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TRIINU SIIBAK

Effect of antibiotics on ribosome assembly is indirect

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LIST OF ORIGINAL PAPERS

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

- I **Siibak, T., Peil, L., Xiong, L., Mankin, A., Remme, J., Tenson, T.** 2009. Erythromycin- and chloramphenicol-induced ribosomal defects are secondary effects of protein synthesis inhibition. Antimicrob Agents Chemother. 53(2):563–71.
- II **Siibak, T., Remme, J.** 2010. Subribosomal particle analysis reveals the stages of bacterial ribosome assembly at which rRNA nucleotides are modified. RNA. 16(10):2023–32.
- III **Siibak, T., Peil, L., Dönhöfer, A., Tats, A., Remm, M., Wilson, D.N., Tenson, T., Remme, J.** 2011. Antibiotic-induced ribosomal assembly defects result from changes in the synthesis of ribosomal proteins. Mol Microbiol. 80(1):54–67

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My contribution to the articles is as follows:

Ref. I – I conducted the experiments, except the one carried out with clinical strains of *S. aureus.* I participated in writing the manuscript.

Ref. II – I designed and preformed all the experiments, analyzed the data and participated in writing the manuscript.

Ref. III – I designed and carried out most of laboratry experiments, analyzed the data and participated in writing the manuscript.

LIST OF ABBREVIATIONS

A-site	ribosomal site for aminoacyl-tRNA
GTPase	GTP hydrolase
IC50	drug concentration needed to give 50% inhibition of measured process
mRNA	messenger RNA
P-tRNA	peptidyl-tRNA
RI	reconstitution intermediate
RNase	ribonuclease
r-proteins	ribosomal proteins
rRNA	ribosomal RNA
tRNA	transfer RNA

INTRODUCTION

Ribosome biogenesis is a complex process that involves in *Escherichia coli* coordinated synthesis of 54 proteins and 3 rRNAs. Ribosome assembly starts cotranscriptionally and involves processing, modification and folding of rRNAs and ribosomal proteins and also their assembly into functional ribosome. During exponential growth phase majority of the total energy production is consumed by ribosome biogenesis (Bremer and Dennis, 1996); therefore assembly must be highly coordinated, fast and efficient.

E. coli ribosome sediments as 70S particle and consists of two subunits: large (50S) subunit contains two rRNA molecules (23S and 5S rRNA) and 33 ribosomal proteins, small subunit contains one rRNA (16S rRNA) and 21 ribosomal proteins. rRNA is dominating as in ribosome as it constitutes about two-thirds in prokaryotic ribosome.

Already more than 50 years ago protein synthesis inhibitor chloramphenicol was shown to induce accumulation of ribosomal particles sedimenting more slowly than mature ribosomal subunits (Dagley and Sykes, 1959; Nomura and Watson, 1959). These particles contain rRNA precursor and incomplete set of ribosomal proteins (Adesnik and Levinthal, 1969; Sykes *et al.*, 1977). During chloramphenicol treatment, ribosomal proteins are produced in nonstoichiometric amounts (Dennis, 1976) and rRNA is expressed in excess over ribosomal proteins (Lazzarini and Santangelo, 1968; Midgley and Gray, 1971; Shen and Bremer, 1977). It has been proposed that this unbalanced synthesis of components is responsible for the chloramphenicol-induced defects in ribosomal assembly (Dodd *et al.*, 1991). About 15 years ago it was suggested that erythromycin and several other antibiotics inhibit ribosomal assembly directly by binding to 50S subunits precursor particle (Champney, 2006).

The first part of the thesis describes briefly synthesis of the ribosomal components and their assembly into mature subunits. Also rRNA modification during ribosome is reviewed. The last chapter of literature overview will focus on the effect of antibiotics on the ribosome assembly.

The experimental part will focus on the erythromycin- and chloramphenicol-induced ribosomal assembly defects. We reveal that assembly of both 50S and 30S subunit is affected in the presence of chloramphenicol as well as erythromycin. In addition, we show that assembly defects, present in cells treated with either of those drugs, are secondary effects due to protein synthesis inhibition (Ref. I).

Results of analysis of posttranscriptional rRNA modifications in incomplete ribosomal particles were analyzed in quantitative way. This work revealed the stages of bacterial ribosome assembly at which certain rRNA nucleotides are modified (Ref. II).

Thirdly, we determined the protein content in the ribosomal precursor particles accumulating in the presence of chloramphenicol or erythromycin. We show that production of ribosomal proteins in the presence of the antibiotics correlates with the amounts of the individual ribosomal proteins within the precursor particles (Ref. III).

3

REVIEW OF LITERATURE

1. Ribosomal genes and their regulation

Ribosomes can account for as much as 50% of the cell dry mass in bacteria; it is thought that about 50% of the total energy production is consumed by ribosome biogenesis (Bremer and Dennis, 1996). It is also known that faster growing cells contain more ribosomes per unit cell mass than do more slowly growing cell. *E. coli* ribosomes consist of three rRNAs and 54 proteins. Two main purposes must be achieved in regulating ribosomal component synthesis; firstly, a balanced synthesis of rRNAs and ribosomal proteins in stoichiometric amounts and secondly, adaptation of ribosomal synthesis to the cells nutritional environment.

In this section, short overview of ribosomal rRNA and protein genes will be given.

1.1. Ribosomal RNA transcription and *rrn* operons

Ribosome assembly begins with synthesis of ribosomal components: rRNA and proteins. 16S, 23S, and 5S rRNA are synthesized as one primary transcript. Folding, processing and modification of rRNA, as well as assembly of ribosomal proteins to rRNA start cotranscriptionally.

There are seven ribosomal RNA operons in *E. coli*: *rrnA*, *rrnB*, *rrnC*, *rrnD*, *rrnE*, *rrnG*, and *rrnH*. Operons start with two functional promoters and the gene order in operons is 16S-tRNA-23S-5S-(distal tRNA) (Lindahl and Zengel, 1986). Ribosomal RNA promotors are among the strongest known, accounting for more than half the cell's transcriptional activity at high growth rates, even though rRNA operons only account for 0.5% of the total genome. Ribosomal RNA promotors are subjected to a number of regulatory mechanisms. In addition to regulation by growth rate, rRNA operons are governed by stringent control, factor-dependent and factor-independent activation, and antitermination (Lindahl and Zengel, 1986; Schneider *et al.*, 2003; Paul *et al.*, 2004; Kaczanowska and Rydén-Aulin, 2007).

None of the single deletions of the seven *E. coli* operons is lethal when tested for logarithmic growth on minimal or rich media (Condon *et al.*, 1992). In strains carrying multiply inactivated *rrn* operons, the expression of the remaining copies can increase significantly to compensate for the deficit, suggesting that *E. coli rrn* operons do not operate at maximal capacity (Condon *et al.*, 1993). It was observed that *E. coli* requires five out of seven operons to maintain growth rates comparable to wild-type strains in rich media (Condon *et al.*, 1995). However, deletion of more than two operons significantly reduced growth rate and the cells became more and more elongated in their shape (Asai *et al.*, 1999). It has been suggested that the significance of seven *rrn* operons is

probably to allow fast adaptation from one growth environment to the next (Condon *et al.*, 1995). The difference in structure and transcription factor binding of seven *E. coli* ribosomal operon upstream regulatory region suggest differential regulation of the individual rRNA operons (Hillebrand *et al.*, 2005).

RNase III is the first endoribonuclease to cleave primary transcript. As a result of RNase III cleavage several types of precursor RNAs are formed: precursor 16S rRNA (17S rRNA), precursor 23S rRNA, precursor 5S rRNA (9S rRNA), and depending on the rRNA operon, a few tRNA precursors (Srivastava and Schlessinger, 1990). 17S rRNA has 115 nucleotides in the 5′end and 33 nucleotides in the 3′end which will be removed by three different enzymes: RNase E, RNase G, and one still uncharacterized RNase (Srivastava and Schlessinger, 1990). Precursor of 23S rRNA contains 3 or 7 additional nucleotides at the 5′ end and 7 or 9 at the 3′end after RNase III cleavage (Srivastava and Schlessinger, 1990), final processing of the 5′ end is carried out by still unknown enzyme and RNase T is responsible for maturation of the 3′ terminus (Srivastava and Schlessinger, 1990). The 9S rRNA includes 84 additional nucleotides at the 5′ end and 42 at the 3′ end (Srivastava and Schlessinger, 1990), both will be cleaved by RNase E, that leaves 3 nucleotides at each end (Srivastava and Schlessinger, 1990) Final maturation is carried out by still unknown RNase and RNase T (Srivastava and Schlessinger, 1990).

1.2. Structure and regulation of ribosomal protein operons

Escherichia coli ribosome contains 54 proteins; each of them is present in single copy except for L7/L12, which is present in four copies. Therefore, stoichiometry between rRNA and ribosomal proteins and stoichiometry among the various ribosomal proteins must be achieved. Genes of ribosomal proteins are organized into at least 16 operons and 4 monocistrons. Several ribosomal protein operons contain genes for nonribosomal proteins, usually other proteins important in transcription or translation, such as the subunits for RNA polymerase and translation elongation factors (Zengel and Lindahl, 1994). Structures of selected ribosomal operons are represented in figure 1.

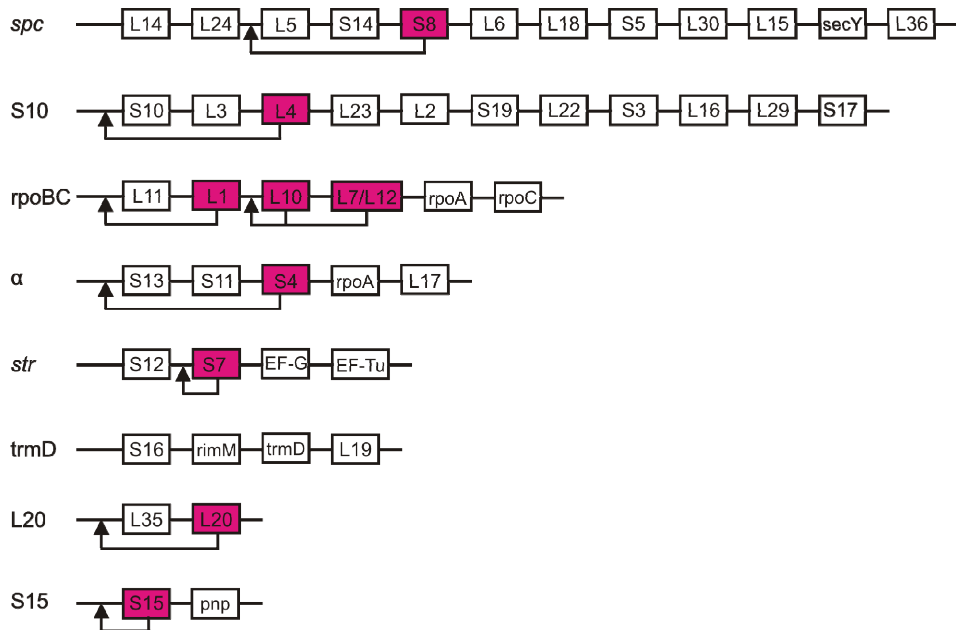

Figure 1. Ribosomal protein operons. Individual genes of the operon are shown as white boxes. The regulatory product is indicated by a pink box and the respective mRNA binding site is shown by an arrow.

The boundaries of ribosomal protein operons are often ambiguous, because of multiple promoters and transcription read-through between adjacent operons (Zengel and Lindahl, 1994). For example, because there is no efficient transcription terminator between *spc* and alpha operons, the alpha operon is transcribed from both *spc* and alpha promoters (Cerretti *et al.*, 1983). It has also been observed for the other operons that some transcription terminators are "leaky" and that several of the ribosomal protein operons contain internal secondary promoters (Zengel and Lindahl, 1994).

One mechanism that contributes to stoichiometric synthesis of ribosomal proteins is translational coupling. First cistron of polycistronic mRNA is usually accessible for the 30S initiation, whereas the second and following initiation sites are hidden within secondary structure. When the first initiation site has been recognized, translation commences and the translating ribosome can unfold secondary structure to reveal the second initiation site. The second and all downstream cistrons are translationally coupled, meaning if one cistron is translated, all downstream ones are translated and vice versa if the first cistron is not translated, then the whole polycistronic mRNA cannot be translated.

In the late 1970s regulation of protein synthesis by selective mRNA inactivation that is regulated by one or several proteins from given operon was suggested (Fallon *et al.*, 1979; Lindahl and Zengel, 1979). It was defined as

"autogenous control" – one gene in an operon encodes a ribosomal protein, which serves both as a structural component of the ribosome and as a regulatory protein controlling the expression of itself and other genes in the operon (Zengel and Lindahl, 1994). One common feature of the repressor proteins is that they are all "primary binding proteins", meaning they can bind to rRNA independent of other proteins. Repressors for most operons have been found. However, when overexpressing each of the small subunit proteins individually and examining levels of ribosomal proteins in these cells relative to wild-type reference, some new and interesting correlations were found that could extend the network of ribosomal protein regulation (Sykes *et al.*, 2010b).

Surprisingly, in some operons the synthesis of ribosomal proteins is not coordinated. For example, *trmD* operon, that codes for S16, RimM, TrmD, and L19, was observed to have differential and noncoordinated expression (Wikström and Björk, 1988).

S10 and *spc* operons are the longest operons containing 11 and 12 genes respectively, S15 and L20 operons contain both two genes, but are the best described examples on molecular level. Repression mechanisms of these four operons have been studied thoroughly, but there are still many interesting questions to ask.

S10 operon contains 11 genes coding for ribosomal proteins. L4 is unique among the regulatory ribosomal proteins because it regulates not only translation but also transcription of S10 operon mRNA. Translation regulation results from inhibition of translation of the proximal gene of the S10 operon and consequent inhibitory effect to downstream genes via translational coupling (Freedman *et al.*, 1987). Transcriptional regulation results from L4 mediated premature termination of transcription within the S10 leader (Freedman *et al.*, 1987). The model of transcription inhibition suggested that NusA causes transcription pause at the attenuator site, L4 role is to stabilize the paused complex that leads to termination of transcription (Zengel and Lindahl, 1990, 1992). There is remarkable flexibility in the features of the 80 nucleotide region in S10 leader required for transcription control, suggesting there could be other determinants, perhaps multiple interaction between L4 and the paused transcription complex, to acquire high specificity (Zengel *et al.*, 2002). It was found that L4 can bind to the S10 leader in mRNA in the absence of NusA, the effect of NusA thus being not related to L4 binding (Stelzl *et al.*, 2003). After observing changes in the RNA leader structure induced by L4 binding, it was suggested that NusA might be required for propagation this signal to the RNA polymerase during the transcription termination event (Stelzl *et al.*, 2003). It has been proposed that regulation of ribosomal proteins by repressor is based on competition between rRNA and mRNA for those proteins and that the same structural features of the ribosomal proteins are used in their interaction with both rRNA and mRNA (Nomura *et al.*, 1980). Surprisingly, mutant forms of ribosomal protein L4 have been found, that fail to become incorporated into ribosome, but are functional in regulation of S10 operon and vice versa mutant

forms of L4, that are incorporated into 50S subunit, but fail to regulate S10 operon (Li *et al.*, 1996). It was suggested that the C-terminal part of L4 is necessary for regulation, but dispensable for ribosome incorporation, whereas a central region of L4 is required for ribosome incorporation, but is dispensable for regulation (Li *et al.*, 1996). Although the secondary structures of the mRNA and rRNA binding sites appear different, footprinting results indicate structural changes of the leader region upon L4 binding, and reveal structural similarities between the RNA and mRNA L4 binding sites (Stelzl *et al.*, 2003).

***Spc* operon** was first described as operon consisting of 10 ribosomal genes, but later 2 additional open reading frames coding for SecY and L36 were found (Cerretti *et al.*, 1983). Surprisingly it was found that majority of transcription originated in the *spc* operon continues into the alpha operon. It was suggested that alpha operon promoter activity is suppressed in exponentially growing cells when transcription from the upstream genes is strong (Cerretti *et al.*, 1983). *Spc* operon is feedback regulated by S8, which binds near translation initiation site of the third cistron, L5, and inhibits translation of L5 directly and that of the distal genes indirectly (Mattheakis and Nomura, 1988). It was suggested that inhibition of L5 synthesis leads to inhibition of distal protein synthesis because of translational coupling and because of decreased translation leads to increased mRNA degradation (Mattheakis and Nomura, 1988). It has been suggested that first two genes, encoding ribosomal proteins L14 and L24, are regulated by a mechanism called retroregulation (Mattheakis *et al.*, 1989). It was shown that L14 and L24 synthesis is regulated by S8 binding at the same site as in the case of L5 regulation, and it was suggested that retroregulation probably involves degradation of L14-L24 mRNA by 3′ to 5′ exonucleases (Mattheakis *et al.*, 1989). Comparison of the S8 binding site in *spc* operon mRNA and in 16S rRNA revealed striking similarities in both primary and secondary structure, however it was observed that S8 has 5 times higher affinity towards 16S rRNA (Gregory *et al.*, 1988). It was shown that a single bulged base is capable of modulation the affinity of RNA for S8 (Wu *et al.*, 1994). However, crystal structure for *E. coli* S8 bound to an RNA representing the autogenous regulation site within *E. coli spc* operon mRNA showed that two bulged nucleotides in the stem of the mRNA binding site had no effect on conformation of the parts of the RNA that interact with S8 (Merianos *et al.*, 2004). It was also demonstrated that the site to which the regulatory ribosomal protein binds in its mRNA is indeed similar to the site that it binds in 16S rRNA (Merianos *et al.*, 2004). Extensive modeling studies reveled large uncertainties: the structures suggested that the stem to which S8 binds is likely to impede translation initiation whether S8 is bound or not (Merianos *et al.*, 2004).

S15 operon consists of 2 genes coding for S15 and polynucleotide phosphorylase. The operon is regulated with S15 binding to its own mRNA. In 16S rRNA S15 contacts three sites, whereas site 1 and 2 are the main binding sites. Similarly, S15 binds to two sites in the mRNA in each organism studied. Reviewing structures important for S15 binding to its mRNA target in three

organisms and comparing S15 binding to mRNA and rRNA, it was suggested that each of them has found its own solution that is related to the way in which the protein binds to 16S rRNA (Springer and Portier, 2003). In *E. coli* mRNA site 2 resembles that of 16S rRNA, whereas in *Thermus thermophilus*, a site similar to site 1 of 16S rRNA is found in the mRNA; in *Bacillus stearothermophilus* site 2 contains motif found in 16S rRNA site 2 (Springer and Portier, 2003). The regulation mechanism appears to differ in the organisms studied. *E. coli* S15 traps a complex of 30S subunit, mRNA, and initiator tRNA in an inactive form *in vitro* (Philippe *et al.*, 1993). In *T. thermophilus* displacement mechanism with molecular mimicry model has been suggested: S15 triggers formation of the mRNA junction that partially mimics 16S rRNA and, in doing so, masks the ribosome-binding site (Serganov *et al.*, 2003). The affinity of *T. thermophilus* S15 for its mRNA target is 100-fold higher than that of *E. coli* S15 (Ehresmann *et al.*, 2004). In the case of *B. stearothermophilus* displacement mechanism was suggested (Scott and Williamson, 2005). While comparing the two mechanisms, it has been suggested that the entrapment mechanism only needs to stabilize unproductive initiation complex, and does not require high affinity. In contrast in the competition mechanism, the repressor should bind the mRNA with an affinity much higher than the 30S subunit, for efficient competition (Schlax and Worhunsky, 2003; Ehresmann *et al.*, 2004). Molecular details of the ribosome entrapment by mRNA-S15 complex in *E. coli* have been characterized using cryo-electron microscopy (Marzi *et al.*, 2007). It was found that in the stalled state, when the mRNA structure is blocked by repressor protein S15, the folded mRNA prevents the start codon from reaching the peptidyl-tRNA site in the ribosome (Marzi *et al.*, 2007). Upon repressor release, the mRNA unfolds and moves into the mRNA channel allowing translation initiation (Marzi *et al.*, 2007).

L20 operon consists of genes coding for L35 and L20. L20 represses directly translation of the first cistron and, through translational coupling, translation of its own gene (Haentjens-Sitri *et al.*, 2008). Double molecular mimicry has been described in the case of L20 (Guillier *et al.*, 2005a). It has been demonstrated that L20 recognizes two distinct sites in its leader mRNA in similar manner as its binding site in 23S rRNA (Guillier *et al.*, 2005a). Both sites are required for control, however, only one molecule of L20 binds to the L20 mRNA despite the presence of two potential binding sites (Guillier *et al.*, 2005a). L20 consist of globular C-terminal domain that sits on the surface of the large subunit, whereas the N terminal domain has an extended shape and penetrates deep into RNA-rich core of the subunit (Guillier *et al.*, 2005b). Surprisingly, it was found that N-terminal tail of L20 is important for ribosome assembly *in vivo*, but dispensable for autogenous control *in vivo* and *in vitro* (Guillier *et al.*, 2005b). It was shown with isolated C-terminal globular domain of L20 that translation inhibition of L20 operon is regulated by competition mechanism (Haentjens-Sitri *et al.*, 2008). L20 competes with the small ribosomal subunit during the two initial steps of translation *in vitro*: the formation

of a binary initiation complex between the 30S subunit and the mRNA and the formation of a ternary initiation complex between the 30S subunit, the mRNA, and the initiator tRNA (Haentjens-Sitri *et al.*, 2008).

2. Ribosome assembly

Majority of the earlier knowledge about ribosome assembly comes from *in vitro* ribosome studies, where ribosome is assembled from purified components in a process called reconstitution. However, *in vitro* reconstitution needs conditions that are nonphysiological: higher temperature and higher salt concentration are used to overcome kinetically trapped intermediates. Assembly *in vivo* is much faster and more efficient, as there are many other proteins involved besides ribosomal proteins. In spite of that, many findings from reconstitution experiments have proven to be true also *in vivo*, but many *in vivo* experiments have also shown us that assembly is much more complex than previously thought.

Ribosome assembly *in vivo* involves many processes that are occurring successively or in parallel. Ribosome assembly starts with rRNA transcription and the last steps are carried out on translating 70S ribosome. rRNAs are transcribed as a single transcript, rRNA processing, carried out by specific RNases, starts cotranscriptionally and is finished on mature 70S ribosomes (Kaczanowska and Rydén-Aulin, 2007). Similarly, modification of 23S and 16S rRNA starts cotranscriptionally by methyltransferases and pseudouridine synthases, last modification reaction being made on 70S ribosome (Kaczanowska and Rydén-Aulin, 2007; Ero *et al.*, 2008). Ribosomal proteins are synthesized in a highly coordinated manner, 10 proteins are known to contain posttranslational modifications (Kaczanowska and Rydén-Aulin, 2007). RNA secondary structures form very rapidly and partial folding of rRNA occurs cotranscriptionally, being assisted by the cotranscriptional binding of several ribosomal proteins (Kaczanowska and Rydén-Aulin, 2007). Many nonribosomal factors, called assembly factors or ribosomal maturation factors participate in ribosome assembly (Kaczanowska and Rydén-Aulin, 2007; Wilson and Nierhaus, 2007; Connolly and Culver, 2009). There are at least three classes of proteins that may help manage RNA folding: RNA chaperones, RNA helicases, and ribosome-dependent GTPases. In addition, rRNA modification enzymes can have functions in ribosome structure formation in addition to their role in rRNA chemical modification steps.

2.1. Assembly of 30S subunit *in vitro*

More than 40 years ago it was shown that functionally active 30S subunit can be reconstituted from 16S rRNA and free small subunit proteins (Traub and Nomura, 1968). Therefore it was suggested that the entire information for the

correct assembly of the ribosomal particles is contained in the structure of their components and not in some other nonribosomal factors (Traub and Nomura, 1968). Later it was demonstrated that functional subunits could be reconstituted *in vitro* using natural 16S rRNA and individual purified natural or recombinant ribosomal proteins (Held *et al.*, 1973; Culver and Noller, 1999). In addition, it has been shown that 16S rRNA, synthesized *in vitro* using T7 RNA polymerase, could be reconstituted into functional 30S subunit (Krzyzosiak *et al.*, 1987).

Based on *in vitro* reconstitution experiments performed with various combinations of the small subunit proteins, a model for 30S assembly, called "assembly map", was proposed (Mizushima and Nomura, 1970; Held *et al.*, 1974). The assembly map demonstrated that 30S subunit assembles in an ordered and cooperative manner *in vitro*. Original assembly map and changes introduced during 35 years have been thoroughly reviewed by G. M. Culver (Culver, 2003). According to assembly map proteins can divided into three: primary, secondary and tertiary binding proteins (Figure 2). The primary binding proteins (S4, S7, S8, S15, S17, and S20) bind directly and independently to 16S rRNA, and are thought to initiate folding of 16S rRNA (Culver, 2003). The secondary binding proteins (S5, S6, S9, S11, S12, S13, S16, S18, and S19) require at least one of the primary proteins, and the tertiary binding proteins (S2, S3, S10, S14, and S21) require at least one protein from both of the previous sets to be bound (Culver, 2003). It has been suggested that only two proteins (S4 and S7) can initiate 30S subunit assembly and each assembly initiator protein initiates its own assembly domain (Nowotny and Nierhaus, 1988). Based on reconstitution experiments it was suggested that there is a predominant 5′ to 3′ polarity to *in vitro* assembly, even though it is uncoupled from transcription (Powers *et al.*, 1993).

In vitro reconstitution of 30S subunit proceeds through three steps. At low temperatures (0–15º) a 21S reconstitution intermediate (RI) is formed, that lacks tertiary binding proteins. Next, temperature is elevated to about 40ºC and second reconstitution intermediate (RI*) is formed, that contains the same protein composition as RI, but sediments at 26S. Large conformational changes and compaction must take place to achieve activated RI*. Five late binding small subunit proteins bind to RI* completing the 30S subunit assembly. Large conformational changes from one intermediate to another which can be facilitated by increased temperature, changes in 16S rRNA architecture associated with this assembly pathway have been described in detail (Holmes and Culver, 2004; Holmes and Culver, 2005).

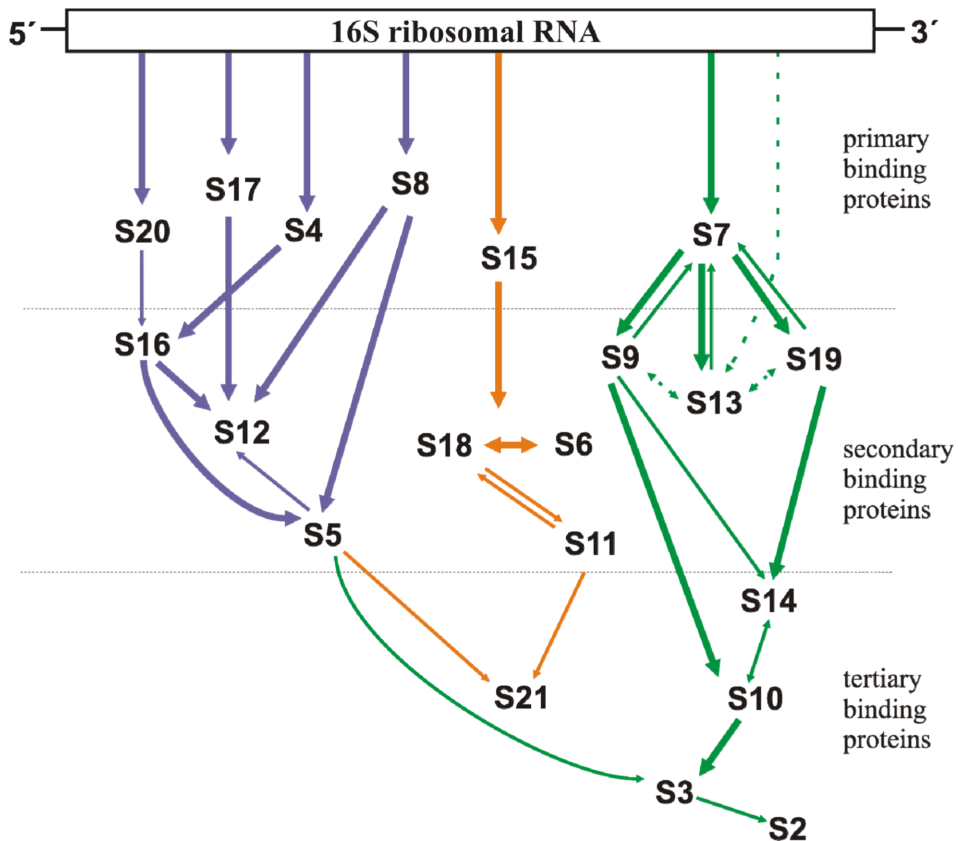

Figure 2. Assembly map of the 30S subunit. The arrows symbolize the direction dependence of ribosomal protein binding with the width symbolizing the strength of dependencies. Purple, orange and green arrows indicate 5´-domain, central domain and 3´-domain assembly proteins.

More recently new advanced technologies and clever experimental design have lead to interesting findings and new ideas concerning 30S assembly. It appears that *in vitro* assembly is more flexible and complex than previously thought. Interesting addition in the fields of 30S subunit self-assembly *in vitro* came from work of Megan Talkington and colleagues(Talkington *et al.*, 2005). They suggested that assembly proceeds by various local transitions in complex assembly landscape rather than a global rate-limiting conformational change (Talkington *et al.*, 2005). They used a method called PC/QMS (pulse-chase monitored by quantitative mass spectrometry) to measure the binding kinetics of the individual proteins during the assembly (Talkington *et al.*, 2005). The reconstitution was performed using 16S rRNA and uniformly ^{15}N-labelled 30S proteins. In various time points, binding of ^{15}N-proteins was chased with an excess of unlabelled (^{14}N) proteins (Talkington *et al.*, 2005). The ^{15}N/^{14}N ratio

for each protein from completely formed subunits was determined by mass spectrometry (Talkington *et al.*, 2005). It was found that the classic RI → RI* mechanism is not adequate to explain the rates and activation energies observed for binding of the individual proteins (Talkington *et al.*, 2005). It was suggested that the 21S particle formed in the low-temperature assembly reaction is a diverse collection of unstable particles (Talkington *et al.*, 2005). This model was called "assembly landscape" (Talkington et al. 2005).

It has been shown that 16S rRNA 5′ domain forms all the expected tertiary interactions in the absence of ribosomal proteins, demonstrating that the 5′ domain folds independently (Adilakshmi *et al.*, 2005). Based on these findings it was been suggested that the folding pathway of the rRNA guides the initial phase of RNA-protein interactions and establishes the structural platform for next steps in small subunit assembly (Adilakshmi *et al.*, 2005). By mapping changes in the structure of the rRNA within 20 milliseconds after the addition of all 30S subunit proteins it was discovered that nucleotides contacted by the same protein are protected at different rates, indicating that the initial RNA-protein contacts are reorganized during assembly (Adilakshmi *et al.*, 2008). For many nucleotides multiphase folding kinetics was observed: an initial fast RNA folding or protein binding event was followed by a second, slower event (Adilakshmi *et al.*, 2008). Interestingly, nucleotides with similarly fast back-bone protection did not map to single domains, but were distributed throughout the 16S rRNA, suggesting concurrent nucleation of assembly from many points along the rRNA in agreement with the "assembly landscape" model (Adilakshmi *et al.*, 2008).

Lately, an induced fit model has been used to describe RNA-protein recognition (Williamson, 2000). There are three basic classes of induced fit mechanism: the RNA undergoes a conformational change upon protein binding, but the conformation of protein is relatively unchanged; the protein undergoes significant change and the RNA undergoes little conformational change; mutually induced fit, where both the RNA and protein components change conformation (Williamson, 2000). While studying temperature-dependent conformational rearrangements in binary complexes of primary binding proteins with 16S rRNA at least two types of induced fit were observed: when only the RNA is changing its conformation after protein binding and, when both the RNA and the protein are changing conformation at binding (Dutcă *et al.*, 2007). An interesting dual role in ribosome assembly has been proposed for secondary binding protein S16, in addition to stabilizing the native conformation, S16 also destabilizes certain rRNA interactions at early stages of assembly (Ramaswamy and Woodson, 2009).

In 2010 it was suggested that individual domains of 16S rRNA have different assembly properties: assembly of the 5′ and central domain appears to be more plastic, while assembly of the 3′ major and minor domains is more complex (Xu and Culver, 2010). A set of rRNA nucleotides that are critical for small subunit assembly was defined and it was observed that changes in the 3′

domain are least tolerated (Xu and Culver, 2010). Still assembly between different regions of 16S rRNA was found to be interdependent, both within a domain and across domains (Calidas and Culver, 2011). For example, the assembly of the junction of helices 25–26a appears to be dependent upon the establishment of appropriate structure at the junctions of helices 20–23, also the orientation of the neck is dependent upon the assembly of the head and body (Calidas and Culver, 2011).

Evidence for parallel assembly pathways during *in vitro* reconstitution was visualized using single particle electron microscopy image analyzes that can resolve heterogeneous population of molecules and classify them into homogeneous subpopulations (Mulder *et al.*, 2010). 30S reconstitution was stopped at time points ranging from 0–120 min, 14 distinct assembly intermediates were identified, composition of individual particles were determined by 3D difference mapping, comparison to the x-ray crystal structure, and subcomplex reconstitution experiments (Mulder *et al.*, 2010).

There have been attempts to determine cotranscriptional protein addition to the rRNA in ribosome assembly *in vivo*. Bacterial folded chromosomes, which contain nascent ribosomal RNA, were isolated and analyzed for the presence of ribosomal proteins (de Narvaez and Schaup, 1979). The protein binding order to the ribosome characterized *in vivo* was found to be similar to *in vitro* assembly map, but there were also some differences (de Narvaez and Schaup, 1979). The ribosome assembly plasticity has been characterized and proved by investigating S15 deletion strain (Bubunenko *et al.*, 2006). S15 is a primary binding protein that is required for S6, S18, S11 and S21 binding in 30S subunit assembly *in vitro* (Held *et al.*, 1974). However, deletion of the gene encoding S15, *rpsO*, yields a viable *E .coli* strain, demonstrating that functional ribosomes lacking S15 are able to assemble *in vivo* (Bubunenko *et al.*, 2006).

In conclusion, based on the 30S model system, it appears that ribosome assembly can follow multiple pathways and contain several different precursors, rather than one discrete rate-limiting step and well-defined intermediate, as previously observed.

2.2. Assembly of 50S subunit *in vitro*

The assembly of the large subunit is much more complex than the assembly of the small subunit. As the 23S rRNA is almost twice the size of the 16S rRNA, it binds nearly twice as many proteins, and it must form correct interactions with 5S rRNA. This is the main reason why 30S subunit has been chosen as a model in studies of ribosome assembly and relatively less is known about 50S subunit assembly.

Assembly map by Herold and Nierhaus published in 1987 shows that assembly of the large subunit is not organized by structural domains so clearly as assembly of the 30S subunit and has many more proteins with more complex

binding hierarchy (Herold and Nierhaus, 1987). 50S subunit assembly map is shown in figure 3.

50S reconstitution requires four steps with different reaction conditions (Herold and Nierhaus, 1987). First 23S rRNA, 5S rRNA and subgroup of proteins are incubated at 0ºC with 4 mM Mg^{2+}, which results in $RI_{50}(1)$ particle that sediment at 33S. Next temperature must be increased to 44ºC to enable conformational change that leads to formation of $RI^*_{50}(1)$ sedimenting at 41S. The addition of remaining proteins results in formation of 48S particle, $RI_{50}(2)$. Incubation at 50ºC with 20 mM Mg^{2+} leads to formation of 50S subunit.

According to the assembly map 20 of large subunit proteins can bind to the naked 23S rRNA *in vitro*, however only L24 and L3 were shown to initiate the assembly process (Nowotny and Nierhaus, 1982). Surprisingly, a temperature-sensitive mutant which lacks the assembly initiator protein L24 was described (Dabbs, 1982). Later it was shown that L20 can replace L24 for the initiation of assembly at permissive temperatures *in vitro* (Franceschi and Nierhaus, 1988).

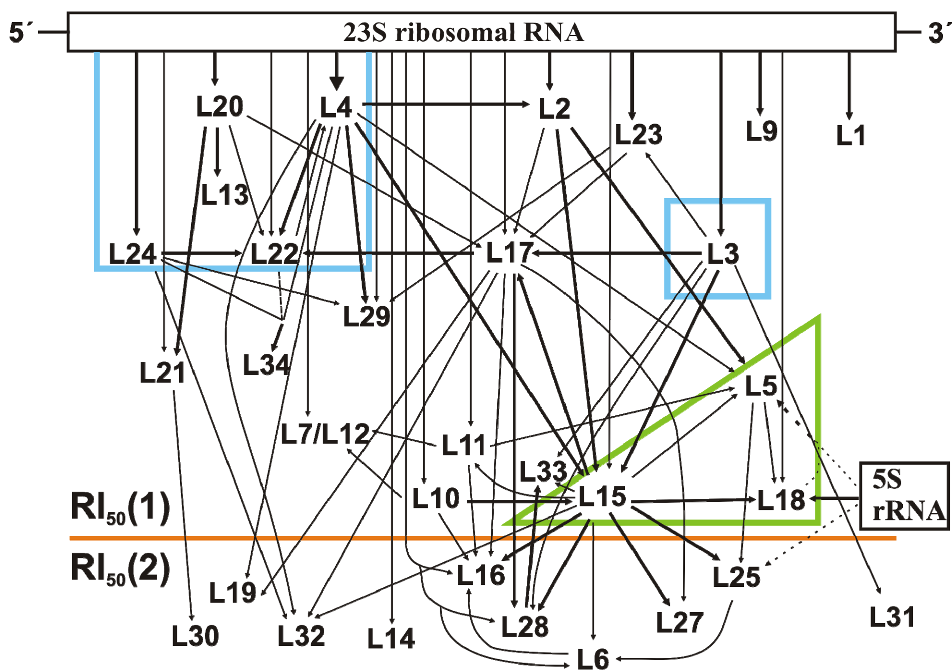

Figure 3. Assembly map of the 50S subunit. The arrows symbolize the direction of dependence of ribosomal protein binding with the width symbolizing the strenght of the dependence. The protein boxed with blue line are required for RI50* formation. Proteins in green triangle are important for mediating the binding of 5S rRNA to 23S rRNA.

6

3. rRNA modification during ribosome assembly

It is known that modification of rRNA starts cotranscriptionally and last modifications are carried out on mature 70S ribosome (Kaczanowska and Rydén-Aulin, 2007; Connolly *et al.*, 2008; Ero *et al.*, 2008). 16S rRNA contains 11 and 23S rRNA contains 25 modified nucleosides, most of these modifications are located in functionally important regions of the ribosome (Figure 4).

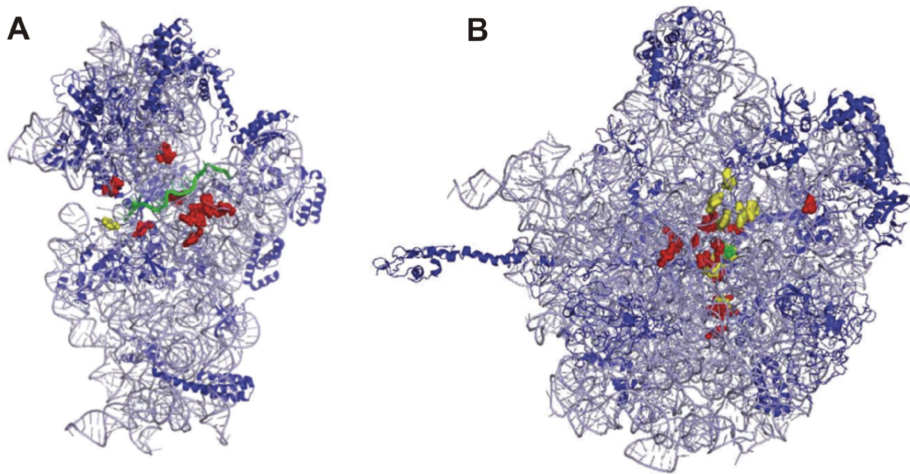

Figure 4. Methylation(red) and pseudouridinylation (yellow) sites of the E. coli small (A) and large (B) ribosomal subunits. rRNA and proteins are shown as ribbons in light and dark blue, respectively. In (A) a green ribbon indicates the path of the mRNA through the small subunit, whereas in (B) the antibiotic chloramphenicol (green) acts as reference for peptidyltransferase centre on the large subunit (adapted from Wilson and Nierhaus, 2007).

There have been many attempts to find out if a certain modification enzyme works in early, middle or late stages of ribosome assembly. So far most of the data comes from *in vitro* modification experiments, with only few exceptions. Conclusions from both *in vitro* and *in vivo* experiments are gathered in table 1. As seen from table 1, modifications occur mostly late in 30S subunit assembly and early in 50S subunit assembly. For most of 30S modification enzymes the substrate in ribosomal assembly has been characterized as opposed to the enzymes modifying the large subunit of which about half have a substrate in assembly characterized.

Tabel 1. Modified nucleosides in *Escherichia coli* rRNAs and the specificities of the corresponding enzymes

Modi-fication	Enzyme	Stage of assembly	Comment	Reference
16S rRNA				
Ψ516	RsuA	Intermediate	Some proteins	(Wrzesinski *et al.*, 1995)
m^7G527	RsmG	Late	30S	(Okamoto *et al.*, 2007)
m^2G966	RsmD	Late	Requires S7 and S19	(Weitzmann *et al.*, 1991)
m^5C967	RsmB	Late	Blocked by S7 and S19	(Weitzmann *et al.*, 1991)
m^2G1207	RsmC	Late	30S	(Tscherne *et al.*, 1999)
m^4C1402	RsmH	Late	30S	(Kimura and Suzuki, 2010)
Cm1402	RsmI	Late	30S	(Kimura and Suzuki, 2010)
m^5C1407	RsmF	Late	30S	(Andersen and Douthwaite, 2006)
m^3U1498	RsmE	Late	30S	(Basturea and Deutscher, 2007)
m^6_2A1518	RsmA	Late	30S *in vitro*	(Poldermans *et al.*, 1979)
m^6_2A1519		Late	30S *in vivo*	(Connolly *et al.*, 2008)
23S rRNA				
m^1G745	RlmA	Early	23S rRNA	(Hansen *et al.*, 2001)
m^6A1618	RlmF	Intermediate	3,5M LiCl particle	(Sergiev *et al.*, 2008)
m^2G1835	RlmG	Early	23S rRNA	(Sergiev *et al.*, 2006)
Ψ1911	RluD	Late	50S *in vitro*	(Vaidyanathan *et al.*, 2007)
Ψ1915		Late	50S *in vivo*	(Leppik *et al.*, 2007)
Ψ1917				
$m^3\psi1915$	RlmH	Late	70S	(Ero *et al.*, 2008)
m^5C1962	RlmI	Early	23S rRNA	(Purta *et al.*, 2008b)
m^2G2445	RlmL	Early	23S rRNA	(Lesnyak *et al.*, 2006)
Cm2498	RlmM	Early	23S rRNA	(Purta *et al.*, 2009)
Um2552	RlmE	Late	50S, 70S	(Bügl *et al.*, 2000; Caldas *et al.*, 2000a)

3.1. 16S rRNA modification during ribosome assembly

The substrate specificity of modification enzymes has been mostly studied by cell-free experiments using purified enzymes in simple experiments with 16S rRNA or 30S subunit from modification deletion strains tested as substrates. However it is possible that in cell many of these enzymes have different substrates as the activity of some enzymes studied *in vitro* is quite low. Possibly some other factors can be important during assembly that help to prepare the right substrate for modification enzyme.

In the case of RsuA that isomerizes U516 of 16S rRNA to pseudouridine it was shown that the enzyme is inactive on the free 16S rRNA or 30S subunits, the preferred substrate being an RNA fragment containing residues from 1 to 678 which has been complexed with 30S ribosomal proteins, suggesting that the

modification event occurs during intermediate stages of ribosome assembly (Wrzesinski *et al.*, 1995).

RsmG is able to methylate 70S ribosomes and 30S subunits, but not naked 16S rRNA, implying that the enzyme requires 16S rRNA to be properly folded or certain protein to be assembled to 16S rRNA for efficient reaction (Okamoto *et al.*, 2007).

Similar experiments to find out preferred substrates for modification enzymes have been carried out with RsmC (m^2G1207), RsmH (m^4C1402), RsmI (Cm1402), RsmF (m^5C1407) or RsmE (m^3U1498) RsmA ($m^6{}_2$A1518, $m^6{}_2$A1519) all showed preference towards 30S subunit compared to 16S rRNA suggesting that all of these enzymes work in later steps of 30S subunit assembly (Poldermans *et al.*, 1979; Tscherne *et al.*, 1999; Andersen and Douthwaite, 2006; Basturea and Deutscher, 2007; Kimura and Suzuki, 2010).

More surprisingly it has been found in reconstruction experiments that the presence of ribosomal proteins S7 and S19 plays an important and opposite roles when two neighboring nucleosides G966 and C967 are modified (Weitzmann *et al.*, 1991). m^5C967 formation is an early event in ribosome assembly as it can occur on free rRNA but is blocked by the time S7 and S19 are added. In contrast, m^2G966 formation is a relatively late event in ribosome assembly requiring the incorporation of S7 and S19 (Weitzmann *et al.*, 1991). It was proposed that S19 shuts down C967 methylation by altering the conformation of the stem-loop structure containing C967 so that it no longer can be recognized by the methyltransferase (Weitzmann *et al.*, 1991).

In the case of RsmA (also known as KsgA), the most thoroughly studied and universally conserved 16S rRNA modification enzyme, results from *in vivo* experiments are available. Quite recent *in vivo* studies suggest that KsgA functions as a late assembly ribosome binding factor and that the methylation triggers release of KsgA from the assembling subunit, allowing it to finally mature and enter the translation, KsgA mediated methylation can be a checkpoint in 30S subunit assembly (Connolly *et al.*, 2008). KsgA and initiation factor 3 compete for overlapping binding sites on the 30S subunit (Xu *et al.*, 2008). KsgA binding prevents incompletely assembled 30S subunit from engaging in translation initiation prior to methylation by KsgA (Xu *et al.*, 2008). However, in 2011 Desai et al. proposed very interesting model; KsgA binds pre-16S rRNA at some early to intermediate time point in the ribosome biogenesis cascade and awaits a late assembly event, which is governed by the close approach of helix 45 and specifically A1518 and A1519 to the active site of KsgA (Desai *et al.*, 2011). From the X-ray crystal structure of the 30S subunit from a *ksgA* deletion strain of *Thermus thermophilus*, it was suggested that methylation by KsgA plays an additional direct role in establishing a fully active 30S subunit conformation (Demirci *et al.*, 2010). The authors conclude that the methylation facilitates the formation of a packing interaction between helix 45 and helix 44 in the vicinity of the decoding site (Demirci *et al.*, 2010). Loss of this packing interaction perturbs surrounding rRNA structure in both the

A and P sites of the ribosome (Demirci *et al.*, 2010). In addition to its rRNA adenine methyltransferase activity, KsgA was demonstrated to contain a novel DNA glycosylase activity to repair C/oxidized T-mispairs in DNA that prevents mutations (Zhang-Akiyama *et al.*, 2009).

3.2. 23S rRNA modification during ribosome assembly

Five out of nine 23S rRNA modification enzymes, RlmA (m^1G745), RlmG (m^2G1835), RlmI (m^5C1962), RlmL (m^2G2445), and RlmM (Cm2498), are reported as early assembly factors according to the *in vitro* methylation experiments, as they can methylate 23S rRNA but not purified 50S particles or 70S ribosomes (Hansen *et al.*, 2001; Lesnyak *et al.*, 2006; Sergiev *et al.*, 2006; Purta *et al.*, 2008b, 2009).

RlmF (m^6A1618) is the only 23S rRNA modification enzyme studied so far that appears to work in intermediate stage of ribosome assembly, as protein-free 23S rRNA as well as 50S subunit are poor substrate for this enzyme (Sergiev *et al.*, 2008). However, RlmF could efficiently methylate A1618 of the LiCl core particles that contain only 23S rRNA and proteins L2, L3, L4, L13, L17, L20, L21, L22, L23, L29 and L34 (Sergiev *et al.*, 2008).

Another interesting 23S rRNA modification enzyme, RlmE (Um2552), has been suggested to have an important role in ribosome assembly, because *rlmE* deletion strain shows severe ribosome assembly defect (Bügl *et al.*, 2000). *In vitro* methylation studies showed that RlmE was unable to methylate 23S rRNA or 40S ribosomal particles from *rlmE* deletion strain, but methylated efficiently mature 50S subunit from the same strain (Bügl *et al.*, 2000). In addition methylation of 70S ribosomes has been demonstrated (Caldas *et al.*, 2000a). *RlmE* deletion strain shows slow growth, higher sensitivity to lincosamycin, decreased protein synthesis rate and increased translational accuracy (Caldas *et al.*, 2000b; Widerak *et al.*, 2005).

There are 11 pseudouridine modifications in *Escherichia coli* 23S rRNA but not much is known about their relation to the ribosome assembly. Only one of these pseudouridine synthases has been studied in more details, namely RluD, that converts uridines at positions 1911, 1915, and 1917 of 23S rRNA to pseudouridines. Analysis of the pseudouridylation pattern in ribosome assembly precursor particles has shown that these three pseudouridines are formed by RluD during late assembly (Leppik *et al.*, 2007). With *in vitro* experiments it has been shown, that the best substrate for RluD is 50S subunit (Vaidyanathan *et al.*, 2007). The RluD activity on free 23S RNA is at least fivefold lower and is likely nonspecific (Vaidyanathan *et al.*, 2007). Low level activity was observed also in 70S ribosome (Vaidyanathan *et al.*, 2007). Inactivation or deletion of *rluD* has been reported to have effects on cell growth, 50S subunit assembly, subunit association, and elevated levels of stop codon readthrough (Gutgsell *et al.*, 2005; Ejby *et al.*, 2007). However, recent study by O'Connor and Gregory demonstrate that the slow growth, impaired subunit association,

and increased readthrough phenotypes associated with *rluD* inactivation are limited to *Escherichia coli* K-12 strain carring a mutant RF2 protein (O'Connor and Gregory, 2011).

One of the pseudouridines made by RluD is further methylated by RlmH (Kowalak *et al.*, 1996; Ero *et al.*, 2008; Purta *et al.*, 2008a). *In vitro* studies have shown that RlmH can methylate 50S subunits and 70S ribosomes (Ero *et al.*, 2010). Methylation of 70S ribosomes is faster, suggesting that 70S could be the preferred substrate also *in vivo* (Ero *et al.*, 2010). This finding is supported by docking data of the RlmH crystal structure into the 70S ribosome showing extensive contacts of RlmH with both ribosome subunits (Purta *et al.*, 2008a). It has been suggested that methylation of pseudouridine 1915 by RlmH is very likely the last modification incorporated into ribosomes (Ero *et al.*, 2010).

Interestingly most 16S rRNA modification enzymes work late in ribosome assembly and most 23S rRNA modification enzymes seem to work early in ribosome assembly. The reason could be that the 30S subunit is smaller and has simpler structure as compared to the 50S subunit, which is more complex. It is evident from crystal structures that many nucleotides are not accessible later in the 50S assembly path.

4. Antibiotics and ribosome assembly

In addition to the extremely important role in medicine, antibiotics have been very useful in studies on ribosomal structure and function. Of the antibiotics targeting the large ribosomal subunit erythromycin and chloramphenicol are among the most thoroughly studied. These antibiotics inhibit ribosomal activity and also ribosome assembly. In next chapters of the thesis short overview of chloramphenicol and erythromycin action mechanisms will be given, followed by a review on the effects of antibiotics on ribosomal assembly.

4.1. Chloramphenicol and erythromycin

Chloramphenicol was originally isolated from *Streptomyces venezuele* in 1947 and displays a broad-spectrum activity, inhibiting a wide range of Gram-positive and Gram-negative bacteria, but not eukaryotes (Schwarz *et al.*, 2004; Wilson, 2009). The relative simplicity of chloramphenicol structure (Figure 5A) made one of the first antibiotics to be marketed as the product of chemical synthesis and has been produced this way since 1950 (Schwarz *et al.*, 2004).

Chloramphenicol binds directly to the A-site on the 50S ribosomal subunit, occupying the same location as the aminoacyl moiety of an A-site tRNA (Tu *et al.*, 2005). Seven highly conserved nucleotides (G2061, A2451, A2503, U2504, G2505, U2506, and U2585) comprise the chloramphenicol binding site (Schlünzen *et al.*, 2001; Wilson, 2009). Chloramphenicol can potentially form

hydrogen bond interaction with the base of G2061 and the ribose of G2505 (Schlünzen *et al.*, 2001; Wilson, 2009). Recent findings with chloramphenicol bound to *E. coli* ribosomes suggest that the nitrobenzene ring of chloramphenicol is stacked on C2452 and one chlorine atom in chloramphenicol is in a position to contact the exocyclic amine of A2062 (Dunkle *et al.*, 2010).

Addition of chloramphenicol to growing bacterial cells blocks the ribosomes on the mRNA and protects the peptidyl-tRNA from hydrolysis, enabling the visualization of polysomes on sucrose gradients (Wilson, 2009). It has been suggested that P-tRNAs bearing bulky aromatic side chains are less prone to inhibition than P-tRNAs bearing smaller or charged amino acids, such as glycine or lysine (Wilson, 2009). Chloramphenicol has also been shown to influence translational accuracy, promoting stop codon readthrough and frameshifting (Thompson *et al.*, 2004).

The first and still most frequently encountered mechanism of bacterial resistance to chloramphenicol is enzymatic inactivation by acetylation of the drug via different types of chloramphenicol acetyltransferases (Schwarz *et al.*, 2004). There are also other mechanism of chloramphenicol resistance, such as efflux systems, inactivation by phosphotransferases, mutations of the target site and permeability barriers (Schwarz *et al.*, 2004). Methylation of A2503 by the methyltransferase Cfr is sufficient to confer resistance to chloramphenicol in *E. coli* (Kehrenberg *et al.*, 2005). It was suggested that methyltransferase Cfr confers resistance by methylating m^2A2503, resulting in m^2m^6A2503, and thereby perturb drug binding (Kehrenberg *et al.*, 2005). Mutation that confer resistance to chloramphenicol are generally located in the binding site, e.g. 2451, 2503 and 2504, or are located relatively close by, such as 2447 and 2452 (Wilson, 2009).

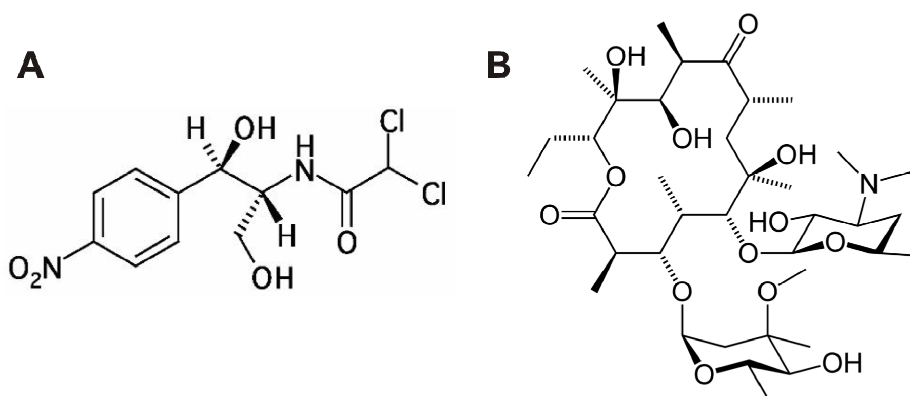

Figure 5. Structures of chloramphenicol (A) and erythromycin (B).

Erythromycin (Figure 5B) belongs to the macrolide class of antibiotics. Macrolides represent a large class of polyketide compounds, that are composed of a 12-member to 16-member macrolactone ring decorated with various amino-sugars (Katz and Ashley, 2005; Tenson and Mankin, 2006; Mankin, 2008; Wilson, 2009). In nature macrolides are synthesized by actinomycetes and inhibit eubacterial, but not archaeal nor eukaryotic, protein synthesis (Wilson, 2009). The inhibition of protein synthesis results from the rapid drop-off of the peptidyl-tRNA from the ribosome during early rounds of translation (Tenson *et al.*, 2003).

Multiple structures of a variety of macrolides bound to bacterial and archaeal large ribosomal subunit reveal a common binding model, such that the general orientation and conformation of the lactone ring and C5-sugar is placed to establish interactions with A2058 and A2059 of the 23S rRNA (Schlünzen *et al.*, 2001; Hansen *et al.*, 2002; Tu *et al.*, 2005; Wilson *et al.*, 2005; Bulkley *et al.*, 2010; Dunkle *et al.*, 2010). The macrolides binding pocket consist of RNA from domains II, IV, and V, with the majority of the pocket being composed of residues from domain V (Katz and Ashley, 2005). There are some stabilizing contributions to the binding pocket from ribosomal proteins L3, L4, L22, and L24 (Katz and Ashley, 2005). The binding pocket lies in the peptide exit tunnel 10–15 Å distal from the peptidyltransferase site; macrolide binding appears to block progression of the peptide chain upon contact between the growing peptide chain and macrolide (Katz and Ashley, 2005). Different macrolides leave different amounts of space available for the newly synthesized peptide, for example, erythromycin starts inhibiting translation only when the *nascent* peptide is at least six amino acid long (Tenson *et al.*, 2003). Surprisingly, it has been proposed recently that at least some nascent peptides are able to squeeze through the opening left by the macrolide molecule in the exit tunnel (Mankin, 2008).

Resistance to erythromycin was first reported in 1952, the same year erythromycin was introduced into clinical practice (Katz and Ashley, 2005). N6 mono- or dimethylation of A2058, as well as mutation of A2058/9, reduce the affinity of the drug for the ribosome and confer resistance to macrolides (Katz and Ashley, 2005; Poehlsgaard and Douthwaite, 2005; Tenson and Mankin, 2006; Wilson, 2009). Dimethylation of A2058 is carried out by Erm-type methyltransferases and would lead to steric clashes with the C5-mono-saccharide, whereas mutation to G would encroach on the binding position of lactone ring (Wilson, 2009). Resistance to macrolides also arises from alteration in ribosomal proteins L4 and L22 (Chittum and Champney, 1994; Gregory and Dahlberg, 1999). Resistance to macrolides can also be achieved with specialized or broad-spectrum drug-efflux transporters that can efficiently reduce the intracellular drug concentration.

Macrolide resistance can also be attained with specific short peptides that act *in cis* on the ribosome on which they have been translated (Tenson and Mankin, 2001). In the case of erythromycin optimal length of resistance peptide was

observed to be 5–6 amino acids (Tenson *et al.*, 1997). “Bottle brush” model of the peptide action has been suggested (Tenson and Mankin, 2001). The ribosome with bound macrolide can still translate resistance peptide, as macrolides do not block the translation initiation or first few rounds of elongation. The N-terminal segment of the peptide reaches the site where antibiotic is bound and during termination of peptide synthesis, or during last translocation event, the peptide “kicks” the drug out of its binding site (Lovmar *et al.*, 2006). Once the antibiotic is removed the ribosome can translate cellular proteins, if it has time to polymerize the first 4–6 amino acids, then the binding site will be occupied by the nascent polypeptide and the ribosome can successfully finish translation of the protein (Lovmar *et al.*, 2006).

4.2. Effect of chloramphenicol on ribosome assembly

In 1959 Nomura and Watson observed that in the cell grown in the presence of high concentration of chloramphenicol large amount of rRNA is accumulating as incomplete ribosomal particles, so-called „chloramphenicol particles“ (Nomura and Watson, 1959). rRNA/protein ratio in these particles is variable and usually higher than the same ratio in mature ribosome (Nomura and Watson, 1959; Nomura and Hosokawa, 1965; Hosokawa and Nomura, 1965; Yoshida and Osawa, 1968).

After removal of chloramphenicol, a major part of the RNA contained in “chloramphenicol particles” is converted into RNA in normal ribosomes without extensive breakdown, although some of the RNA contained in the “chloramphenicol particles” is converted to ribosomes through breakdown and re-utilization (Nomura and Hosokawa, 1965). It was suggested by Nomura and Hosokawa, that breakdown of some of the “chloramphenicol particles” after the removal of chloramphenicol may be due to unstable characteristics of those particles, and the particles are normal precursors of the 50S subunit (Nomura and Hosokawa, 1965).

Chloramphenicol particle size, sedimentation, and quantity are dependent of chloramphenicol concentration in culture. in *E. coli* the effect of various concentrations of chloramphenicol (0 to 1000 μg/ml) on ribosome formation was studied thoroughly by Osawa and others (Osawa *et al.*, 1969). It was found that chloramphenicol at concentrations above 3 μg/ml inhibited ribosome formation completely and considerable amounts of the typical 18S and 25S “chloramphenicol particles” were found in cell extract (Yoshida and Osawa, 1968). At chloramphenicol concentrations between 0.6 and 1.5 μg/ml, various ribonucleoprotein particles of sedimentation coefficients higher than typical chloramphenicol particles were detected in the cell extracts together with some mature 50S and 30S subunits (Osawa *et al.*, 1969). Chloramphenicol at concentrations below 0,5 μg/ml allowed the formation of mature ribosomes although small amounts of immature particles were still detected with 0.5 μg/ml of chloramphenicol (Osawa *et al.*, 1969).

In the presence of low concentration of chloramphenicol (0,8 µg/ml) 4 peaks ware present in sedimentation analyses in addition to 70S ribosomes. 50S and 40S peaks both contained 23S rRNA, 30S region contained both 23S and 16S rRNA suggesting that this fraction is heterogeneous, 22S peak contained 17S rRNA (Osawa *et al.*, 1969). By further fractionation it was observed that 30S region consists of 3 components; firstly 28S to 30S particles containing 23S rRNA, secondly 26S particles containing 17S rRNA and thirdly 30S subunits (Osawa *et al.*, 1969). It was concluded, that 30S and 40S peaks each represent a heterogeneous population of particles having different composition but similar sedimentation properties (Osawa *et al.*, 1969). These so called "low chloramphenicol particles" are convertible to 50S and 30S ribosomal subunits (Osawa *et al.*, 1969). 30S and 40S particles were found to contain 5S rRNA in 30% as compared to 50S ribosomes (Osawa *et al.*, 1969). It was also observed that the extent of methylation of 23S rRNA component of the 30S and 40S particles was about 60% of the 23S rRNA derived from mature 50S ribosomal subunit (Osawa *et al.*, 1969).

Sykes et al showed that particles accumulating in the presence of quite high concentration of chloramphenicol (50 µg/ml) contain mature and precursor form of 16S and 23S rRNA, the proteins in the particle are entirely ribosomal and the number of ribosomal proteins incorporated increases as the sedimentation coefficient increases (Sykes *et al.*, 1977). Therefore they suggested that the particles are arrested ribosome precursors and not artefacts of preparation, as previously thought (Sykes *et al.*, 1977).

Hosokawa and Nomura observed accumulation of ribosomal particles with similar properties as the chloramphenicol particles also in puromycin-treated cells; they concluded that the formation of incomplete ribosomes having the properties of "chloramphenicol particles" is not restricted to inhibition of protein synthesis by a particular antibiotic (Hosokawa and Nomura, 1965) Similar assembly defects have been also observed for example in the case of streptomycin (Dubin, 1964), chlortetracycline (Holmes and Wild, 1967) and virginiamycin (Cocito, 1979).

In conclusion, in the presence of chloramphenicol or some other protein synthesis inhibitors precursors to both subunits are accumulating. Size, sedimentation properties and quantity of these particles are dependent on the drug concentration in the culture.

4.3. Effect of antibiotics on synthesis of ribosomal components

It was observed already in 1960s that the treatment of cells with chloramphenicol or other protein synthesis inhibitors stimulates rRNA synthesis (Kurland and Maaloe, 1962). Growing cells at 25ºC in minimal medium and incubating cells for one generation with 0, 1, 10 and 100 µg/ml of chloramphenicol in the presence of [^{14}C]uracil, Kurland and Maaløe observed that the initial rate of RNA synthesis is 15–20 times greater than that of control cells

(Kurland and Maaloe, 1962). In the medium supplemented with amino acids very little acceleration of RNA synthesis (5–15%) was seen (Kurland and Maaloe, 1962), as also supported by later work (Midgley and Gray, 1971). The concentration-dependent effect of chloramphenicol was studied more thoroughly using antibiotic concentrations ranging from 0 to 1000 μg/ml (Kurland and Maaloe, 1962). The acceleration effect of chloramphenicol was seen at lower concentrations of the drug, but at the higher concentrations of the drug the amount of RNA synthesis was decreased (Kurland and Maaloe, 1962). Using approximately 50 μg/ml of chloramphenicol a constant increase of 50 to 60% was reached (Kurland and Maaloe, 1962).

Shen and Bremer tried to characterize the changes in the synthesis of rRNA, tRNA and mRNA in the presence of chloramphenicol (Shen and Bremer, 1977). A significant (1,8 to 8-fold) stimulation of rRNA and tRNA synthesis was observed in the presence of high drug concentration (100 μg/ml) in every growth medium tested (Shen and Bremer, 1977). The stimulation decreased with increasing growth rate of the bacteria measured before the addition of chloramphenicol (Shen and Bremer, 1977). Addition of chloramphenicol always produced an increase in the fraction of rRNA and tRNA synthesis and a corresponding decrease in the fraction of mRNA synthesis (Shen and Bremer, 1977). It has been also observed that half of the mRNA produced in drug-inhibited cultures was unstable (Midgley and Gray, 1971) showing that some of the overproduced RNA ends up in ribosomes and some is degraded.

As quantification and identification of proteins was difficult, not so much information about the effects of chloramphenicol on ribosomal protein synthesis was available in early years. P.P. Dennis Used various concentrations (0, 0,2, 1, 4 and 20 μg/ml) of chloramphenicol to study the effects of chloramphenicol on the transcriptional activities of ribosomal RNA and ribosomal protein genes (Dennis, 1976). During steady-state growth r-proteins represent about 13% of total protein synthesis rate while in the presence of more than 1 μg/ml of chloramphenicol complete and mature r-proteins account for 25 to 40% of the residual protein synthesis (Dennis, 1976). Although ribosomal operons were not completely characterized by 1976 P. P. Dennis observed that in chloramphenicol-treated (4 μg/ml) cells proximal genes in ribosomal protein transcription units had a significantly greater synthesis rate than most other ribosomal proteins (Dennis, 1976).

Similar results were obtained by Dodd et al.; using chloramphenicol concentration ranging from 0,4 μg/ml to 4 μg/ml differential accumulation of almost all r-proteins was stimulated (Dodd *et al.*, 1991). 0.4 μg/ml chloramphenicol, which inhibited total protein synthesis by about 40%, stimulated slightly accumulation of r-proteins compared to control cells (Dodd *et al.*, 1991). However, when chloramphenicol concentrations were increased above 1 μg/ml, ribosomal protein accumulation started to become inhibited, reaching 88% inhibition at 4 μg/ml chloramphenicol (Dodd *et al.*, 1991).

In conclusion, in the presence of chloramphenicol RNA synthesis is stimulated. The level of increase in RNA synthesis is dependent of drug concentration and is more pronounced in cells with slower growth. Secondly, in the presence of chloramphenicol differential accumulation of ribosomal proteins is stimulated, this effect is also dependent of chloramphenicol concentration. Unbalanced synthesis of rRNA and ribosomal proteins as well as differential synthesis of individual ribosomal proteins is the main reason for accumulation of chloramphenicol particles. In other words, ribosome assembly is inhibited in cells treated with chloramphenicol due to protein synthesis inhibition.

4.4. Direct effect of antibiotics on ribosome assembly

Over last 15 years more than 30 papers have been published by Scott Champney and coworkers to introduce a new and alternative theory about antibiotic effects on ribosome assembly. They have found over 40 drugs that inhibit 50S subunit assembly specifically and 3 drugs that affect 30S subunit assembly specifically. The effects of antibiotics were studied in six different species: *Escherichia coli*, *Bacillus subtilis*, *Staphylocuccus aureus*, *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Streptococcus pyogenes*; while most work has been carried out in *S. aureus* and *E. coli.* In addition, different strains more sensitive or on the contrary resistant to some antibiotics were studied. The different organisms, strains, antibiotics and antibiotic concentrations studied are reviewed in additional tables 1 and 2.

According to 50S assembly inhibition model, antibiotic can bind to a precursor of the subunit and stop the assembly process. The incomplete intermediates are degraded by the activity of ribonucleases and proteases and 50S particle formation is prevented (Champney, 2003). Similar model was suggested for 30S assembly inhibition by aminoglycosides (Mehta and Champney, 2002).

All this extensive work was carried out to find antibiotics that target ribosome assembly more strongly than translation and to find structural elements needed for ribosomal assembly inhibition to enable the design of novel antimicrobial agents (Champney, 2003). Champney suggested that the compounds which preferentially inhibit particle assembly over translation would very likely be bactericidal agents for most cells (Champney, 2003).

4.4.1. Methods used to examine the direct effect of antibiotics on ribosome assembly

To study antibiotic effect on ribosome assembly Champney and colleagues have developed and improved a set of experimental procedures that are somewhat different from previously used methods (Champney, 2003).

Most often a four component experimental procedure is used which includes measurements of cell growth rates, viable cell numbers, ^{35}S-amino acid incorpo-

ration into proteins and ^{3}H-uridine labeling of subunits. Cells growing with and without an antibiotic are labeled with ^{3}H-uridine and after 2 cell doublings the isotope is chased with unlabelled uridine; ^{35}S-Methionine is added to measure protein synthesis rates and cell samples are diluted and plated to measure viable cell numbers; growth rates are determined by the increase in cell density (Champney, 2003). Over a concentration range for a given antibiotic, IC50 measurements are made for each of four parameters listed. The IC50 compares the antibiotic concentration needed to give 50% inhibition of the process measured.

A second method frequently used by this group involves determining the kinetics of particle formation. Pulse and chase labeling of cells with ^{3}H-uridine has been used to examine ribosome assembly in the cells. Cells are labeled in the presence or absence of antibiotic for 90 seconds and chased with unlabelled uridine, samples taken in different time points, separated by sucrose gradient and ^{3}H-rRNA in the ribosomal particles measured.

Both methods employ low magnesium ion concentration (0.2 mM) in the sucrose gradients that dissociates 70S ribosome into 30S and 50S subunits. In these conditions some ribosomal precursors can be left hidden in 30S or 50S peak. Assembly inhibition was evaluated only by increase or reduction in 30S or 50S peaks (or the 50S/30S ratio). However, no efforts were made to characterize 70S and free subunits separately, as have been done in many studies concerning ribosome assembly. It has been observed by several groups that the most time consuming stages in assembly are the late steps, where the precursor particles have obtained the sedimentation properties similar to mature subunit, but they are inactive in translation and most likely not finally processed. Therefore it is probable that dissociating the 70S ribosomes creates high amounts of free subunits that can mask some of the assembly defective particles.

4.4.2. Direct inhibition of 50S subunit assembly

First indication for direct inhibition of 50S subunit assembly was published in 1995, when erythromycin and azithromycin were suggested to inhibit 50S particle formation without an effect on 30S subunit assembly in *E. coli* cells (Chittum and Champney, 1995). Cells were grown with [^{3}H] uridine for several generations in the presence or absence of erythromycin or other macrolides, and the radiolabeled subunits were separated from cell lysates by sucrose density gradient centrifugation (Chittum and Champney, 1995). A constant specific activity (^{3}H cpm/A_{260}) was found for ribosomal subunits from cells grown without erythromycin, and the 50S/30S specific activity ratio was found to be 1.0 (Chittum and Champney, 1995). Reduction in 50S peak was observed in cells grown in the presence of erythromycin (75 μg/ml), whereas the level of 30S did not change (Chittum and Champney, 1995). No 50S precursor particles were observed as new peaks in the sucrose gradients (Chittum and Champney, 1995).

Using four-fold higher antibiotic concentration, assembly could also be prevented in two erythromycin-resistant mutant strains with alterations in ribosomal proteins L4 and L22 (Chittum and Champney, 1995). It was proposed that erythromycin interferes with the binding of L4 to 23S rRNA early in the assembly process (Chittum and Champney, 1995).

Later, a 50S subunit precursor particle was revealed in the 30S region of the sucrose gradients from erythromycin-treated *E. coli* lysates (Usary and Champney, 2001). It was discovered by detection of 23S rRNA, 5S rRNA and 18 of the 34 large subunit proteins in 30S peak (Usary and Champney, 2001). In addition, the 30S region was capable of binding radiolabeled erythromycin (Usary and Champney, 2001). A strain with a temperature-sensitive defect in RNase E was more severely inhibited by erythromycin as compared to wild-type cells (Usary and Champney, 2001). In this strain large amounts of ribosomal RNA were found in the 25-30S region of sucrose gradient (Usary and Champney, 2001). These particles were similar to the ones found in 30S peak of wild-type cells (Usary and Champney, 2001)

A model for the 50S assembly inhibition process was proposed. Without the antibiotic 50S subunit assembly proceeds through two intermediate precursors that contain along with 23S and 5S rRNA different protein subsets (Usary and Champney, 2001). In the presence of erythromycin, a proportion of the nascent 50S subunits escapes assembly inhibition and becomes normal 50S particle (Usary and Champney, 2001). These are then capable of binding erythromycin with a subsequent effect on translation (Usary and Champney, 2001). According to the model, a subset of the developing 50S subunits bind erythromycin and fail to mature (Usary and Champney, 2001). These stalled assembly intermediates then become substrate for ribonucleases which degrade the particle (Usary and Champney, 2001).

In both papers and in all other works listed in additional tables 1 and 2, low magnesium ion concentration (0.2 mM) was used in the sucrose density gradients that dissociate 70S ribosome into 30S and 50S subunits. In these conditions precursor to both subunits can sediment with 30S peak. Using higher magnesium ion concentration (10 mM) in sucrose density gradients B. A. Maguire observed accumulation of precursor particles to both subunits in cell treated with erythromycin (Maguire, 2009). In addition the pulse-labeling experiments were conducted differently by the two groups. In their first experiments Chittum and Champney labeled *E. coli* cells for 10 h with [^{3}H] uridine in the presence of antibiotics (Chittum and Champney, 1995). B.A. Maguire grew cells in the presence of long-term [^{14}C]uracil label, erythromycin was added 10 min before the [^{3}H]uracil pulse-labeling for 70 s or 4 min (Maguire, 2009). The latter method enables detection of short-termed precursor particles that will be undetectable with long-term labeling. In sucrose density gradient B. A. Maguire saw precursors to both subunits in ^{3}H pulse-labeling profile and precursor of 50S in ^{14}C-labeling profile from erythromycin-treated cells (Maguire, 2009). He suggested that the assembly of both subunits is impaired by protein limitation,

but because of its greater complexity the 50S subunit is more affected (Maguire, 2009).

Other drugs that have been proposed to have direct effect on 50S subunit assembly are listed in additional table 1 and 2. These include 14-, 15- or 16-membred macrolides, ketolides, lincosamides and B-type streptogramins.

Streptogramin A or mixture of A and B, and chloramphenicol were shown to have an equivalent inhibitory effect on the formation of both subunits in *S. aureus* (Champney and Tober, 2000b). Surprisingly, Champney and Tober did not observe additional peaks in sucrose density gradients, when cells were grown in the presence of chloramphenicol, as observed by several other groups previously (Hosokawa and Nomura, 1965; Osawa *et al.*, 1969; Champney and Tober, 2000b). One of the reasons could be afore mentioned low magnesium ion concentration in gradients.

4.4.3. Direct inhibition of 30S subunit assembly

30S subunit assembly as a target for antibiotics was first suggested in 2002, when it was proposed that aminoglycosides like paromomycin and neomycin inhibit 30S subunit assembly specifically, later hygromycin B was added to the list (Mehta and Champney, 2002; McGaha and Champney, 2007). Growth in the presence of each antibiotic led to reduction in 30S amounts and to accumulation of a 21S particle (Mehta and Champney, 2002). With increasing drug concentration, there was a decrease in 30S subunit amounts and an increased accumulation of the 21S particle (Mehta and Champney, 2002). At higher concentrations of both drugs, an inhibition of 50S particle formation was observed (Mehta and Champney, 2002). Authors suggested that stalling of 30S synthesis could have a nonspecific downstream effect of slowing 50S synthesis as transcription of 16S rRNA precedes transcription of 23S and 5 S rRNA, and concomitant 30S assembly precedes 50S particle formation (Mehta and Champney, 2002).

The same effect was also studied in *S. aureus* cells (Mehta and Champney, 2003). A substantial reduction in 30S amounts was apparent when cells were grown in the presence paromomycin or neomycin (Mehta and Champney, 2003). In sucrose density gradient profiles from cells grown in the presence of each antibiotic a very large reduction in the amount of 30S particle and accumulation of slower sedimenting material was observed (Mehta and Champney, 2003). As in *E. coli* cells decline in 50S subunit amounts was also observed in *S. aureus* cells grown in the presence of aminoglycosides (Mehta and Champney, 2003). No further studies were carried out to characterize the 30S peak or the putative 30S precursor peak (Usary and Champney, 2001; Mehta and Champney, 2002, 2003). It is possible that there could be 50S precursors sedimenting with 30S peaks as observed in the case of erythromycin (Usary and Champney, 2001).

Further studies were carried out to characterize the 21S particle accumulating in paromomycin- or neomycin-treated *Escherichia coli* cells (Foster and Champney, 2008). It was found that both drugs were capable of binding to the 21S particle, but also to the 30S subunit and 50S subunit (Foster and Champney, 2008). 16S rRNA was identified in the region corresponding to the 21S precursor and in the 30S region (Foster and Champney, 2008). Surprisingly no hybridization experiments were carried out to exclude the presence of 23S or 5S rRNA in the region (Foster and Champney, 2008). When analyzing total cellular RNA, an increase in the amount of 17S rRNA species in the drug-treated samples was found (Foster and Champney, 2008). The precursor particle isolated from cells treated with neomycin was found to contain 12 out of 21 30S ribosomal proteins (Foster and Champney, 2008).

The effect of aminoglycosides was also investigated *in vitro*. Reconstitution in the presence of tenfold excess of neomycin or paromomycin resulted in the formation of a 21S particle and a small amount of 30S subunit (Foster and Champney, 2008). The aminoglycosides are positively charged which is critical for their attraction to the negatively charged RNA backbone. Therefore it is possible that in reconstitution experiments performed in the presence of high concentrations of the drug, the assembly is inhibited nonspecifically. The effect of aminoglycosides on the conformational changes in the three-way junction in the central domain of the 16S rRNA and on the binding of S15 were studied using fluorescence resonance transfer assay with two fluorophores on the RNA and one on the S15 (Klostermeier *et al.*, 2004). All of seven aminoglycosides tested induced folding of the junction, but did not inhibit binding of S15 (Klostermeier *et al.*, 2004). It was suggested that aminoglycosides bind to the RNA and act in a manner similar to Mg^{2+} ions that stabilize the compact folded form by electrostatic screening of negatively charged phosphates in the junction region (Klostermeier *et al.*, 2004). These results are not contrary to Foster and Champney, as the 21S precursor particle in their study also contained the protein S15, indicating that S15 can bind to 16S rRNA in the presence of aminoglycosides (Foster and Champney, 2008).

Later J. R. Williamson's group discovered that in *E. coli* grown in the presence of sublethal concentrations of neomycin precursors to both the 30S and 50S subunit accumulate contrary to Mehta and Champney (Mehta and Champney, 2002; Sykes *et al.*, 2010a). In the treated cells, the 30S precursor is visible as a distinct 21S peak in the sucrose density gradient as reported previously (Mehta and Champney, 2002; Sykes *et al.*, 2010a). A previously unreported 50S precursor was found to co-sediment with the 30S subunit, as revealed by agarose gel electrophoresis that demonstrated the presence of 23S rRNA in the 30S peak (Sykes *et al.*, 2010a). The precise levels of all ribosomal proteins in fractions collected through the entire sucrose gradient were measured using liquid chromatography-coupled mass spectrometry (LC/MS) (Sykes *et al.*, 2010a). Particles were found to be heterogeneous within

each sucrose gradient fraction as indicated by the low and varied protein levels measured (Sykes *et al.*, 2010a). These results suggest that effects of neomycin on ribosome assembly are general secondary effects as in the case of chloramphenicol (Dodd *et al.*, 1991; Sykes *et al.*, 2010a).

RESULTS AND DISCUSSION

Ref I. Erythromycin- and chloramphenicol-induced ribosomal assembly defects are secondary effects of protein synthesis inhibition

Several protein synthesis inhibitors are known to inhibit assembly of the prokaryotic ribosome (Nomura and Watson, 1959; Chittum and Champney, 1995). Two different explanations have been suggested to describe this „side-effect" of ribosome-targeting antibiotics. Chloramphenicol has been demonstrated to cause loss of coordination in the synthesis of ribosomal components due to protein synthesis inhibition. In the presence of chloramphenicol precursors to 30S as well as 50S precursors are accumulating, showing that the assembly of both subunits is affected. However, erythromycin, and later several other antibiotics, have been suggested to bind the ribosomal precursor particle and in doing so inhibit ribosome assembly directly (Champney, 2006). In this model, assembly of only one subunit is expected to be affected, 50S subunit in the case of erythromycin (Champney, 2006). We decided to investigate the effect of antibiotics on ribosome assembly and to differentiate between these two alternative hypotheses.

Two drugs were used in all further studies: chloramphenicol and erythromycin. Though other antibiotics, like puromycin or streptomycin are thought to inhibit ribosome assembly in similar manner as chloramphenicol (Dubin, 1964; Hosokawa and Nomura, 1965), the effect of chloramphenicol on the ribosome assembly and on the synthesis of ribosomal components is the best described. Erythromycin was chosen as this was the first drug that was suggested to have direct effect on ribosome assembly. Erythromycin is also the most widely and thoroughly studied macrolide antibiotic. The drug concentrations used were chosen to reduce cell growth for about 50% as compared to untreated cells. Our first experiments were designed to characterize the ribosomal particles accumulating in the presence of erythromycin or chloramphenicol.

Ribosome assembly in *E. coli* cells proceeds via several ribosomal precursor particles. The assembly of 30S subunit is thought to go through two intermediates. The first intermediate sediments between 21S and 27S and the second intermediate sediments with 30S (Mangiarotti *et al.*, 1968; Lindahl, 1975). The second intermediate (30S) contains all small subunit proteins, but rRNA in the particle is immature "17S rRNA", which is extended at both its 5´ and 3´ends (Srivastava and Schlessinger, 1990). The assembly of 50S subunit proceeds through three intermediate particles sedimenting at 30 to 36S, 40 to 43S and 50S (Mangiarotti *et al.*, 1968; Lindahl, 1975). The third ribosomal particle in 50S subunit assembly pathway that sediments together with mature subunit contains immature rRNA and is inactive in translation (Lindahl, 1975; Peil *et al.*, 2008).

Most often the first indication of ribosome assembly defects can be observed when fractionating ribosomal particles by sucrose density gradient centrifugation. In the presence of chloramphenicol accumulation of incomplete ribosomal particles can be observed. Composition and quantity of these particles are dependent on drug concentration. Previously it was observed that at higher drug concentration (3–1000 µg/ml) ribonucleoprotein particles sedimenting slower than 30S subunit (18S, 25S) are accumulating (Osawa *et al.*, 1969). At concentrations of chloramphenicol between 0,6 and 1,5 µg/ml, various ribonucleoprotein particles with sedimentation coefficients 50S, 40S, 30S and 22S were detected together with some mature 50S and 30S subunits (Osawa *et al.*, 1969). In our experimental system (chloramphenicol 7 µg/ml) three types of particles, accumulating between 45S and 25S were observed in addition to mature 70S ribosomes, no 50S or 30S subunit peaks were found (I, Figure 1E).

In previous studies it was suggested that erythromycin inhibits specifically 50S subunit assembly. This was based on the observations that only reduction in 50S peak was observed, 30S peak was not reduced and no additional peaks were observed in the cells treated with 75 µg/ml of erythromycin (Chittum and Champney, 1995). Surprisingly, in *Staphylocuccus aureus* cells grown in the presence of 20 µg/ml of chloramphenicol Champney and colleagues did not see additional peaks, but only a reduction in both 30S and 50S peaks (Champney and Tober, 2000b), which is in contrast with the result published previously by others (Osawa *et al.*, 1969; Champney and Tober, 2000b). It is possible that chloramphenicol affects ribosome assembly in *E. coli* and in *S. aureus* in different ways. However, most of the macrolide antibiotics investigated by Champney group were suggested to inhibit ribosome assembly in a similar way in the different bacteria studied (Champney, 2006). We found previously uncharacterized ribosomal particles in cells treated with erythromycin (100 µg/ml). Those particles sedimented as a broad peak between 45S and 25S region, no 50S or 30S subunit peaks were found (I, Figure 1C). It is known that assembly defects are usually more pronounced at lower growth temperatures, so we decided to investigate antibiotic effects on ribosome assembly also at 25ºC. In the case of both antibiotics three types of ribosomal particles were observed sedimenting with 25S, 35S and 45S. The 30S and 50S subunit peaks were not found (I, Figure 1D, F).

When finding ribosomal particles sedimenting slower than 30S in cells treated with erythromycin or chloramphenicol, we suspected the presence of 30S assembly precursor particles. To test this prediction we isolated RNA from sucrose gradient fractions of untreated and drug-treated cell lysates and hybridized it with 16S or 23S rRNA-specific probes (I, Figure 1). Presence of 30S precursor particles in antibiotic treated cells was confirmed by finding 16S rRNA in particles sedimenting slower than 30S. Similarly, 23S rRNA was found in particles sedimenting more slowly than 50S. This experiment revealed that both drugs affect assembly of 30S subunit as well as 50S subunit. Our

findings are in agreement with previously published results showing accumulation of precursors of both subunits in the presence of chloramphenicol (Osawa *et al.*, 1969), and in contrast to papers showing specific inhibition of 50S subunit assembly, in the presence of erythromycin (Chittum and Champney, 1995). Soon after we had published these results, accumulation of precursors to both 30S and 50S in the presence of erythromycin was confirmed by B. A. Maguire (Maguire, 2009). Furthermore, accumulation of precursors to both 30S and 50S was also demonstrated in the presence of neomycin by J. R. Williamson group (Sykes *et al.*, 2010a). Neomycin is an aminoglycoside antibiotic that was suggested to inhibit specifically the 30S subunit assembly (Mehta and Champney, 2002).

It has been reported that so called "chloramphenicol particles" contain precursors of 16S and 23S rRNA (Sykes *et al.*, 1977). Mutant strains with ribosome assembly defects often contain precursors with immature rRNA (Jiang *et al.*, 2006; Peil *et al.*, 2008), but there still exists a possibility that particles accumulating in erythromycin-treated cells could be degradation products of mature subunits. It is known that final processing of the 23S and 16S rRNA are late events in ribosome assembly; therefore, we expect assembly intermediate particles to contain immature rRNA while degradation products derived from mature ribosomes contain mature rRNA. RNase III cleaves pre23S rRNA at the positions –3 and –5 relative to the 5′end of mature 23S rRNA, final maturation occurring in functional ribosomes (Allas *et al.*, 2003). We analyzed ribosomal rRNA isolated from sucrose gradient fractions of drug-treated and untreated cells grown at 25ºC or 37ºC (I, Figure 2). 70S ribosomes from untreated cells grown at 37ºC contain solely mature 23S, free 50S particles from the same cells contain 23S rRNA with three extra nucleotide as a minor component (I, Figure 2B). At lower temperature ribosome assembly is slower; therefore rRNA processing would be expected to be slower as well. Accordingly, we observed molecules cleaved at –3 position in the 70S ribosomes of cells grown at 25ºC. In the 50S particles from the same cells only about half of the 23S rRNA molecules have mature 5′end, both types of processing intermediates (–3 and –7) being found. In antibiotic-treated cells processing is severely affected at both temperatures (I, Figure 2B). 70S particles of drug-treated cells contain mature 23S rRNA, but all precursor particles contain mainly the intermediate cleaved at position –7, only a small fraction of mature 23S rRNA being found in particles from 50S region of the gradient.

We observed similar effects for 16S rRNA processing (I, Figure 2A). It is known that the first cleavage by RNase III is at position –115. This is followed by maturation steps creating heterogeneous 5′ end (Srivastava and Schlessinger, 1990). We observed a small amount of processing intermediate cleaved at position –115 in 30S subunits of untreated cells grown at 37ºC (I, Figure 2A). At lower temperature the processing intermediate was present at higher extent, as expected. Incompletely processed 16S rRNA was found in the 30S and 20S regions of erythromycin- or chloramphenicol-treated cells, but at higher extent

in the slower sedimenting 20S particles. Low temperature increased the percentage of incompletely processed 16S rRNA as compared to its mature form in 30S and 20S regions of both untreated and drug-treated cells. Surprisingly, traces of processing intermediate cleaved at position –115 were found in the 70S ribosomes of antibiotic-treated cells at both temperatures and in the 70S ribosomes of untreated cells grown at 25ºC. It is known from 1970s that 30S subunits reconstituted from precursor 16S rRNA is inactive in translation (Wireman and Sypherd, 1974). However, recently it was demonstrated that immature 16S rRNA can be assembled into 70S ribosomes and it was proposed that incorporation of immature 30S ribosomes into translating population alters translational fidelity and cellular fitness (Roy-Chaudhuri *et al.*, 2010).

We demonstrated that precursor particles to 30S as well as 50S subunit are accumulating in cells treated with erythromycin, supporting the hypothesis that ribosome assembly is inhibited due to protein synthesis inhibition. However, it has been suggested that some macrolides, (flurithromycin, roxithromycin) but not others (erythromycin, clarithromycin, azithromycin), cause inhibition of 30S assembly as an indirect effect of the drugs upon both 50S synthesis and translation in *Haemophilus influenzae* (Mabe *et al.*, 2004). However, it was conclude that 50S subunit inhibition is preferential target for these drugs (Mabe *et al.*, 2004). We were interested to differentiate between the two alternative hypothesis described in the beginning of this chapter. We hoped that expression of erythromycin resistance peptides in erythromycin-treated cells will give answer to the question. The peptide-mediated erythromycin resistance mechanism acts in *cis* only upon actively translating mature ribosomes and does not influence availability of erythromycin in the cell (Tenson *et al.*, 1996; Lovmar *et al.*, 2006). Erythromycin resistance results from synthesis of specific small peptide that promotes the dissociation of erythromycin from the ribosome synthesizing those peptides (Lovmar *et al.*, 2006). If ribosome assembly is inhibited due to protein synthesis inhibition we should see relief in ribosome assembly defect, if assembly is inhibited directly by drug binding to 50S precursor particle, the opposite would be true. We investigated erythromycin effect on cells carrying multicopy plasmid with erythromycin resistance peptide (MRLFV) by monitoring ribosomal particles in sucrose density gradients. When peptide expression was not induced large amounts of incompletely assembled subunits accumulated in the 40S and 30S regions in the presence of the drug (I, Figure. 3). When expression of the resistance peptide was induced in the absence of the drug low level accumulation of precursor particles sedimenting at about 40S was observed. This may indicate inhibitory effect on the translational capacity of the cell due to the massive translation of resistance peptide from multicopy plasmid. Similar distribution of ribosomal particles was observed when the erythromycin resistance peptide was expressed and erythromycin was added to the culture, indicating the relief of assembly defect caused by erythromycin. Although expression of the erythromycin resistance peptide causes accumulation of small amount of 40S precursor particles, it is evident

that this peptide can relieve assembly inhibition caused by erythromycin. We conclude that erythromycin inhibits ribosome assembly through its general effect on protein synthesis.

The time course of inhibition of ribosome assembly in drug-treated cells would give additional insight into the inhibition mechanisms involved. First we analyzed incorporation of newly synthesized rRNA into ribosomal particle after antibiotic treatment. Exponentially growing *E. coli* cells were treated with antibiotic for 5, 10, 20, 40 or 60 minutes, after that RNA was labeled by adding [^{3}H]uridine to the medium during 5 minutes followed by addition of rifampicin to stop RNA synthesis. Rifampicin is an antibacterial agent that binds near the RNA polymerase active center and blocks initiation of RNA transcription (Villain-Guillot *et al.*, 2007; Ho *et al.*, 2009). Cells were grown for another five minutes to allow incorporation of newly synthesized rRNA into ribosomes. Cells were cooled with ice and collected rapidly. Ribosomal particles were fractionated by centrifugation in sucrose density gradients, fractions containing ribosomal particles were precipitated and radioactivity was counted. In untreated cells most radioactivity is found in 30S and 50S subunits after 5 minutes of labeling (I, Figure 5A), in agreement with previously published results (Peil *et al.*, 2008). Newly transcribed rRNA was incorporated into 70S ribosomes after another 5 minutes of incubation in the presence of rifampicin (I, Figure 5B). When erythromycin or chloramphenicol was added to the culture before labeling, ribosome assembly was significantly retarded and broad peaks of assembly intermediates accumulated between the 50S and 20S regions (I, figure 5). Assembly inhibitory effect of both drugs is very fast, 5 minutes of incubation in the presence of antibiotics is enough to cause significant accumulation of precursor particles. After prolonged (10 min, 60 min) with chloramphenicol prior RNA labeling, most ribosomal particles are still accumulating in particles sedimenting slower than 30S. However, if cells are treated with erythromycin for 60 minutes before labeling, most precursors are accumulating in 40S and 30S regions of the sucrose gradient. These slight differences could refer to different inhibitory effect of erythromycin and chloramphenicol on specific ribosomal protein synthesis (III, Figure 5). We find that the heterogeneity in the size of the intermediate particles is in better agreement with a model claiming that assembly is inhibited due to protein synthesis inhibition. If erythromycin would inhibit assembly by binding to a specific assembly precursor, accumulation of the precursor particle as a defined peak would be expected.

In conclusion, we have shown that in the presence of erythromycin or chloramphenicol precursors to both 50S and 30S subunits are accumulating, indicating that assembly of both subunits is inhibited. Ribosomal particles accumulating in the presence of erythromycin or chloramphenicol contain mainly immature pre-16S rRNA or pre-23S rRNA. We observed that ribosomal RNA processing is slower at lower temperatures both in untreated and drug-treated cells. We demonstrated that expression of a small erythromycin

resistance peptide acting *in cis* on mature ribosomes relieves the erythromycin-mediated assembly defect, indicating that assembly is inhibited due to protein synthesis inhibition and not by erythromycin binding to an assembly precursor particle.

Ref II. Subribosomal particle analysis reveals the stages of bacterial ribosome assembly at which rRNA nucleotides are modified

Modification of 16S and 23S rRNA is important part of ribosome assembly. *E. coli* 16S rRNA contains 11 and 23S rRNA contains 25 modified nucleosides. Each rRNA modification is made by specific enzyme; some modification enzymes are responsible for more than one modification. Cell-free experiments using purified enzymes have shown that some enzymes need the presence of ribosomal proteins while other enzymes can modify only protein-free rRNA (summarized in II, Table 1). The shortcoming of most of these *in vitro* studies is the limited number of substrates tested; usually comparison between mature subunit and free rRNA is made. In addition, the activity of a purified enzyme is often quite low and an optimal condition for *in vitro* modification is difficult to find.

Our aim was to find out at which ribosome assembly stages different rRNA nucleotides are modified. We had previously demonstrated that erythromycin and chloramphenicol inhibit assembly of both subunits and precursor particles to both subunits are accumulating in the presence of these antibiotics (Ref I). It has been shown that upon removal of chloramphenicol the precursor rRNA from the “chloramphenicol particles” is converted into normal 30S and 50S ribosomes without prior degradation (Nomura and Hosokawa, 1965). As both chloramphenicol and erythromycin inhibit ribosome assembly due to the unbalanced synthesis of ribosomal components, it is reasonable to assume that the “erythromycin particles” will mature into 70S ribosomes as well. Indeed, we demonstrated later, that both the “erythromycin particles” and the “chloramphenicol particles” are able to mature into 70S ribosomes (Ref III). We decided to characterize the rRNA modification pattern of incompletely assembled ribosomal particles from drug-treated cells and to compare it to the rRNA modification pattern of free subunits and mature 70S ribosomes from untreated cells. Secondly, we were interested to find out if erythromycin and chloramphenicol have different effect on the rRNA modification pattern during ribosome assembly.

Accumulation of assembly defective ribosomal particles was induced by adding chloramphenicol or erythromycin to the exponentially growing *E. coli* culture. Cells were grown for 2 hours in the presence of antibiotic at 25ºC. Addition of chloramphenicol or erythromycin to the growth medium leads to

the appearance of three unusual ribosomal particles sedimenting at 25S, 35S, and 45S (II, Figure 1B, C). The 35S and 45S particles contain mostly immature 23S rRNA and are related to the 50S subunit, the 25S particles contain mostly immature 16S rRNA and are related to the 30S subunit (Ref I). 25S, 35S and 45S particles were separated by sucrose gradient centrifugation. In order to get more resolved samples, sucrose gradient fractions containing particles of interest were combined, concentrated by ultrafiltration, and repurified by a second sucrose gradient (II, Figure 1). rRNA was purified from ribosomal particles by phenol extraction. We have shown that the 35S region of the sucrose gradient from erythromycin- or chloramphenicol-treated cells contain both 16S rRNA and 23S rRNA (Ref I). The two rRNA species were separated by sucrose gradient centrifugation (II, Figure 2B).

Free subunits and 70S ribosomes were isolated from *E. coli* cells growing exponentially at 25ºC. It has been shown that 60% of free 50S subunits from *E. coli* cells growing exponentially at 25ºC are inactive in peptidyltransferase assay and contain immature 23S rRNA (Peil *et al.*, 2008). Therefore, majority of the free subunits from cells grown at low temperature could be considered as late stage assembly precursor particles. Free subunits were collected by two consecutive sucrose gradient centrifugation steps. Mature 16S and 23S rRNA species were isolated from 70S ribosomes by phenol extraction followed by sucrose gradient centrifugation (II, Figure 2A). Modification pattern of the rRNA species was analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC). This method allows identification and quantitative estimation of all nucleosides of *E.coli* 16S rRNA and nearly all nucleosides of 23S rRNA (Gehrke and Kuo, 1989).

All modified nucleosides of the 16S rRNA were present at lower level in the 25S particles as compared to the mature 16S rRNA from 70S ribosomes (II, Figure 3 and Table 2). 16S rRNA from "erythromycin 25S particles" and "chloramphenicol 25S particles" shows similar modification pattern, in agreement with the similar protein composition of these particles (Ref III) Low levels (3–60%) of modified nucleosides are compatible with the heterogeneous protein compositions of the 25S particles (Ref III). It would be interesting to find out if certain proteins are required for or if certain proteins block addition of specific modifications as suggested for m^2G966 and m^5C967 (Weitzmann *et al.*, 1991). However, 25S particles contain most of the small subunit proteins at very low or negligible levels and direct comparison with the modification pattern is therefore difficult to perform (Ref III).

Surprisingly, most modifications were also under represented in the free 30S subunits (II, Table 2). Levels of specific modifications in the 16S rRNA from free 30S subunits range from 20–90% as compared to the mature 16S rRNA from 70S ribosomes. This finding is in agreement with our results showing that in the bacteria grown at 25ºC, 70–80% of the free 30S subunits contain the 16S rRNA precursor with 115 extra nucleotides at the 5′end (Ref I). It has been suggested that KsgA modifies its substrate nucleosides the latest in the 30S

subunit assembly and prevents incompletely assembled 30S subunits from engaging in translation initiation (Xu *et al.*, 2008). Indeed, free 30S subunits contain only 20–25% of KsgA product $m^6{}_2A$. Therefore we can conclude that about 20–30% of the 30S subunits are mature and 70–80% are incompletely processed and incompletely modified precursor particles.

23S rRNA was isolated from the 35S and 45S particles of both erythromycin- and chloramphenicol treated cells as well as from the free 50S subunits of untreated cells. All 5 types of 23S rRNAs were found to be undermethylated as compared to 23S rRNA from 70S ribosomes (II, Figure 4 and Table 3). The modification patterns of the 23S rRNA from "chloramphenicol particles" and "erythromycin particles" sedimenting in the same sucrose gradient regions show only minor differences (0–20%). Therefore, slight differences in protein composition of the "erythromycin 35S particle" as compared with the "chloramphenicol 35S particle" cannot be correlated with any specific modification (Ref III). Interestingly, although the 35S particle contains only very low or negligible levels of most large subunit proteins, most modifications are present at quite high levels (40–90%). These results indicate that most of the 23S rRNA modifications are made during early-assembly stages, in contrast to the 16S rRNA where most modifications are late-assembly specific events. The only exception is Um2552 that was not detected in the 23S rRNA from the 35S particles.

It has been demonstrated previously that the 50S subunit precursor particles accumulating in the presence of chloramphenicol (10 μg/ml) are submethylated and that the degree of methylation in different precursor particles may reflect the order of rRNA modification in ribosome assembly pathway (Gordon *et al.*, 1964). Indeed, the levels of all modifications are increasing with the increasing sedimentation coefficients of particles; being lower in 35S particles and the highest in 50S subunits. The level of methylation of 23S rRNA from 30S and 40S precursor particles accumulating in the presence of chloramphenicol (0.8 μg/ml) has been shown to be at about 60% of mature 23S rRNA from 50S ribosomal subunits (Osawa *et al.*, 1969). Based on theses findings, it was suggested that around 60% of methyl groups are inserted into 23S rRNA before 50S ribosomal precursor particles are formed, and the reminder 40% were incorporated during the conversion of the 40S ribosomal precursor particles into the 50S subunits, or immediately after the completion of the subunit assembly (Osawa *et al.*, 1969). In general, our results are in good agreement with the previously published observations. Monitoring of the different modifications allows us to follow the gradual addition of specific modifications during ribosome assembly. For example, Um2552 is missing in the 35S particle, present at low levels in 45S particle (~35%), and free 50S subunits containing already about 60% of the modification as compared to the 70S ribosomes (II, Table 3). Whereas some other methylations like m^1A and m^2A are present at high levels (80–100%) in all particles.

Based on our results we have divided rRNA modifications into three major groups: early, intermediate, and late assembly modifications (II, Figure 6). In general, the assembly dependence of rRNA modifications *in vivo* is in very good agreement with the published specificities of modification enzymes determined *in vitro* (II, Table 1). In addition, the data obtained allow to define the specificity of several previously uncharacterized enzymes (6 pseudouridine synthases and for 6 methyltransferases) in respect of ribosome subunit assembly (II, Figure 6).

In conclusion we have demonstrated that the modification pattern of rRNA is similar in both erythromycin and chloramphenicol-induced particles of the same size. Modifications are added gradually during association of ribosomal proteins with the rRNA and thereby with the growing S-value indicating that the synthesis of rRNA modified nucleosides depends on the progression of ribosome assembly. In addition, we have demonstrated that the free subunits of exponentially growing bacteria contain undermodified rRNA. We have observed that most modifications in 16S rRNA occur during late-assembly and most modifications in 23S rRNA occur during early-assembly.

Ref III. Antibiotic-induced ribosomal assembly defect result from changes in the synthesis of ribosomal proteins

After we had shown that both erythromycin- and chloramphenicol-induced ribosome assembly defects are secondary effects due to protein synthesis inhibition we were interested to examine the assembly inhibition mechanism in more detail. Analyzing protein content of the precursor particles and comparing to ribosomal protein availability in cells treated with antibiotics could give additional information about ribosome assembly inhibition and ribosome assembly in general.

It has been demonstrated that upon removal of chloramphenicol (100 μg/ml), a major part of rRNA contained in "chloramphenicol particles" is converted into rRNA in normal ribosomes without extensive breakdown (Nomura and Hosokawa, 1965), demonstrating that "chloramphenicol particles" are assembly intermediates rather than misassembled particles. We were interested to find out whether or not the particles accumulating in the presence of erythromycin are able to mature into 70S ribosomes as well. RNA pulse-labeling strategy was used to analyze the kinetics of ribosome assembly in antibiotic-treated cells. To allow better resolution of the intermediate particles cells were grown at 25ºC where the ribosome assembly is slower as compared to the assembly at 37ºC (Peil *et al.*, 2008). Exponentially growing cells were treated with erythromycin or chloramphenicol for 5 minutes. This is sufficient time for inhibition of both translation and ribosome assembly in cells growing at 25ºC (Ref I Figure 5).

RNA was labeled with $[^3H]$uridine for 5 minutes, after which transcription was blocked with rifampicin and time-points were taken for ribosome profile analysis. Fate of precursor particles was followed by monitoring incorporation of freshly synthesized rRNA into ribosomes (III, Figure 1). The optical curve shows the positions of the 70S ribosomes and the free 30S and 50S subunits and the radioactive diagram represents the newly synthesized ribosomal particles. In the first time point of untreated cells, collected at the time of rifampicin addition, the majority of labeled rRNA was found in the 50S and 30S fractions, indicating fast rate of subunit assembly (III, Figure 1A). 5 minutes later the radioactivity was distributed equally between the free subunits and the 70S fraction and 10 minutes after rifampicin addition most of the radioactively labeled rRNA was incorporated into 70S ribosomes, in accordance with the previously published observations (Peil *et al.*, 2008). Further incubation in the presence of transcription inhibitor rifampicin did not change the distribution of radioactive RNA in sucrose gradient.

Addition of erythromycin or chloramphenicol to the culture significantly reduced the rate of ribosome assembly; after 5 minutes of labeling most of radioactivity was found in the 30S and 25S regions (III, Figure 1B, C). Radioactive rRNA moved slowly into particles with higher sedimentation properties, being almost equally represented in 70S, 50S and 30S regions 40 minutes after stopping the transcription. Most of the radioactivity was in 70S region of erythromycin-treated cells after 60 minutes. In cells treated with chloramphenicol the radioactivity was approximately equally divided between 70S, 50S and 30S regions after 20 minutes of incubation in the presence of rifampicin and most of radioactivity had moved into 70S region in 60 minutes after stopping the transcription. These results show that the majority of the rRNA accumulating in the presences of either erythromycin or chloramphenicol is finally incorporated into 70S ribosomes without significant rRNA degradation. Our results are in accordance with previous results for chloramphenicol (Nomura and Hosokawa, 1965). Slow incorporation of rRNA into 70S ribosomes is in agreement with our previous findings showing that rRNA processing and modification slowed down in antibiotic-treated cells growing at low temperature (Ref I, Ref II).

In addition, we performed a similar experiment at 37ºC. The results are shown in Figure 6. Ribosome assembly is very fast at 37 ºC and therefore difficult to analyze. In the untreated cells most of radioactivity is found in 70S peak already 5 minutes after the $[^3H]$uridine label is added to the culture in agreement with earlier reports (Lindahl, 1975). Five minutes after stopping the transcription only traces of radioactivity can be found in the 50S and 30S regions. As expected, ribosome assembly in antibiotic-treated cells is also much

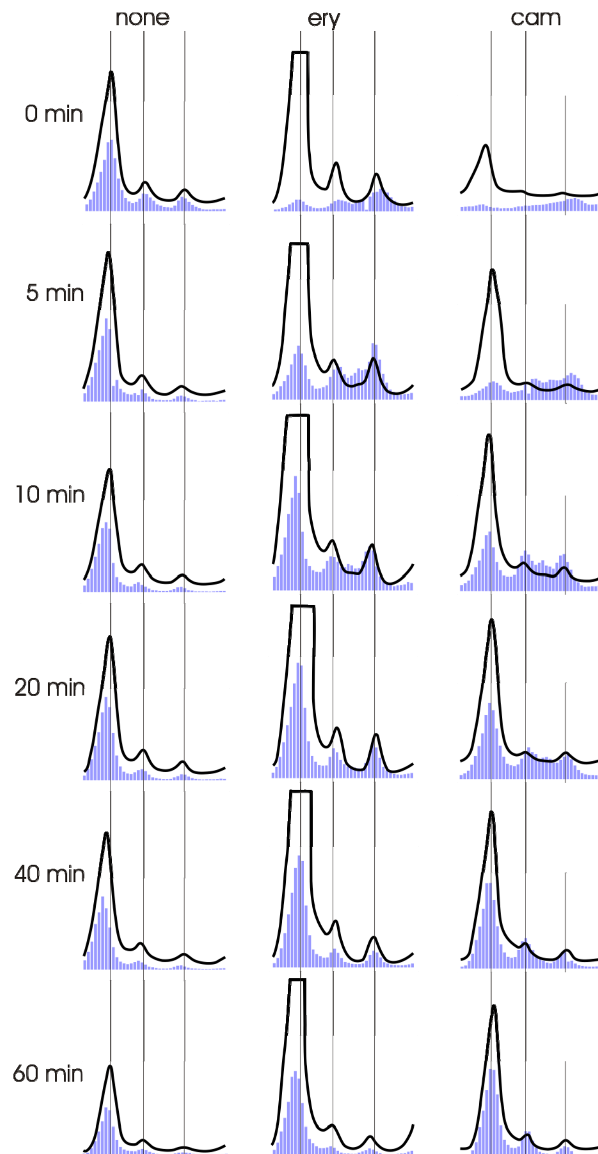

Figure 6. Time-course of ribosome assembly shows that the drug-induced 25S, 35S and 45S particles are assembled into 70S ribosomes. (A) No antibiotics (none), (B) erythromycin (Ery, 100 µg ml−1) or (C) chloramphenicol (Cam, 7 µg ml−1) was added 5 min before labelling and RNA was labeled for 5 min with [3H]uridine, after which the transcription initiation was blocked with rifampicin. Cells collected at different time points (0, 5, 10, 20, 40 and 60 min) were lysed, ribosomes were fractionated by centrifugation in sucrose density gradients, and the fractions were counted for radioactivity. The optical density profiles are shown by black lines and radioactivity profiles by blue bars.

faster at 37ºC than at 25ºC. In erythromycin-treated cells a remarkable amount of newly synthesized 70S ribosomes can be found already after 5 minutes and in 10 minutes after stopping the transcription most radioactivity is found in the 70S peak. Assembly in the presence of chloramphenicol at 37ºC is slightly slower than in the presence of erythromycin at the same temperature as a remarkable amount of new 70S ribosomes is formed during 10 minutes and the assembly of 70S is completed in 20 minutes time after rifampicin addition. In conclusion, we have shown that the rRNA transcribed in the presences of either erythromycin or chloramphenicol is incorporated into 70S ribosomes with a much faster rate in cells grown at 37ºC compared to the same cells grown at 25ºC.

Champney and colleagues have studied ribosome assembly in the presence of several different drugs, including erythromycin and chloramphenicol, using pulse labeling strategy (Champney, 2006). However, their experimental design and results are somewhat different compared to our findings. First, all sucrose density gradient centrifugations have been performed in dissociation favoring conditions where 70S ribosomes are divided into free 30S and 50S subunits. Ribosome assembly is very fast and most time consuming stage in ribosome assembly is when subunits have gained sedimentation properties similar to mature subunits, but are yet unable to be involved in translation. If ribosomal particles are separated in dissociating conditions, ribosomal subunits from 70S ribosomes, polysomes and free immature subunits are all sedimenting in 30S and 50S peaks. Furthermore, in conditions like these it would be difficult to detect new precursor particles sedimenting slower than 30S or 50S.

The first experiment suggesting that erythromycin inhibits 50S subunit specifically was published in 1995 (Chittum and Champney, 1995). The exponentially growing cells were treated with erythromycin, 1 h later [^{3}H]uridine was added and labeling was continued up to 6 or 10h. In the same paper breakdown of the existing ribosomes was investigated. [^{3}H]uridine labeled cells were chased with "cold" uridine for 8.5 h before addition of erythromycin; the cells were grown for next 9 h in the presence of erythromycin. Altogether, starting from labeling cells were grown for 18 h (Chittum and Champney, 1995). During such a long incubation time the radioactive rRNA is incorporated into 70S and possibly already re-utilized. In our experiments, most of rRNA has been incorporated into 70S ribosomes in erythromycin-treated cells grown at 37ºC already in 10 minutes (Fig. 6).

Champney´s group has also obtained results comparable to ours, however they have interpreted their results differently (Usary and Champney, 2001). In this study the cells were labelled with [^{3}H]uridine for 90 seconds and chased with an excess of unlabelled uridine for 30 minutes (Usary and Champney, 2001). This experiment is quite similar to our 20 minutes time point with exception that cells were grown in the presence of erythromycin for two generation as compared to 5 minutes in our experiments. We observed quite similar radioactive profile as Usary and Champney in either cells grown at 37ºC

or 25ºC (27ºC) in the presence of erythromycin. Nevertheless, differences in sucrose gradient buffer composition allow us to see maturation of ribosomal particles into 70S ribosomes, while in their experiments 70S ribosomes were dissociated into free subunits, that complicates analysis of the assembly kinetics. They have evaluated ribosome subunit formation by calculating radioactivity of corresponding subunit peak area. This approach is not always justified. For example, in the cells grown at 27ºC ribosomal particles are accumulating as a broad peak and it is difficult to separate 30S subunits from the precursors of 50S particles.

When we had proven that ribosomal particles accumulating in the presence of chloramphenicol and erythromycin are able to mature into 70S ribosomes, we were interested to find out protein composition of these particles and compare it to previously known assembly precursors. We observed three peaks besides 70S ribosome peak in sucrose density gradient analysis of both erythromycin- and chloramphenicol-treated cells grown at 25ºC as previously described (Ref I, Ref II). We separated ribosomal precursor particles by two consecutive sucrose density gradient centrifugation steps. Firstly, we were interested if ribosomal precursors accumulating in the drug-treated cells are homogeneous in their structure and similar to 30S and 50S subunits. Negative-stain electron microscopy analysis showed that 25S and 35S particles isolated from chloramphenicol- and erythromycin-treated cells are highly heterogeneous, tend to aggregate and do not show much morphological similarity to either 30S or 50S subunit (III, Figure 2). However, 45S particles show higher homogeneity and higher similarity to the 50S subunit. The heterogeneous nature of ribosomal precursor particles is in agreement with the observations made by others (Sykes *et al.*, 2010a). According to the model proposed by Champney, accumulation of particles with homogeneous morphological properties would be expected.

The levels of ribosomal proteins in assembly precursor particles were determined by quantitative mass-spectrometry analysis. Ribosomal precursor particles were mixed with equimolar amount of the corresponding ribosomal subunit containing uniformly [^{15}N]-labeled ribosomal proteins. Ribosomal proteins were digested with trypsin and the [^{14}N]/[^{15}N] ratio in tryptic peptides was determined by mass spectrometry. Subribosomal particles showed a great degree of protein deficiency and heterogeneity, in accordance with our results from negative-stain electron microscopy analysis. The most abundant rRNA primary binding protein in the 45S and 35S samples was L3 with the average occupancy of 100% in the 45S particles and 90% in the 35S particles. The most abundant rRNA primary binding protein in the 25S samples was S15 with the average occupancy of 70%. We decided to calculate relative amounts of ribosomal proteins in precursor particles by taking the [^{14}N]/[^{15}N] ratio of rRNA primary binding proteins L3 and S15 as 100% for large and small subunit proteins respectively (III, figure 3).

Precursor of small subunit, the 25S particle, contained proteins S15, S16 and S18 in nearly equal high amounts (III, Figure 3A). Other proteins are found in

low or negligible levels, with the late assembly proteins S1, S2, S3 and S21 being present in the lowest amounts. Both chloramphenicol and erythromycin cause accumulation of 25S particles with similar protein composition, with the exception of proteins S4 and S11 which are more abundant in the "chloramphenicol 25S particles" as compared to the "erythromycin particles".

When comparing the 25S particle from erythromycin- and chloramphenicol-treated cells with previously known assembly precursor particles and reconstitution intermediates, we observed more differences than similarities. Protein composition is not similar in the 25S particles as compared to the previously characterized *in vivo* assembly precursor particle sedimenting at 21S (Homann and Nierhaus, 1971; Nierhaus *et al.*, 1973). The 21S precursor particles have been shown to contain S1, S4, S5, S8, S13, S15, S16, S17 and S20 in normal amounts. In response to erythromycin treatment, the 25S particles from drug-treated cells contain most of these proteins in negligible levels with the exception of S15. In the 25S particles of chloramphenicol-treated cells only S16 is present in amount comparable to S15; proteins S4, S8, S13 and S17 are found in low levels and proteins S1, S5 and S20 in negligible levels.

Protein composition of the 25S particles is not in good agreement with previously known reconstitution intermediate particles. First of all, the assembly initiator proteins S4 and S7 are present in very low amount in 25S particles accumulating in the presence of erythromycin. Although S4 is present in higher level in the 25S particles from chloramphenicol treated cells as compared to the same particle from erythromycin-treated cells, the level of the S4 protein is still only about half of that of the primary binding protein S15. Other primary binding proteins like S8, S17 and S20 are present around 50% as compared to the primary binding protein S15. Proteins S4, S7, S8, S16 and S19 are essential for reconstitution intermediate formation and for enabling further progression of the 30S ribosome assembly. These proteins are found in low or negligible levels in the 25S particles from cell treated with either erythromycin or chloramphenicol. In addition, the proteins in the 25S particle do not follow the suggested sequential assembly of ribosomal proteins with a 5′ to 3′ polarity along the 16S rRNA chain (Powers *et al.*, 1993).

Protein composition of the 21S particles accumulating in the presence of neomycin has been determined by two independent studies (Foster and Champney, 2008; Sykes *et al.*, 2010a). With qualitative study by two-dimensional polyacrylamide gel electroforesis 12 proteins were found to be present in the 21S particle (Foster and Champney, 2008; Sykes *et al.*, 2010a). In the 25S particles most of these proteins were present in low levels with the exception of S15, S16 and S18. In the 21S particles of neomycin-treated cells S7 was found, while S6 and S8 were not observed (Foster and Champney, 2008). In contrast, we found S6 and S8 at higher levels than S7 in the 25S particle from cells treated with either erythromycin or chloramphenicol. With a quantitative study by mass spectrometric approach protein levels in the 21S particles accumulating in the presence of neomycin were measured more precisely (Sykes *et al.*,

2010a). In the 21S particle from neomycin treated cells S8 and S15 were found to be present at the highest levels (~13–14%). In comparison, the 25S particles from erythromycin- or chloramphenicol-treated cells contained S15 and S18 in much higher level (~70%). However, levels of single ribosomal proteins as compared to the primary binding protein S15 in the 21S particle from neomycin-treated cells and in the 25S particle from chloramphenicol-treated cells seem to be quite similar with the exception of S8 and S18 (Sykes *et al.*, 2010a).

In the 35S particles most proteins are present in low or negligible amounts relative to the level of the primary binding protein L3, with the exception of L11 being present in similar amounts with L3 (III, figure 3B). The biggest difference concerns the large subunit assembly initiator protein L24, which is present at equal level with L3 in the "chloramphenicol 35S particles", but four times less in the "erythromycin 35S particles" (~25%). Proteins L4 and L23 are also present in erythromycin particles in considerably lower amounts as compared to the chloramphenicol particles. In contrast, proteins L6 and L9 are present in "erythromycin particles" at considerably higher levels as compared to the "chloramphenicol particles".

Three precursor particles sedimenting at 32S, 43S and 50S have been identified during 50S subunit assembly *in vivo* (Lindahl, 1975). The 35S particles from antibiotic-treated cells are similar to the 32S particles in sedimentation properties, but protein composition of the two particles is slightly different. 32S particle have been shown to contains 15 proteins in normal amounts, 1 protein in reduced amount and 4 proteins present in traces (Nierhaus *et al.*, 1973). Most proteins that are present in normal amounts in the 32S particle are present in reduced amounts or in traces in both the "erythromycin 35S particle" and "chloramphenicol 35S particle". In contrast, L11 and L3 that are present in the highest amounts in both erythromycin and chloramphenicol particles are present only in traces in the 32S precursor particle. We conclude that protein composition of the 35S particle accumulating in drug-treated cells is not similar to the previously known assembly precursor particle sedimenting at 32S.

Reconstitution experiments have identified L3 and L24 as the assembly initiator proteins. Both proteins are present at the highest level in chloramphenicol 35S particle, but unexpectedly L24 is present in 4 fold lower amount in the "erythromycin 35S particle". However, it has been demonstrated that L20 can replace L24 at low temperatures (Franceschi and Nierhaus, 1988). Indeed, L20 is present at higher amount in erythromycin particle (~60%) than in chloramphenicol particle (~40%). Proteins L4, L13, L20, L22 and L24 are important for 50S reconstitution precursor particle formation and for assembly progression. Only two out of the five proteins were present at levels over 50% in 35S particles. These proteins are L13 and L20 in the "erythromycin 35 particle" and "L4 and L24 in chloramphenicol 35S particle". Thus the two drugs affect early stages of ribosome large subunit assembly in different ways.

Protein composition of the 50S precursor particle sedimenting with 30S in erythromycin-treated cells has been characterized previously by two-dimensional gel electrophoresis (Usary and Champney, 2001). The particle was found to contain 18 large subunit proteins (Usary and Champney, 2001). The relative amount of each protein is not in good agreement with the relative amount of the same protein in our “erythromycin 35S particle”. For example, proteins L6, L7/12, L9 and L10, most highly represented in the particle studied by Usary and Champney (2001), were significantly reduced in our “erythromycin 35S particle”. In the “erythromycin 35S particle” most abundant protein is L3. In the 50S precursor particle characterized by Champney group the most abundant protein is L6; L3 was found at four fold lower level. Another protein present in nearly 100% in the erythromycin 35S particle, L11, was also significantly reduced in 50S precursor particle characterized by Champney group. In conclusion, we have identified 17 previously observed and 15 additional proteins in the precursor particle accumulating in response to erythromycin treatment. However, protein composition of the precursor particles is not in good agreement with the previously published results. The differences might be caused by the higher accuracy of the mass spectrometric analysis as compared to the two-dimensional gel electroforesis.

It has been shown that neomycin inhibits assembly of both the small and the large subunit. The precursor to 50S subunit sediments together with 30S subunit (Sykes *et al.*, 2010a). 50S proteins were found to be present in the 30S peak at low levels (6–12% compared to levels in 50S subunits) that varied from protein to protein (Sykes *et al.*, 2010a). Surprisingly, proteins L3, L11 and L24 that are present in nearly 100% in the “chloramphenicol 35S particle” are also among the highest represented in the “neomycin 30S particle”. However, Proteins L13, L21, L22 and L25 that are present at similar levels with previously mentioned proteins in “neomycin 30S particle” are present in significantly reduced amounts in the “chloramphenicol 35S particle”. In conclusion, protein composition of the 50S precursor particles accumulating in the presence of neomycin is qualitatively more similar to the 35S precursor particles accumulating in response to chloramphenicol-treatment than to precursor particles accumulating in response to erythromycin-treatment.

The 45S particle contains quite high levels of most of the large subunit proteins. Only proteins L15, L25, L27, L30, L31, L33 and L34 are present at levels from 30 % to 50% and L16, L35 and L36 are present at levels less than 30% (III, figure 3C).

Comparison of the 45S particle with previously known assembly and reconstitution intermediates is difficult as most proteins were found in 45S particle although at variable levels. Proteins L15 and L16 that play decisive role in late assembly process are present in low amounts in both 45S particles. Absence of these proteins could be the reason for accumulation of 45S particles and slowing down assembly into 50S subunits and into 70S ribosomes. Proteins essential for 5S rRNA integration (L5, L15, L18) are present ~35–45% in the

"chloramphenicol 45S particle" and ~50–60% in the "erythromycin 45S particle".

Sometimes accumulation of ribosomal precursor particles can be observed in ribosome assembly factor deletion or mutant strains. Ribosome assembly factors are nonribosomal proteins involved in ribosome assembly. In the absence of functional DnaK chaperone particles sedimenting at 21S, 32S and 45S are accumulating at 44ºC (El Hage and Alix, 2004). However, protein composition of these particles is different from the 25S, 35S and 45S particles accumulating in the presence of either chloramphenicol or erythromycin. Deletion of RNA helicase CsdA or RNA helicase SrmB leads at low temperatures to accumulation of a particle sedimenting around 40S (Charollais *et al.*, 2003; Charollais *et al.*, 2004). Protein composition is different in each particle: CsdA 40S; SrmB 40S; DnaK 45S; and 45S particles of antibiotic-treated cells. Differences in the protein compositions of assembly precursor particles accumulating in the absence of assembly factors could be related to functioning of the factors during various stages of ribosome subunit assembly. In contrast, differences in the protein compositions of assembly precursor particles accumulating in the presence of protein synthesis inhibitors could be related to differential synthesis of ribosomal proteins in response to different antibiotics.

Coordinated synthesis of ribosomal components should assure fast and efficient ribosome assembly. Ribosome assembly depends on the availability of ribosomal proteins. Therefore it is reasonable to assume that the ribosome assembly defects caused by protein synthesis inhibitors are related to unbalanced synthesis of ribosomal components. Secondly, when we observed slight differences in protein composition of precursor particles accumulating in response to erythromycin- or chloramphenicol-treatment, we were interested to find out if these two antibiotics affect availability of ribosomal proteins differently.

We used pSILAC approach to measure the production of individual ribosomal proteins in the presence of erythromycin or chloramphenicol. In "stable isotope labeling by amino acids in cell culture" or SILAC, cells are metabolically labeled by cultivating them in medium containing heavy stable isotope version of essential amino acids. Samples from heavy and light labeled cells can be mixed and analyzed together. Pulsed SILAC (pSILAC) makes direct comparison of protein translation rates between two samples by pulse labeling with two different stable isotopes possible. Cells were first cultivated in growth medium with the normal "light" (L) amino acids. Exponentially growing cells were divided into two parts. In one sample the medium was changed against medium containing "heavy" (H) amino acids, antibiotic was added and the cells were collected after 4 hours. The control sample was diluted with equal amount of fresh unlabeled medium, after 2h the medium was changed against medium containing "medium-heavy" (M) amino acids and the cells were collect after 2h. As the antibiotics inhibit protein synthesis and thereby label incorporation, different labeling period of untreated and drug-treated samples were important

to achieve similar label incorporation in both samples. Both samples were combined and analyzed together. The measured H versus M ratio reflects differences in translation of the corresponding protein in antibiotic-treated cells versus untreated cells.

Inhibition of translation by chloramphenicol or erythromycin caused differential synthesis of many cellular proteins. Synthesis of most cellular proteins became inhibited, but increased synthesis of certain ribosomal and nonribosomal proteins was observed as well. Relative differential synthesis rates of individual ribosomal proteins were observed in the presence of chloramphenicol or erythromycin, a phenomenon previously observed by P. P. Dennis in the case of chloramphenicol (Dennis, 1976). It has been suggested that operon feedback mechanisms are responsible for differential synthesis of individual ribosomal proteins during chloramphenicol-treatment (Dennis, 1976; Dodd *et al.*, 1991).

We mapped the synthesis levels of ribosomal proteins onto the longer operon structures (III, Figure 5). In the S10 operon the first protein in the operon is synthesized at the highest level, followed by gradual loss of production over the following cistrons. All cistrons in S10 operon are translationally coupled. First cistron of polycistronic mRNA is accessible for the 30S initiation, whereas the second and following initiation sites are hidden within secondary structure. When the first initiation site has been recognized, translation commences and the translating ribosome can unfold secondary structure to reveal the second initiation site. Therefore, in the presence on protein synthesis inhibitors higher levels of first proteins in the cistron would be expected.

In the *spc* operon, protein encoded by the third cistron (L5), is present at the highest amount, followed by gradual decrease of proteins from the next cistrons. Lower levels of the proteins L14 and L24 encoded by the first two cistrons are in good agreement with the previously suggested retroregulation mechanism for these two cistrons (Mattheakis *et al.*, 1989).

In alpha operon the first protein (S13) is synthesized at the highest level, followed by gradual decrease of proteins from the next three (S11, S4, rpoA) cistrons. Surprisingly, the product of the last cistron (L17) of the operon is synthesized at similar level as the product of the first cistron, indicating that the L17 gene might not be translationally coupled with previous genes. Previously it has been suggested that the first three cistrons (S13, S11, S4) are translationally coupled and the fourth (rpoA) cistron is not translationally coupled to the other cistrons (Thomas *et al.*, 1987). The L17 was thought to be translationally coupled to the three upstream genes, even though the unregulated gene is in between (Thomas *et al.*, 1987).

The levels of proteins from rpoBC operon are in good agreement with previously characterized operon regulation mechanisms. The first and the third gene in operon are expressed at the highest level, while the second gene is expressed at slightly lower level. These results are in agreement with the previously described promoter locations in the operon. The first promoter is located at the beginning of the gene cluster and the second promoter between

the second and third gene (Steward and Linn, 1991). The remarkable reduction in synthesis of RNA polymerase subunits as compared to ribosomal proteins from the same operon is in good accordance with the previous results showing that the polymerase subunit coding genes are transcribed only about one-fifth as frequently as ribosomal protein genes (Steward and Linn, 1991).

Based on our results trmD operon seems to be translationally uncoupled in accordance with pervious publications by others (Wikström *et al.*, 1988).

If the ribosome assembly inhibition is caused by limitation of certain ribosomal proteins, the differences in the levels of proteins in the subribosomal particles accumulating in the presence of erythromycin versus chloramphenicol would be in correlation with differences in ribosomal protein synthesis rates. We observed only a few remarkable differences in protein composition of subribosomal particles accumulating in response to erythromycin versus chloramphenicol. Similarly we observed only few examples of differences between the levels of individual proteins produced in the cells treated with different antibiotics. Notably, 50S subunit initiator protein L24 is present at 4 times lower level in "erythromycin 35S particle" as compared to "chloramphenicol 35S particle"; correspondingly L24 translation rate is remarkably lower in the presence of erythromycin. Proteins L2, L4, L14 and L23 are present at lower levels in erythromycin-treated cells and "erythromycin 35S particle" as compared to chloramphenicol-treated cells and "chloramphenicol 35S particle". Although 45S particle is more similar to the 50S subunit and contain much higher levels of most large subunit proteins as compared to the 35S particle, proteins L2, L4, L14, L23 and L24 are still present in slightly lower amount in the "erythromycin 45S particle" as compared to the "chloramphenicol 45S particle". We conclude that the small difference between protein composition of erythromycin and chloramphenicol particles is caused by the differences in production of individual ribosomal proteins in cells in response to erythromycin and chloramphenicol accordingly.

In conclusion, we have shown that most ribosomal particles accumulating in response to chloramphenicol- or erythromycin-treatment can mature into functional ribosomes. Erythromycin and chloramphenicol cause accumulation of very heterogeneous particles, a finding we have confirmed by electron microscopy analysis of subribosomal particles and mass spectrometry analysis of ribosomal protein composition of these particles. Erythromycin particles and chloramphenicol particles have similar, but not identical, protein composition. Production of ribosomal proteins in the presence of the antibiotics correlates with the amounts of the individual ribosomal proteins within the precursor particles. The treatment of cells with chloramphenicol or erythromycin leads to an unbalanced synthesis of ribosomal proteins, providing the explanation for formation of assembly-defective particles.

CONCLUSIONS

I. We have shown that in the presence of erythromycin or chloramphenicol precursors to both 50S and 30S subunits are accumulating, indicating that assembly of both subunits is inhibited. Ribosomal particles accumulating in the presence of erythromycin or chloramphenicol contain mainly immature pre-16S rRNA or pre-23S rRNA. We demonstrated that expression of a small erythromycin resistance peptide acting *in cis* on mature ribosomes relieves the erythromycin-mediated assembly defect, indicating that assembly is inhibited due to protein synthesis inhibition and not by erythromycin binding to an assembly precursor particle.

II. We have demonstrated that modification pattern of rRNA is similar in both erythromycin and chloramphenicol-induced particles of the same size. Modifications are added gradually during association of ribosomal proteins with the rRNA and thereby with the growing S-value indicating that the synthesis of rRNA modified nucleosides depends on the progression of ribosome assembly. In addition, we have demonstrated that free subunits of exponentially growing bacteria contain undermodified rRNA. We have observed that most modifications in 16S rRNA are late-assembly specific and most modifications in 23S rRNA are early-assembly specific.

III. We have shown that most ribosomal particles accumulating in response to chloramphenicol- or erythromycin-treatment can mature into functional ribosomes. Erythromycin and chloramphenicol cause accumulation of very heterogeneous particles, a finding we have confirmed by electron microscopy analysis of subribosomal particles and mass spectrometry analysis of ribosomal protein composition of these particles. Erythromycin particles and chloramphenicol particles have similar, but not identical, protein composition. Production of ribosomal proteins in the presence of the antibiotics correlates with the amounts of the individual ribosomal proteins within the precursor particles. The treatment of cells with chloramphenicol or erythromycin leads to an unbalanced synthesis of ribosomal proteins, providing the explanation for formation of assembly-defective particles.

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ADDITIONAL TABLES

Additional table 1. Cell growth rates, amount of ^{3}H-uridine labeled subunits and ^{35}S-amino acid incorporation into proteins in different organisms.

Organism/Strain	Media	Tempe-rature	Antibiotic	Conc. (µg/ml)	Doubling time (h)	50S/30S	% total cpm		Protein synthesis	Ref
							30S	50S		
Escherichia coli										
SK 901	TB	37ºC	none	0	1.4	1.02	nd	nd	nd	(Chittum and Champney, 1995)
			erythromycin	75	8	0.52				
			azithromycin	2.5	12	0.7				
			clarithromycin	20	12	1.03				
			oleandomycin	150	14	0.97				
			spiramycin	150	7.5	1.08				
			lincomycin	75	4	1				
			virginiamycin M1	20	12.5	0.93				
N281	TB	37ºC	none	0	1.2	0.9	nd	nd	nd	(Chittum and Champney, 1995)
			erythromycin	75	1.6	0.88				
			erythromycin	300	7.5	0.7				
			erythromycin	450	7.5	0.65				
			none	0	1.1	0.95				
			erythromycin	75	2	0.96				
			erythromycin	300	4	0.92				
			erythromycin	450	5.5	0.88				
SK 901	TB	37ºC	none	0	0.75	1.9	12	23	nd	(Usary and Champney, 2001)
			erythromycin	75	6	1.6	15	24		
		27ºC	none	0	15	1.2	14	17		
			erythromycin	75	8	0.5	16	9		
		37ºC	none	0	1.5	1.4	20	27		
			erythromycin	18	8	0.3	25	8		
		27ºC	none	0	1.7	1.3	20	24		
			erythromycin	18	8	0.3	24	7		

Organism/Strain	Media	Tempe-rature	Antibiotic	Conc. (µg/ml)	Doubling time (h)	50S/30S	% total cpm		Protein synthesis	Ref
							30S	50S		
SK 901	TB	32ºC	azithromycin	0	0.6	1.8	18	33	100	(Silvers and Champney, 2005)
				2.5	0.9	0.7	25	17	83	
MRE 600 (RNase I)	TB	32ºC	azithromycin	0	0.6	2	21	41	100	
				1	2.5	0.3	22	6	11	
D10 (RNase I)	TB	32ºC	azithromycin	0	0.8	2	17	34	100	
				2.5	1.1	0.7	26	19	76	
SK7622 (RNase III)	TB	32ºC	azithromycin	0	1.1	1.8	19	34	100	(Silvers and Champney, 2005)
				2.5	1.6	1.3	18	23	48	
SK5665 (RNase E)	TB	32ºC	azithromycin	0	0.9	1.9	22	41	100	
				2.5	1.1	1.3	24	32	15	
CA244 (PNPase)	TB	32ºC	azithromycin	0	0.8	1.2	14	17	100	
				2.5	2.3	1.1	13	14	13	
SK4803 (RNase II)	TB	32ºC	azithromycin	0	2	2	21	42	100	
				2.5	2.6	1	19	19	68	
N7060 (RNase I,	TB	32ºC	azithromycin	0	0.7	1.7	19	33	100	
RNase II, PNPase)	TB	32ºC	azithromycin	2.5	1.2	0.5	22	11	29	
SK5704 (RNase II,	TB	32ºC	azithromycin	0	1.4	1.8	21	37	100	
RNase E, PNPase)	TB	32ºC	azithromycin	1	2.9	0.9	21	18	35	
Bacillus subtilis										
BD464 (pBD9, *ermC*)	TB	37ºC	none	0	1.2	1.09	nd	nd	nd	(Champney and Burdine, 1995)
			erythromycin	0.4	4.3	0.69				
			clarithromycin	0.4	4.2	0.64				
			azithromycin	1.2	4.8	0.31				
			oleandomycin	30	4.6	1.03				
			erythromycin	1500		0.55				

Organism/Strain	Media	Tempe-rature	Antibiotic	Conc. (µg/ml)	Doubling time (h)	50S/30S	% total cpm		Protein synthesis	Ref
							30S	50S		
Staphylococcus aureus										
RN 1786	TB	37ºC	none	0	0.7	1.04	nd	nd	nd	(Champney and Burdine, 1995)
			erythromycin	0.4	4.2	0.65				
			clarithromycin	0.4	4.7	0.57				
			azithromycin	1.5	3.5	0.67				
			14-hydroxyclarithromycin	2	6	0.70				
			flurithromycin	2	4.8	0.74				
			roxithromycin	2	4.5	0.89				
			oleandomycin	30	3.9	0.80				
			lincomycin	0.4	2.3	1.08				
			virginiamycin M1	0.5	1.8	0.97				
			virginiamycin S	75	2.7	1.03				
RN 1786	TSB	37ºC	none	0	0.7	1.7	20	34	100	(Champney *et al.*, 1998)
			3-deoxyclarithromycin	0.5	0.8	1.8	19	34	99	
			11,12-carbonate-3-deoxycl.	0.5	0.9	1.3	19	24	91	
			azithromycin	0.5	1.2	1.9	17	33	79	
			oleandomycin	0.5	1.2	1.5	19	28	69	
			flurithromycin	0.5	1.7	1.1	21	24	59	
			roxithromycin	0.5	1.5	1.3	19	24	40	
			14-hydroxyclarithromycin	0.5	1.7	1.3	19	24	40	
			erythromycin	0.5	2.4	0.9	22	19	30	
			clarithromycin	0.5	7	0.6	11	7	6	

Organism/Strain	Media	Tempe-rature	Antibiotic	Conc. (µg/ml)	Doubling time (h)	50S/30S	% total cpm		Protein synthesis	Ref
							30S	50S		
RN 1786	TSB	37ºC	none	0	0.8	2.1	17	35	100	(Champney and Tober, 1998)
			A22 (Abott 156785)	1	0.8	2	19	38	98	
			A53 (Abott 170585)	1	0.9	2	20	40	73	
			A51 (Abott 174620)	1	0.9	1.8	20	36	74	
			A11 (Abott 182026)	1	1.7	1.4	20	28	51	
			A23 (Abott 163685)	1	3.3	1.4	17	23	32	
			A52 (Abott 165695)	1	2.7	1.3	19	25	20	
			A24 (Abott 157395)	1	5.4	1.5	11	16	11	
			A54 (Abott 177511)	1	5.7	1.6	7	11	5	
			A55 (Abott 161741)	1	4.2	1.3	8	11	5	
			HMR 3647	1	7.3	1	8	8	3	
			HMR 3004	1	7.3	0.8	5	4	3	
RN 1786	TSB	37ºC	none	0	0.8	2.1	16	34	100	(Champney and Tober, 1999)
			clarithromycin (cl.)	0.2	3.9	1.3	20	25	18	
			11,12-carbonate cl.	0.2	4.6	1.2	17	20	13	
			3-keto cl.	0.5	1.7	1.9	16	30	45	
			3-keto-11,12-carbonate cl.	0.5	3.4	0.7	18	12	13	
			3-deoxy cl.	10	1.4	1.8	16	29	58	
			3-deoxy-11,12.carbonate cl.	10	2.7	1.4	16	23	26	
	TB	37ºC	none	0	1.5	1.8	17	30	100	
			clarithromycin (cl.)	0.2	4.7	1.1	20	22	16	
			11,12-carbonate cl.	0.2	6.3	1	19	19	12	
			3-keto cl.	0.5	2.7	1.3	19	25	34	

Organism/Strain	**Media**	**Tempe-rature**	**Antibiotic**	**Conc. (µg/ml)**	**Doubling time (h)**	**50S/30S**	**% total cpm**		**Protein synthesis**	**Ref**
							30S	**50S**		
RN 1786	TB	37ºC	3-keto-11,12-carbonate cl.	0.5	4.3	0.8	17	13	16	(Champney and Tober, 1999)
			3-deoxy cl.	10	2.3	1.3	19	24	57	
			3-deoxy-11,12.carbonate cl.	10	3.6	1.1	16	17	33	
SK 1786	TSB	37ºC	evernimicin	0	0.8	1.9	20	38	100	(Champney and Tober, 2000a)
				0.02	1.3	1.6	21	34	59	
				0.04	2.5	1.4	23	33	35	
				0.08	2.9	1.4	22	31	24	
				0.16	3.5	1.4	22	30	19	
				0.32	4.7	1.1	22	24	17	
				0.64	7.8	0.9	18	16	14	
RN 4220 (*ermC*)	TSB	37ºC	evernimicin	0	0.6	1.4	23	33	100	
				0.32	3.3	1.1	22	25	17	
SK 983 (*ermC*)	TSB	37ºC	evernimicin	0	0.6	1.5	23	35	100	
				0.32	4.3	0.9	22	19	16	
A 1018 (MRSA *ermA*)	TSB	37ºC	evernimicin	0	0.6	1.9	19	37	100	
				0.04	1.4	1.4	23	33	33	
A 1024 (MRSA)	TSB	37ºC	evernimicin	0	0.75	1.7	21	35	100	
				0.04	1.9	1.2	22	26	23	

Organism/Strain	Media	Tempe-rature	Antibiotic	Conc. (µg/ml)	Doubling time (h)	50S/30S	% total cpm		Protein synthesis	Ref
							30S	50S		
RN 1786	TSB	37ºC	none	0	0.75	2.1	18	38	100	(Champney and Tober, 2000b)
			tylosin	0.5	1	1.2	21	25	85	
			spiramycin	10	3.3	1.3	16	21	13	
			lincomycin	0.2	1.8	1.1	19	21	73	
			clindamycin	0.1	3.2	0.7	22	16	53	
			pristinamycin IA	0.75	1.1	1.4	20	28	87	
			virginiamycin S	10	2	1.6	21	33	40	
			CP37277	10	2.4	1.1	22	25	46	
			virginiamycin M1	2	2.3	2.4	14	33	12	
			CP36926	0.5	1.1	2	13	26	46	
			Chloramphenicol	20	3.6	2	13	26	14	
			daltopristin/	0.02	1	2.1	19	40	42	
			quinupristin	0.035	2.2	2.4	14	34	31	
			(70/30)	0.05	2.5	1.3	9	12	19	

Additional table 2. IC_{50} values (µg/ml) for antibiotic inhibition in different organisms

Organism/Strain	Antibiotic	growth	transl.	50S	30S	Reference
Staphylococcus aureus						
RN 1786	Erythromycin	0.17	0.38	0.36	nd	(Champney and Burdine, 1996)
RN 4220 (*ermC*)	Erythromycin	nd	0.75	nd	nd	(Champney *et al.*, 2003)
RN 1786	Clarithromycin	0.075	0.15	0.15	nd	(Champney and Burdine, 1998)
	Azithromycin	2.5	5	5		
RN 1786	Clarithromycin	nd	0.075	0.15	nd	(Champney and Tober, 2001)
	HMR 3832		0.055	0.11		
	HMR 3647		0.04	0.08		
	HMR 3787		0.03	0.06		
	HMR 3562		0.03	0.06		
	HMR 3004		0.025	0.05		
	ABT-773		0.02	0.035		
RN 1786	TAN-1057A	6	4.5	9		(Champney *et al.*, 2001)
RN 1786	Linezolide	0.6	0.3	0.6		(Champney and Miller, 2002b)
RN 1786	Paromomycin	7.5	3	nd	2.75	(Mehta and Champney, 2003)
	Neomycin	3	1.25		3	
RN 1786	Retapamulin	nd	0.005	0.027	nd	(Champney and Rodgers, 2007)
A1024 (meth.-resist.)	Retapamulin	nd	0.017	0.02	nd	
A1024 (meth.-resist.)	Quin./dalf.	0.55	0.22	0.4	nd	(Mabe and Champney, 2005)
	XRP2868	0.14	0.12	0.2		
Escherichia coli						
SK 901	Paromomycin	4	3.2	nd	6.4	(Mehta and Champney, 2002)
	Neomycin	3	3.6		7.2	
D10-1	Hygromycin B	25	16	nd	65	(McGaha and Champney, 2007)

Organism/Strain	Antibiotic	growth	transl.	50S	30S	Reference
Haemophilus influenzae						
G79-89	Erythromycin	1.5	1.5	8	nd	(Champney and Miller, 2002a)
	Azithromycin	0.4	0.4	0.9		
G79-89	Clarithromycin	7.8	5.6	9	42.3	(Mabe *et al.*, 2004)
	Flurithromycin	5.4	6	8	11.5	
	Roxithromycin	7.9	9	12.5	12.2	
G79-89	Telithromycin	1.2	1.25	2.5	2.5	(Champney and Tober, 2003)
	ABT-773	1.2	1.25	2.5	2.5	
G79-84 (β-lactamase-positive)	Quin./dalf.	3	3	3.6	nd	(Mabe and Champney, 2005)
	XRP2868	0.7	0.6	0.75		
Streptococcus pneumoniae						
ATCC 49619	ABT-773	0.005	0.0025	0.005	nd	(Champney and Pelt, 2002b)
ATCC 49619	Telithromycin	0.015	0.0075	0.0075	nd	(Champney and Pelt, 2002a)
11591 (*ermB*)	ABT-773	0.025	0.035	0.035	nd	(Champney *et al.*, 2004)
	Telithromycin	0.03	0.08	0.05		
11591 (*ermB*)	Quin./dalf.	0.5	0.3	0.3	nd	(Mabe and Champney, 2005)
	XRP2868	0.17	0.12	0.17		
Streptococcus pyogenes						
A7193034	ABT-773	0.009	0.01	0.011	nd	(Champney *et al.*, 2004)
	Telithromycin	0.016	0.012	0.015		
CCC66F (*ermA*)	ABT-773	0.011	0.012	0.015	nd	
	Telithromycin	0.04	0.055	0.055		
1721 (*ermB*)	ABT-773	0.075	nd	0.215	nd	
	Telithromycin	0.21		0.38		

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SUMMARY IN ESTONIAN

Antibiootikumide mõju ribosoomide kokkupakkimisele on kaudne

Ribosoomide kokkupakkimine on keeruline protsess, mis soolekepikeses *Escherichia coli* koosneb 54 valgu ja 3 ribosomaalse RNA koordineeritud sünteesist ja assambleerumisest funktsionaalseks ribosoomiks. Ribosoomide kokkupakkimine algab samaaegselt ribosomaalse RNA transkriptsiooniga ja hõlmab ribosomaalse RNA ja ribosomaalsete valkude protsessimist, modifitseerimist ja ruumilist voltumist.

E. coli 70S ribosoom koosneb kahest subühikust: suur (50S) subühik sisaldab kahte ribosomaalset RNA-d (23S ja 5S rRNA) ja 33 valku ning väike (30S) subühik sisaldab ühte ribosomaalset RNA-d (16S rRNA) ja 21 valku. RNA on domineeriv komponent moodustades eeltuumses organismis kaks kolmandikku kogu massist.

Rohkem kui 50 aastat tagasi näidati, et valgu sünteesi inhibiitor klooramfenikool põhjustab küpsete subühikutega võrreldes aeglasemalt sedimenteeruvate ribosomaalsete partiklite kuhjumist (Dagley and Sykes, 1959; Nomura and Watson, 1959). Need partiklid sisaldasid ribosomaalset RNA-d ja ribosoomi valkude mittetäielikku komplekti (Adesnik and Levinthal, 1969; Sykes *et al.*, 1977). Klooramfenikooli lisamine rakukultuuri põhjustab ribosomaalsete valkude sünteesi stöhiomeetria kadumist (Dennis, 1976) ning lisaks sünteesitakse ribosomaalset RNA-d ülehulgas võrreldes ribosomaalsete valkudega (Lazzarini and Santangelo, 1968; Midgley and Gray, 1971; Shen and Bremer, 1977). Arvatakse, et valgusünteesi inhibeerimine põhjustab ribosoomi komponentide tasakaalustamata sünteesi, mis omakorda põhjustab ribosoomi kokkupakkimise inhibeerimist (Dodd *et al.*, 1991).

Umbes 15 aastat tagasi pakuti välja, et erütromütsiin ning paljud teised antibiootikumid takistavad otseselt ainult suurema subühiku kokkupakkimist (Champney, 2006). Oletati, et erütromütsiin seondub suure subühiku eellaspartiklile ning takistab selle edasist kokkupakkimist funktsionaalseks subühikuks (Champney, 2006).

Antud uuringu põhjal võib järeldada, et:

I. Nii erütromütsiini kui ka klooramfenikooli juuresolekul kasvavates rakkudes kogunevad suurema ja väiksema subühiku eellaspartiklid, mis viitab sellele, et mõlema subühiku kokkupakkimine on defektne. Need eellaspartiklid sisaldavad põhiliselt lõpuni protsessimata pre-16S rRNA-d või pre-23S rRNA-d. Me panime tähele, et RNA protsessimine on aeglasem madalamal temperatuuril nii rakkudes, mis kasvasid antibiootikumide juuresolekul, kui ka rakkudes, mis kasvasid ilma antibiootikumideta. Väikese resistentsuspeptiidi, mis toimib *in cis* küpsel ribosoomil, ekspressioon

leevendab erütromütsiini poolt põhjustatud ribosoomide kokkupakkimise defekti. Viidates sellele, et ribosoomide kokkupakkimine on erütromütsiini juuresolekul inhibeeritud kaudselt ehk valgusünteesi inhibeerimise tõttu.

II. Erütromütsiini ja klooramfenikooli juuresolekul kuhjuvatest partiklitest eraldatud ribosomaalse RNA modifikatsioonimuster on sarnane. Ribosomaalse RNA modifikatsioonide süntees sõltub ribosoomide kokkupakkimise edenemisest. Modifikatsioonid lisatakse järk-järgult ribosomaalse RNA ja ribosomaalsete valkude ühinemisel. Vabad subühikud ilma antibiootikumideta kasvatatud rakkudest sisaldavad vähesemal määral modifitseeritud rRNA-d võrreldes 70S ribosoomiks ühinenud subühikutega. Enamik 16S rRNA modifikatsioone sünteesitakse kokkupakkimise varasemas etapis, kuid suurem osa 23S rRNA modifikatsioone lisatakse kokkupakkimise hilisemas etapis.

III. Suurem osa klooramfenikooli või erütromütsiini juuresolekul kuhjuvatest ribosomaalsetest partiklitest küpsevad funktsionaalseteks ribosoomideks. Ribosomaalsete eellaspartiklite elektronmikroskoopia analüüs ja valgulise koostise määramine mass-spektriomeetrilise meetodiga näitab, et mõlemad antibiootikumid põhjustavad väga heterogeensete partiklite kogunemist. Erütromütsiini partiklite ja klooramfenikooli partiklite valguline koostis on sarnane, kuid mitte identne. Ribosomaalsete valkude produktsiooni tase antibiootikumiga töödeldud rakkudes korreleerub hästi individuaalsete ribosoomivalkude kogusega eellaspartiklites. Rakkude töötlemine erütromütsiini või klooramfenikooliga põhjustab ribosoomi valkude tasakaalustamata sünteesi, mis on seletuseks kokkupakkimise defektiga partiklite kogunemisele.

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PUBLICATIONS

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1. Main research interests:
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2. List of publications:

Siibak, T., Peil, L., Xiong, L., Mankin, A., Remme, J., Tenson, T. 2009. Erythromycin- and chloramphenicol-induced ribosomal defects are secondary effects of protein synthesis inhibition. Antimicrob Agents Chemother. 53(2):563–71.

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Siibak, T., Peil, L., Dönhöfer, A., Tats, A., Remm, M., Wilson, D.N., Tenson, T., Remme, J. 2011. Antibiotic-induced ribosomal assembly defects result from changes in the synthesis of ribosomal proteins. Mol Microbiol. 80(1):54–67

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II. Teaduslik ja arendustegevus

1. Peamised uurimisvaldkonnad:
   Peamiseks uurimisvaldkonnaks on olnud ribosoomide kokkupakkimine antibiootikumide juuresolekul kasvavates bakteri *Escherichia coli* rakkudes.

2. Publikatsioonide loetelu:

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