutations, they were introduced into the *rimP* and *rbfA* single mutants. Neither of the five *rpsE* mutations suppressed the growth defect of the *rimP* deletion mutant, whereas all of them improved growth of the *rbfA* null mutant at 30, 37 or 44°C, with the strongest effect seen at 30°C (Paper III).Further, when the five *rpsE* mutations were introduced into a wild-type strain, no effect on growth was seen. Thus, the suppressor mutations seem to have been isolated towards the lack of RbfA, and

not the lack of RimP, although they were isolated in a strain lacking both proteins. Whether or not the *rpsE* mutations were better suppressors of the slow growth of the double mutant than that of the *rbfA* null mutant was difficult to judge, but since they are the first chromosomal mutations isolated towards the lack of RbfA it is tempting to speculate that the simultaneous lack of RimP facilitated the selection.

The *rpsE* mutations improve the translational status of the *rbfA* null mutant.

As a first characterization of the suppression by the five *rpsE* mutations, their effect on the distribution of free ribosomal subunits and translating ribosomes was examined. The *rbfA* null mutant displayed a severely reduced amount of translating ribosomes with a corresponding higher amount of free 30S and 50S subunits, in comparison to a wild-type strain (Paper III). All five of the *rpsE* mutations decreased the ratio of free 30S to total 70S ribosomes, indicating that the altered forms of S5 improved the 30S subunit maturation and hence increased the amount of 30S subunits taking part in translation.

The *rpsE* mutations improve the processing of 16S rRNA.

To investigate whether the *rpsE* suppressor mutations in fact influenced the maturation of the 30S subunit, their effect on the processing of 16S rRNA in the *rbfA* null mutant was examined by primer extension analysis. All five of the *rpsE* mutations slightly improved the processing of the 16S rRNA in the *rbfA* null mutant, in accordance with their positive effect on the formation of translational capable ribosomes.

The *rpsE* mutations suppress the lethal effect of over-expression of RimP in the *rbfA* null mutant.

Since the *rpsE* mutations suppressed the lack of RbfA we reasoned that they might be able to suppress the lethal effect of over-expressing *rimP* in the *rbfA* null mutant. The introduction of a *rimP* containing plasmid into the *rbfA* null mutant containing the *rpsE* mutations did not have the same deleterious effect as when introduced into the suppressor-free *rbfA* null mutant. At 37 and 44°C, all suppressor mutations except that encoding the A127T substitution

supported growth, however at 44°C the only substitution giving a strong suppression of lethality was G91A, i.e. the one that was the best suppressor of the growth defects of the *rbfA* null mutant. At 30°C, all five *rpsE* mutations suppressed the lethality associated with the over-expression of *rimP* in an *rbfA null* background. The suppression by the *rpsE* mutations of the growth defects of an *rbfA* null mutant and the lethality of over-expressing *rimP* suggests that the alterations in r-protein S5 improve the maturation of 30S ribosomal subunits in the *rbfA* null mutant.

The *rpsE* mutations suppress the growth defects of a C23U 16S rRNA mutant.

RbfA was discovered as a high-copy suppressor of a dominant cold-sensitive C23U mutation in helix 1 of 16S rRNA and the *rbfA* null mutant and a wild-type strain carrying the C23U mutation on a plasmid share many phenotypic properties (Dammel and Noller 1993; Dammel and Noller 1995). These findings lead us to investigate whether the alterations in S5 conferred by the *rpsE* mutations could suppress the growth defects of the C23U rRNA mutant. A wild-type strain carrying the C23U rRNA mutation on a plasmid showed no growth on solid medium after 96 hours at 30°C, whereas at 37°C it displayed very weak growth after 40 hours. At 44°C, growth was initially very weak but improved markedly after prolonged incubation suggesting that the C23U mutation might impose an extended lag period to the cells. Interestingly, all five amino acid substitutions in S5 improved growth of the C23U containing strain at 37°C. At 30°C, the G91A substitution promoted decent growth, G87A, G87S and A127V had weaker effects, whereas A127T had no effects. At 44°C, the growth of the C23U mutant expressing either of the G87A, G87S and A127V S5 variants, was almost the same as that of the control strains. The A127T and G91A had weaker effects although the latter was the strongest of the suppressors at 30°C. In conclusion, the *rpsE* mutations suppressing the slow growth of an *rbfA* null mutant also improve growth, to various degrees depending on the temperature, of a strain harboring the dominant cold-sensitive C23U mutation in 16S rRNA.

The *rpsE* mutations decrease the lag period conferred by a C23U 16S rRNA mutation.

To test if the C23U rRNA mutant had a prolonged lag period and if the *rpsE* mutations could reduce the length of the lag, as inferred from growth on solid medium, the different C23U-containing strains were grown in liquid medium. The *rpsE* strain containing the C23U mutation showed an extended lag period but also a lower growth rate than the control strain. The different *rpsE* mutations shortened the length of the lag period but also increased the growth rate 1.2-to-1.3-fold of the C23U rRNA mutant. Single cell out-streaks from the culture after the growth experiments showed that the strains had retained their original growth phenotype, suggesting that no new suppressor mutations had been selected for. Thus, the major effect of the *rpsE* mutations on the growth of a strain containing the C23U mutation in 16S rRNA on a plasmid, seems to be to reduce the length of the lag period, at least at 37°C. Whether or not this is the case also at 30°C is difficult to judge, since the C23U-containing *rpsE*$^{+}$ strain did not grow at this temperature.

The roles of maturation factors RbfA and RimP in the assembly of the 30S ribosomal subunit.

From the results presented in Papers I-III, a model was formed that sheds some light on the roles of the ribosome maturation factors RbfA and RimP in the maturation of the 30S ribosomal subunit (Paper III). Experimental evidence suggest that the deleterious effects by the *rbfA* null mutation and the C23U mutation in 16S rRNA both stem from a reduced ability to form the mature helix 1 (Dammel and Noller 1993). Intriguingly, the suppressor mutations found towards the absence of RbfA affected amino acids situated on a surface of r-protein S5 that is in close proximity to helix 1 in 16S rRNA (Paper III). We find it likely that the altered forms of S5 change the interaction with helix 1 in such a way that they facilitate the formation of the mature form of helix 1. Helix 1 and helix 2 in 16S rRNA are integral parts of the central pseudoknot, a structure in the core of the 30S ribosomal subunit that connects the individual 5'- (body), central- (platform) and 3'-domains (head) (Brink et al. 1993; Poot et al. 1996). The lethal effect of over-expressing RimP in an *rbfA* null mutant might be explained by an involvement of RimP in the formation of the central

pseudoknot. Over-expression of RimP might promote premature formation of the pseudoknot by incorporating an immature form of helix 1, which seems to be the predominating form when RbfA is missing (Bylund et al. 1998). The correct formation of helix 2 in the central pseudoknot is important for the formation of 70S initiation complexes and influences also the stability of the 30S subunit (Poot et al. 1996). A strain lacking RimP has a disproportionately low amount of free 30S subunits relative to that of free 50S subunits (Paper I), suggesting that the 30S subunits are unstable in the absence of RimP, which is in agreement with a role of RimP in the formation of the central pseudoknot. Moreover, the addition of RimP to the *in vitro* reconstitution of the 30S subunits increased the incorporation rates of r-proteins S12 and S5 (Paper II). These two proteins are found on each side of helix 1 in the pseudoknot structure where S5 interacts directly with helix 1, and S12 interacts with helix 27 and the junction between helix 27 and the central pseudoknot supporting a role for RimP in the maturation of that region of the 30S subunit. We speculate that RimP might help in the formation of the central pseudoknot by bringing helix 1 and the region next to helix 27 together, thereby creating helix 2. RimP contains two domains both of which show structural similarities to RNA-binding proteins. We envision that the joining of helix 1 and helix 27 is facilitated by the binding of one of them to the N-terminal domain of RimP and the other to the C-terminal domain of RimP. Between the two domains there is a highly conserved region that might act as a hinge, which could facilitate the joining of the two helices during the formation of the central pseudoknot. A more detailed discussion regarding this model can be found in Paper III.

Conclusions

- The lack of the RimP protein results in growth defects that are more pronounced at elevated temperatures.
- The lack of the RimP protein leads to a decrease in the amounts of free 30S subunits relative to that of free 50S subunits and a corresponding decrease in 70S ribosomes and polysomes.
- The lack of the RimP protein results in an increase in the amount of immature 16S rRNA.
- The RimP protein binds to free 30S subunits but not to 50S subunits or 70S ribosomes.
- The RimP protein increases the *in vitro* incorporation rates of r-proteins S12, S5 and S9.
- The RimM protein increases the *in vitro* incorporation rates of r-proteins S19 and S9 in accordance with the ability of RimM to bind to r-protein S19. Interestingly RimM at the same time inhibits the incorporation of r-proteins S13 and S12.
- The Era protein increases the *in vitro* incorporation rates of most of the late binding r-proteins. Possibly, it stabilizes the binding of r-protein S2.
- Five *rpsE* mutations resulting in specific amino acid substitutions in the C-terminal domain of r-protein S5 suppress the lack of RbfA.
- The five *rpsE* mutations also suppress the growth defects associated with a C23U mutation in 16S rRNA.
- Overexpression of *rimP* in an *rbfA* null mutant leads to growth arrest.
- The five *rpsE* mutations abolish the lethal effect of over-expressing *rimP* in an *rbfA* null mutant.

Future perspectives

The investigations of the roles of the ribosome assembly factors in the assembly of the ribosome have experienced a reawakening in the last years, partially due to their potential as targets for new classes of antibiotics.

- The localization and analysis of the still unidentified strong suppressor mutation in strain STN188 might result in a better understanding of the role of RbfA and its dependence on S5 in assembly.
- To deepen the understanding of the role of RimM and its effect on the *in vitro* reconstitution it would be beneficial to have a crystal structure of RimM bound to the 30S subunit.
- Much of what is known about ribosome assembly is based on different *in vitro* experiments. The development of *in vivo* methods for the analysis of ribosome assembly would be desirable.
- The investigation of rRNA modification defects in mutants deleted for genes for specific ribosome maturation factors might give an understanding of the progression of ribosomal subunit assembly and the function of rRNA modifications.
- The possibility to use the ribosomal maturation factors as targets for new classes of antibiotics is extremely interesting.

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