Antibiotic-induced ribosomal assembly defects result from changes in the synthesis of ribosomal proteins

Triinu Siibak,1 Lauri Peil,2 Alexandra Dönhöfer,3 Age Tats,1 Maido Remm,1 Daniel N. Wilson,3 Tanel Tenson2 and Jaanus Remme1*

Institutes of 1Molecular and Cell Biology and 2Technology, University of Tartu, Tartu, Estonia. 3Gene Center and Department for Biochemistry, University of Munich, Munich, Germany.

Summary

Inhibitors of protein synthesis cause defects in the assembly of ribosomal subunits. In response to treatment with the antibiotics erythromycin or chloramphenicol, precursors of both large and small ribosomal subunits accumulate. We have used a pulse-labelling approach to demonstrate that the accumulating subribosomal particles maturate into functional 70S ribosomes. The protein content of the precursor particles is heterogeneous and does not correspond with known assembly intermediates. Mass spectrometry indicates that production of ribosomal proteins in the presence of the antibiotics correlates with the amounts of the individual ribosomal proteins within the precursor particles. Thus, treatment of cells with chloramphenicol or erythromycin leads to an unbalanced synthesis of ribosomal proteins, providing the explanation for formation of assembly-defective particles. The operons for ribosomal proteins show a characteristic pattern of antibiotic inhibition where synthesis of the first proteins is inhibited weakly but gradually increases for the subsequent proteins in the operon. This phenomenon most likely reflects translational coupling and allows us to identify other putative coupled non-ribosomal operons in the Escherichia coli chromosome.

Introduction

Bacterial ribosomes are composed of three ribosomal RNAs (rRNAs) and over 50 ribosomal protein (r-proteins). The rRNA and r-proteins are organized into two unequal subunits, termed small (30S) and large (50S) subunits. In addition to the transcription of the rRNA and translation of r-proteins, ribosome biogenesis also involves processing and modification of the constituent components and assembly of them into functional particles. The co-ordinated synthesis of the ~60 different molecules is achieved through a variety of regulatory mechanisms (Nomura et al., 1984; Zengel and Lindahl, 1994; Condon et al., 1995). Ribosome subunit assembly is fast and efficient, occurring within 2–3 min following the transcription of the rRNA (Lindahl, 1975). The high efficiency of ribosome assembly is evident from the negligible turnover of ribosomal components during exponential growth at moderate to fast rates in wild-type Escherichia coli (Bremer and Dennis, 1987). rRNA processing, modification and association with r-proteins is already initiated concomitant with transcription (reviewed by Kaczanowska and Ryden-Aulin, 2007). Short-lived intermediate particles of both subunits are formed during ribosome assembly (Lindahl, 1975). These particles have been extensively analysed using in vitro ribosome reconstitution approaches (reviewed by Nierhaus, 1991; Culver, 2003). One predominant precursor particle, the 21S particle, is observed during the small subunit, whereas two assembly intermediate particles, 34S and 43S, are seen during large subunit assembly. The protein composition of the subunit assembly intermediate particles has been used to define the order of the r-protein association with the rRNA, culminating in the ribosome subunit assembly maps (Held et al., 1974; Herold and Nierhaus, 1987). Assembly maps depict the cooperativity between binding of individual proteins. More recent experiments have identified multiple parallel pathways for association of small ribosome subunit proteins with 16S rRNA during reconstitution of the 30S subunit (Talkington et al., 2005; Mulder et al., 2010).

Chloramphenicol and erythromycin are well-known inhibitors of protein synthesis (reviewed by Wilson, 2009). These antibiotics bind to the large ribosomal subunit (Schlünzen et al., 2001; Bulkley et al., 2010; Dunkle et al., 2010), chloramphenicol inhibiting peptidyl transfer and release (Monro and Marcker, 1967; Tompkins et al., 1970) and erythromycin entrance of the nascent peptide into the ribosome exit tunnel (Tenson et al., 2003; Lovmar et al., 2004). It was observed in the late 1950s that treatment of bacteria with chloramphenicol leads to the accumulation
of defective ribosomal particles (Dagley and Sykes, 1959). After several years of studies it was concluded that the assembly inhibition is indirect, caused by inhibition of r-protein synthesis (Dodd et al., 1991). In the 1990s it was recognized that erythromycin also causes accumulation of defective ribosomal particles (Chittum and Champney, 1995). In this case it was suggested that the drug binds to the ribosomal large subunit precursor particles and thereby directly inhibits the progression of subunit assembly (Usary and Champney, 2001). Our recent observation that both chloramphenicol and erythromycin inhibit assembly of both ribosomal subunits, in combination with the result that a ribosomal resistance mechanism that does not function on the precursor particles can rescue the erythromycin induced assembly defect, suggests that both drugs inhibit assembly indirectly (Siibak et al., 2009). The indirect mechanism suggests that the composition of the assembly-defective particles reflects the amounts of r-proteins synthesized in the presence of the drugs, with production of different r-proteins being suppressed to different extents. Here we use quantitative mass spectrometry to determine the compositions of the precursor particles and measure the amounts of r-proteins produced in the presence of the antibiotics chloramphenicol and erythromycin.

Results

Time-course of ribosome subunit assembly

The assembly-defective ribosomal particles start to accumulate immediately after addition of chloramphenicol or erythromycin to the exponentially growing *E. coli* culture (Siibak et al., 2009). We used a pulse-labelling approach to study whether or not the subribosomal particles are able to mature into 70S ribosomes. *E. coli* culture was grown at 25°C where ribosomal assembly is slower compared with the assembly at 37°C, thus allowing better resolution of the intermediate particles (Peil et al., 2008; Al Refaai and Alix, 2009). The exponentially growing bacterial culture was incubated with either chloramphenicol or erythromycin for 5 min, which is sufficient for inhibition of translation and ribosome subunit assembly (Siibak et al., 2009). RNA was labelled 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), after rifampicin addition, were lysed and the ribosomes were analysed by sucrose density gradient centrifugation.

Figure 1A shows the time-course of ribosome assembly in the untreated cells. The optical curve shows the positions of the 70S ribosomes and the free 30S and 50S subunits. The radioactive diagram depicts the newly made ribosomal particles formed in the presence or absence of drugs. In the first time point, collected at the time of rifampicin addition, the majority of radioactively labelled RNA was found in the 30S and 50S fractions, whereas 5 min later the radioactivity was distributed equally between the free subunits and the 70S fractions. After 10 min, most of the radioactive signal was found in the 70S fraction, indicating that the ribosome assembly was complete and that there was no significant degradation of rRNA. Further incubation did not change the distribution of radioactive RNA in sucrose gradients. Thus, the mean time for entering the newly transcribed rRNA into 70S pool is 5 min at 25°C, in agreement with previous observations (Peil et al., 2008).

When erythromycin was added to the culture, most of the radioactivity was in the 30S and 25S fractions at the starting time point (Fig. 1B). After 40 min, approximately equal amounts of [3H]uridine were found in the 30S, 50S and 70S regions. Upon further incubation the proportion of radioactivity in the 70S fraction continued to grow leaving only a minor peak to the 35S region of the 60 min time point. Similar results were obtained with chloramphenicol, although assembly was a slightly faster in this case: already at 20 min after rifampicin addition, radioactivity is distributed equally in the 70S and free subunits (Fig. 1C). Nevertheless, 1 h after stopping RNA synthesis, most of the radioactivity was also found in the 70S ribosome fractions. In summary, the mean time of ribosome assembly was 40 min in the presence of erythromycin and 20 min in the presence of chloramphenicol. The results show that 70S ribosomes are assembled in the presence of chloramphenicol and erythromycin, but that the rate of ribosome assembly is significantly reduced. The latter finding is consistent with the slow maturation of rRNA observed in the presence of chloramphenicol or erythromycin (Siibak and Remme, 2010). Finally, it is important to note that majority of the rRNA is finally incorporated into 70S ribosomes without significant rRNA degradation being observed.

Protein content of the precursor particles

As a next step, chloramphenicol and erythromycin were added to the exponentially growing *E. coli* culture and the ribosomal particles accumulating in the presence of drugs were separated by two consecutive sucrose density gradient centrifugation steps (Fig. 2). The assembly intermediate particles were initially analysed by negative-stain electron microscopy (EM) (Fig. 2B and C) in comparison with mature 30S and 50S ribosomal subunits and 70S ribosomes (Fig. 2A). 35S and 45S particles are assembly intermediates of the large ribosomal subunit and 25S particles are precursors of the mature 30S ribosomal subunit. The results show that 25S and 35S intermediate particles isolated from chloramphenicol- and
Erythromycin-treated cells are highly heterogeneous, with a tendency for aggregation. In contrast, 45S particles show higher homogeneity, with morphological similarity to mature 50S ribosomal subunits.

Subsequently, the levels of r-proteins in the assembly intermediate particles were determined using a quantitative mass-spectrometry approach. The collected particles were mixed with equimolar amount (according to the

Fig. 1. 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 labelled 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 grey bars.
absorbance at 260 nm) of the corresponding *E. coli* ribosomal subunit containing uniformly \[^{15}\text{N}\]-labelled r-proteins. R-proteins of the mixed particles were digested with trypsin and the \[^{15}\text{N}]/{[^{14}\text{N}]} ratio in tryptic peptides was determined by mass spectrometry, as described previously (Pulk *et al*., 2010). Relative amounts of r-proteins in the subribosomal particles were calculated by taking the \[^{15}\text{N}]/{[^{14}\text{N}]} ratio of primary rRNA-binding proteins L3 and S15 as 100% for the large and small subunit proteins respectively (Fig. 3). The average occupancy of the reference proteins was 70% for S15 in the 25S, 90% for L3 in the 35S and 100% for L3 in the 45S particles (data not shown).

The 25S particles contain proteins S15, S16 and S18 in nearly equal high amounts. 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 particles with similar protein composition (Fig. 3A, correlation in Fig. S1). A difference is observed for proteins S4 and S11, which are more abundant in particles accumulating in response to chloramphenicol as compared with erythromycin (Fig. 3A). The protein composition of 25S particles suggests that the lack of primary 16S rRNA-binding proteins is the reason for their accumulation in response to drug treatment.

In the 35S particles most proteins are present in low or negligible amounts relative to the level of L3. Only L11 is present at \(\sim\)100% in the case of both antibiotics. In general, the two 35S fractions induced by either antibiotic are similar to each other (Fig. 3B, correlation in Fig. S2). The biggest difference concerns the large subunit assembly-initiator protein L24, which is present at \(\sim\)100% in the ‘chloramphenicol 35S particles’, but four times less \((\sim25\%)\) in the ‘erythromycin particles’ (Fig. 3B). The other two proteins being present in erythromycin particles in considerably lower levels as compared with the chloramphenicol particles are L4 and L23. In contrast, L6 and L9 are present in erythromycin particles at considerably higher levels compared with the chloramphenicol particles. It is evident that the protein composition of 35S particles is different from the known early assembly particles (Herold and Nierhaus, 1987; Nierhaus, 1991). The 45S particle contains high levels of most of the large subunit proteins, with only L16, L35 and L36 being present at less than 30%. This is in line with the previous observation that L16 is required for the late events of large ribosome subunit assembly (Franceschi and Nierhaus, 1990). In the 25S and 35S particles most of the proteins

---

**Fig. 2.** Sucrose density gradient centrifugation profiles from (A) control with no antibiotic (none), (B) chloramphenicol (Cam)- and (C) erythromycin (Ery)-treated cells. Fractions from sucrose gradients were collected (grey shaded area) and negative-stain EM was performed on the (A) mature ribosomal subunits (30S and 50S) and 70S ribosomes, in comparison with intermediate particles from the small ribosomal subunit (25S) and the large ribosomal subunit (35S and 45S) from (B) Cam- and (C) Ery-treated cells.
are present in substoichiometric amounts indicating that the particles are heterogeneous, in agreement with the EM images.

Proteins synthesized in the presence of the drugs

Ribosomal subunit assembly depends on the availability of the r-proteins. In particular, the primary binding assembly initiator proteins have an important role during early stages of ribosome subunit assembly. Since the protein composition of 25S and 35S proteins does not coincide with that of the known early assembly intermediate particles (21S and 34S respectively) (Fig. 3), it might reflect instead the availability of r-proteins in the drug-treated cells. Additionally, an important point is whether the drugs inhibit production of r-proteins in a uniform manner or

Fig. 3. Quantification of proteins from (A) 30S subunit precursor particles (25S) and (B and C) 50S subunit precursor particles (35S and 45S). 25S, 35S or 45S particles ([14N]) were mixed with [15N] 30S or 50S subunits and r-proteins were isolated. The ratio of [14N] and [15N] r-proteins was determined by quantitative mass spectrometry and normalized against L3 (L3 = 100%). White bars indicate proteins from ribosomal particles in erythromycin (Ery)-treated cells, whereas grey bars indicate proteins from ribosomal particles in chloramphenicol (Cam)-treated cells. Standard error is indicated.
whether the inhibition is more pronounced for some specific proteins. Therefore we used a pSILAC approach (Schwanhauser et al., 2009) to measure the production of individual r-proteins in the presence of the antibiotics chloramphenicol and erythromycin. Exponentially growing cells were divided into two parts. In one sample ‘light’ medium (unlabelled amino acids, Arg0:Lys0) was changed against isotopically labelled ‘heavy’ medium (Arg10:Lys8) and antibiotic was added; cells were collected after 4 h. To obtain the control cells without antibiotic treatment, the other sample was diluted with equal amount of fresh unlabelled medium, after 2 h the medium was switched to ‘medium’ labelled (Arg6:Lys4) and the cells were collected after 2 h. The different labelling period in the presence (4 h) and absence of the antibiotics (2 h) was important to have similar label incorporation in both samples, as the antibiotics suppress protein synthesis and thereby label incorporation. Equal amounts of cells, as estimated by optical density, were mixed together, lysed and the proteins were protease digested and analysed by mass spectrometry. The measured heavy-to-medium ratio was used in calculations.

Inhibition of translation by chloramphenicol or erythromycin causes enhancement (for example, as seen for L5) or inhibition of r-protein synthesis as related to the level of average protein synthesis. However, production of individual r-proteins differs markedly (Table S1). To test for the possible correlations between synthesis of r-proteins and the compositions of the subribosomal particles, a linear regression analysis was used. The synthesis of large ribosomal subunit proteins correlates with their amounts in the 35S particles, the coefficient of determination, $R^2$, was 0.38 for erythromycin measurements and 0.4 for the chloramphenicol case. In the case of both antibiotic treatments, the L5 level was unproportionally high. Therefore, after removal of the data for L5 the correlations become considerably stronger ($R^2$ around 0.6) (Fig. 4) indicating that the protein composition of 35S particles is largely determined by protein availability in the cells. The correlation of 25S composition with ribosomal small subunit protein synthesis is weaker ($R^2$ being 0.28) in the case of erythromycin, or absent ($R^2$ being 0.08) in the case of chloramphenicol. We conclude that the composition of 35S particles, but not the 25S particles, reflects the protein levels produced in the cell in the presence of the drugs.

**Detection of coupled cistrons using protein levels**

The genes for r-proteins are organized in operons and certain r-proteins can act as a translational inhibitor within their own operon (Nomura et al., 1984). In Fig. 5, the levels of synthesis of r-proteins are mapped onto the operon structures with examples of autogenous regulation (arrowed). Interestingly, in some operons, exemplified by the S10 operon, we observe a general trend where the first protein in the operon is synthesized at the highest level, followed by a gradual loss of production over the following cistrons. In the spc operon, L5 (encoded by the third cistron) is present in the highest amount, followed by a gradual decrease of proteins from the subsequent cistrons. There are some interesting exceptions to that rule, seen for L17, L7/L12 and L19 in the $\alpha$, rpoBC and trmD operons respectively. The pattern of gradual decrease is consistent with the mechanism of translational coupling described previously for r-protein operons (Nomura et al., 1984; Zengel and Lindahl, 1994). As the initiation of translation of a downstream gene depends on the number of ribosomes reaching the termination codon of the upstream gene the inhibition of translation would lead to gradual decrease of translation in the operon. Therefore we considered the gradually decreasing pattern in the operon as an indicator of translational coupling. We screened our data set for other, non ribosomal operons exhibiting this pattern and found several potential candidates (Fig. 6). We note that while the gradual decrease is seen very clearly for ribosomal operons, only a limited number of other operons showed a similar pattern. This
may partly relate to the fact that while r-proteins are expressed at very high levels, other operons often code for proteins expressed at low to very low levels. This in turn hinders reliable mass-spectrometry measurements and creates ‘holes’ in the operon maps of the lower expressed proteins. Nevertheless, a gradual decrease was observed in the his operon, although this was only in the presence of erythromycin. Another example of an operon showing a gradually decreasing pattern starts with yceD. In the lptA operon, the first three genes seem to be

Fig. 5. Levels of r-protein synthesis in the presence of erythromycin (Ery, white bar) and chloramphenicol (Cam, grey bar) respectively. Feedback regulation of operons is shown with arrows, and regulatory r-proteins are highlighted with a grey box (Nomura et al., 1984). Non-r-proteins are marked by white bars and grey-shaped boxes. Standard errors are indicated. (A) spc operon; (B) S10 operon; (C) α operon; (D) rpoBC operon; (E) trmD operon; (F) S6 operon.

Fig. 6. Levels of protein synthesis in the presence of erythromycin (Ery, white bar) and chloramphenicol (Cam, grey bar) respectively. The data are presented on the operon structures. Standard errors are indicated.
A. The well-documented case of translational coupling of atpH, atpA and atpG (boxed) (Hellmuth et al., 1991; Rex et al., 1994).
B–D. Additional cases of translational coupling suggested by the current analysis.
coupled whereas the other genes in the operon do not. Translational coupling between *atpH*, *atpA* and *atpG* genes (Fig. 6A) has been described previously (Gerstel and McCarthy, 1989; Hellmuth et al., 1991; Rex et al., 1994) consistent with the gradual pattern observed in our data set.

**Discussion**

We have confirmed that assembly-defective particles accumulate in response to treatment of *E. coli* cells with either chloramphenicol or erythromycin (Fig. 2). Pulse-labelling experiments show (Fig. 1) that most of the particles can mature into functional ribosomes albeit with a reduced rate, which is qualitatively consistent with previous reports for chloramphenicol particles (Hosokawa and Nomura, 1965; Nomura and Hosokawa, 1965; Adesnik and Levinthal, 1969). The experiments indicate that the drug-induced subribosomal particles represent assembly intermediates. Why do chloramphenicol and erythromycin slow down ribosome subunit assembly? Binding to assembly intermediates causing direct inhibition has been proposed for erythromycin (Usary and Champney, 2001) and an indirect effect through inhibition of r-protein synthesis has been suggested for chloramphenicol (Dodd et al., 1991).

Both erythromycin and chloramphenicol cause accumulation of 25S, 35S and 45S particles. The corresponding particles induced by both drugs have similar, although not identical, protein composition (Fig. 3), suggesting that the subribosomal particles of both antibiotics accumulate due to the similar defect in ribosome biogenesis. The 25S and 35S particles contain individual proteins at very different levels, with a continuum from only trace amounts (e.g. S1, S2 and L15, L16, L32) to apparent full occupancy (S15, S18 and L3, L4, L11). This shows that the particles are very heterogeneous, a finding we have confirmed by EM (Fig. 2). Furthermore, the heterogeneity of the subribosomal 25S and 35S particles indicates that the antibiotics do not block a specific state in the assembly, but rather act on a more general protein synthesis level. R-protein composition of the 45S particles is more complete as compared with that of the 35S (Fig. 3), although several proteins are still present in low amounts. Recently, Williamson and colleagues have analysed r-protein composition of the subribosomal particles formed in the presence of neomycin by using quantitative LC/MS approach (Sykes et al., 2010). The protein composition of 21S particles formed in the presence of neomycin was very similar to that described here for ‘chloramphenicol-25S particles’. Protein composition of the pre-50S subunits formed upon neomycin treatment described by Sykes et al. (2010) is similar to the protein content of the ‘chloramphenicol-induced 35S subunits’ (Fig. 3).

In *E. coli*, ribosomal assembly intermediates have been characterized using cell-free reconstitution systems. Assembly of the small subunit proceeds via one clearly separable precursor particle (21S) and the large subunit has two precursors (34S and 43S) (Lindahl, 1975). It is possible that the 25S, 35S and 45S subribosomal particles formed upon addition of antibiotics are related to the previously described precursor particles. Small subunit reconstitution experiments have revealed the proteins required for early assembly intermediate formation: S4, S7, S8, S15, S17 and S20 (Held et al., 1974; Holmes and Culver, 2004). Large subunit reconstitution experiments identified L3, L4, L13, L20, L22 and L24 as essential proteins for the first assembly intermediate (34S) formation (Herold and Nierhaus, 1987). Proteins S4 and S7 of the small subunit and L3 and L24 of the large subunit were identified by reconstitution experiments as assembly initiator proteins (Nowotny and Nierhaus, 1982; 1988). Our data show that both erythromycin- and chloramphenicol-induced 25S particles contain the assembly essential proteins S4, S7, S8, S17 and S20 at levels < 60% of nominal amount. In the erythromycin-induced 35S particles, the early assembly essential proteins L4, L22 and L24 are present at levels < 50% (Fig. 3B), whereas in the chloramphenicol-induced 35S particles the proteins L13, L20 and L22 are also found at levels < 50%. This shows that several proteins identified previously as being essential for early assembly are present at low levels in the 35S and 25S particles. Since the particles are still formed and can mature into functional ribosomes, this suggests that under certain stress conditions, such as translation inhibition, assembly proceeds via alternative pathways. Precedents are documented; for example, in the absence of assembly initiator protein L24, protein L20 has been shown to take over its role in initiating large ribosome subunit assembly *in vitro* (Francoschi and Nierhaus, 1988). Similarly, multiple pathways of small subunit protein binding to 16S rRNA have been observed during 30S subunit reconstitution and a phenomenon termed the ‘assembly landscape’ explains incomplete cooperativity of small ribosome subunit assembly (Talkington et al., 2005). Unlike the composition of 35S particles, the protein composition of 45S particles is in general agreement with that of the second assembly intermediate (43S particle) and thereby with the large ribosome subunit assembly map (Nierhaus, 1991).

The protein composition of the 35S and 45S particles might reflect direct inhibition of assembly by the drugs or be caused by unbalanced synthesis of r-proteins during antibiotic inhibition. To investigate this issue the proteins produced during antibiotic treatment were determined in relation to the proteins produced in the absence of the drugs. Inhibition of translation by chloramphenicol or erythromycin is expected to cause overexpression of
rRNA in relation to r-proteins. This in turn should lead to the derepression of r-protein operons (Dean et al., 1981; Nomura et al., 1984; Mattheakis and Nomura, 1988; Zengel and Lindahl, 1994). The protein composition of the subribosomal particles was compared with the relative levels of r-proteins produced in the presence of drugs. The synthesis of large subunit r-proteins correlates well with their amounts in the 35S particles (Fig. 4): All r-proteins present in 35S at levels near to 100% are produced at ratios above 1.5 when compared with the uninhibited control. Conversely, proteins produced at ratios around 0.5 are found in the 35S particles at levels < 10% (Table S1). The composition of the 25S particles does not however reflect the proteins produced in the presence of the drugs, but rather the assembly map, with the late assembly proteins being present in negligible amounts. The lack of correlation in the 25S may reflect the lower general exchangeability of small subunit r-proteins compared with large subunit r-proteins (Pulk et al., 2010).

Nevertheless, the observation that composition of the 35S particles reflects the amounts of r-proteins produced, in combination with the fact that the drugs inhibit assembly of both subunits, leads us to the conclusion that during treatment with either chloramphenicol or erythromycin, ribosomal assembly is inhibited through an indirect mechanism, i.e. via inhibition of r-protein synthesis.

In general, the levels of individual proteins produced in the cell are very similar in the presence of both chloramphenicol and erythromycin. The same is true for the protein compositions of the respective accumulating subribosomal particles. Still, there are some interesting differences between the two drugs: production of the proteins L23 and L24 is inhibited considerably stronger by erythromycin as compared with chloramphenicol, the difference being reflected in the composition of the corresponding 35S particles. The low level of the assembly initiator protein L24 can cause relative over-representation of 35S-like particles in the erythromycin-treated cells (Figs 1 and 2). Decreased level of L24 could also account for the apparently specific erythromycin inhibition of 50S subunit assembly, as observed by others (Usary and Champney, 2001). What might cause the erythromycin specific decrease in the L24 level? It has been observed previously that both antibiotics can have inhibitory effects depending on the nascent peptide sequence (Lovett and Rogers, 1996; Tenson and Ehrenberg, 2002; Ramu et al., 2009; Starosta et al., 2010), thus suggesting that this kind of sequence-dependent differences are operational also here. Alternatively, more indirect influences, through differential effects on cell physiology, are also possible.

We observed that the amounts of r-proteins synthesized in the presence of the drugs decrease in the operons, with the first proteins made in the highest amounts followed by a gradual decrease (Fig. 5). Exceptions include the third cistron in the L5 sgc operon, which is produced at higher levels than upstream genes encoding L14 and L24 (Fig. 5A). Interestingly, the regulator protein S8 binds to the mRNA in front of the L5 coding region (Cerretti et al., 1988; Merianos et al., 2004) suggesting that the cistrons under translationally coupled regulation start with the open reading frame for L5, whereas the upstream ones are regulated through the mRNA degradation-mediated retroregulation (Mattheakis et al., 1989). Similarly, S4 and L10 act as regulator proteins for S13/L17 and L10/(L7/L12) in the α and rpoBC operons respectively (Fig. 5C and D) (Yates et al., 1980; 1981). L19 in the trmD operon also appears to be independently regulated from S16-RimM-TrmD (Fig. 5F), although non-autogenous regulation has been suggested for this operon (Wikström et al., 1988). It is also possible that the gradual decrease we describe is not caused by effects at the level of protein synthesis, but effects at the level of mRNA availability. Nevertheless, the data available for chloramphenicol inhibition (Cheung et al., 2003) show that the gradual decrease in protein levels is not reflected in mRNA levels, leaving translational effects as the prime candidate to explain the current results. It is noteworthy that the results of r-protein expression upon chloramphenicol treatment obtained by Dennis (1976) are in agreement with the gradual decrease in operons at variety of drug concentrations.

We have collected the data for about 1400 members of the E. coli proteome, allowing us to ask if other operons respond in a similar manner to the r-protein operons. We note that one limitation was that many operons previously described to be translationally coupled (Schümperli et al., 1982; Aksoy et al., 1984; Harms et al., 1988; Little et al., 1989; Wilson and Macnab, 1990; Gan et al., 1995; Lyngstadaa et al., 1995) are expressed only in specific conditions and therefore not detectable in our analysis. Nevertheless, we could confirm some previously described cases, for example the translational coupling between atpH, atpA and atpG (Gerstel and McCarthy, 1989; Hellmuth et al., 1991; Rex et al., 1994) (Fig. 6). Furthermore, several new cases of translational coupling are suggested by our analysis (Fig. 6): a gradual decrease is observed in the histidine biosynthesis operon in the presence of erythromycin. It is interesting to note that the his operon is induced by erythromycin, but not by chloramphenicol. This induction can be caused by stalling ribosomes at the attenuator open reading frame hisL (Johnston et al., 1980; Chan and Landick, 1993), consistent with the ability of erythromycin to allow synthesis of eight amino acids (Tenson et al., 2003) causing ribosome stalling at the beginning of the histidine codon track required for functional attenuation. Both erythromycin and chloramphenicol cause gradual decreases in the lptA and
iptB genes required for lipopolysaccharide assembly (Sperandeo et al., 2008), followed by rpoN, the sigma factor for nitrogen assimilation (Zhao et al., 2010). A similar decrease is observed in the operon that combines the rpmF gene for r-protein L32 with genes for fatty acid biosynthesis (Podkovyrov and Larson, 1995; Zhang and Cronan, 1998). In general, the gradual decrease characteristic for r-protein genes was clearly seen for only a few non-ribosomal operons suggesting that translational coupling is not very common for the highly expressed genes in E. coli. For ribosomal biosynthesis the translational coupling is expected to lead to stoichiometric production of r-proteins. Similarly, translational coupling has been proposed to be involved in ensuring the stoichiometry of the ATP synthase complex (Rex et al., 1994). The generally low level of translational coupling might indicate the lack of other complexes with such strict control of the stoichiometry of the components or the dominance of other mechanisms regulating protein production.

**Experimental procedures**

**Strains**

*Escherichia coli* strain MG1655 (Blattner et al., 1997) was used in all experiments, except for SILAC labelling, where arginine and lysine auxotrophic strain AT713 was used instead (a generous gift from Dr Matthias Selbach, Max Delbrück Center for Molecular Medicine).

**Isolation of* E. coli* ribosomal particles**

Cells were grown at 25°C in 200 ml 2× YT medium (Sambrook and Russell, 2001) until the $A_{600}$ reached 0.2. At this point, either erythromycin (final concentration, 100 μg ml$^{-1}$) or chloramphenicol (final concentration, 7 μg ml$^{-1}$) was added. The cultures were then incubated for a further 2 h. The cells were collected by centrifugation in a Sorvall GS-3 rotor at 4000 r.p.m. and 4°C for 10 min and were resuspended in 1 ml of lysis buffer [60 mM KCl, 60 mM NH$_4$Cl, 50 mM Tris-HCl (pH 8)], 6 mM MgCl$_2$, 6 mM β-mercaptoethanol, 16% sucrose]; lysozyme and DNase I (Amresco, GE Healthcare) were added to final concentrations of 1 mg ml$^{-1}$ and 20 U ml$^{-1}$ respectively. The cells were incubated for 15 min at −70°C and then thawed in ice-cold water for 30 min. The freeze–thaw cycle was repeated twice, followed by centrifugation at 13 000 g and 4°C for 20 min. The supernatant was diluted twofold with buffer A [60 mM KCl, 60 mM NH$_4$Cl, 10 mM Tris-HCl (pH 8)], 12 mM MgCl$_2$, 6 mM β-mercaptoethanol]. Lysate was first loaded onto a 30 ml, 10–25% (w/w) sucrose gradient prepared in buffer A and then centrifuged at 23 000 r.p.m. in an SW28 rotor (Beckman) at 4°C for 13.5 h. 45S, 35S gradient was then collected into 40 fractions. High-molecular-weight material was precipitated by adding an equal volume of 20% trichloroacetic acid (TCA). The precipitates were collected on glass-fibre filters, and the radioactivity incorporated was measured by scintillation counting.

**Negative-stain electron microscopy**

Ribosomal particles were diluted in Tico buffer [20 mM HEPES-HCl pH 7.6 (0°C), 6 mM MgCl$_2$, 30 mM NH$_4$Cl and 4 mM β-mercaptoethanol] to a final concentration of 1 $A_{600}$ ml$^{-1}$. One drop of each sample was deposited on a carbon-coated grid. After 30 s, grids were washed with distilled water and then stained with three drops of 2% aqueous uranyl acetate for 15 s. The remaining liquid was removed by touching the grid with filter paper. Micrographs were taken using a Morgagni transmission electron microscope (FEI), 40–100 kV, wide angle 1 K CCD at direct magnifications of 80–100 K.

**Pulse labelling**

*Escherichia coli* cells were grown at 25°C in 400 ml of tryptone (10 g l$^{-1}$)–yeast extract (1 g l$^{-1}$)–NaCl (10 g l$^{-1}$) until the $A_{600}$ reached 0.2. Erythromycin (final concentration, 100 μg ml$^{-1}$) or chloramphenicol (final concentration, 7 μg ml$^{-1}$) was added, 5 min later 20 μCi of [3H]uridine (38 Ci mmol$^{-1}$; GE Healthcare) was added, and 5 min later incorporation of the label was stopped by adding rifampicin (rifampicin) (final concentration, 500 μg ml$^{-1}$). Cells were collected at 0, 5, 10, 20, 20 and 60 min after the addition of rifampicin, centrifuged and lysed, and ribosomes were analysed by sucrose gradient centrifugation as described above. Each sucrose gradient was fractionated into 40 fractions. High-molecular-weight material was precipitated by adding an equal volume of 20% trichloroacetic acid (TCA). The precipitates were collected on glass-fibre filters, and the radioactivity incorporated was measured by scintillation counting.

**15N labelling and LC-MS/MS analysis**

*Escherichia coli* strain MRE600 was uniformly labelled by growing the cells in the presence of 15NH$_4$Cl as the only nitrogen source (Kalju Vanatalu, Tallinn University of Technology). Cells were lysed and ribosomes extracted as described above.

For quantitative studies, one $A_{600}$ unit of [15N]-labelled 50S or 30S wild-type ribosome subunits was mixed either with one $A_{600}$ unit of 45S or 35S particles, or with one $A_{600}$ unit of 25S particles from antibiotics-treated cells respectively. Ribosomal particles were precipitated with 10% ice-cooled TCA and proteins dissolved in 7 M urea/2 M thiourea. For protease digestion, disulphide bridges were reduced with 1 mM DTT for 1 h at room temperature and cysteines blocked with 5 mM iodoacetamide (IAA) for 1.5 h at room temperature. The urea concentration was diluted by adding two volumes of 100 mM NH$_4$HCO$_3$, and proteins were digested with endoprotease LysC (1:50 enzyme/protein ratio, Wako, Japan) for 4 h at room temperature. Subsequently, two volumes of 100 mM NH$_4$HCO$_3$ were added, and peptides were further digested with trypsin (1:50 enzyme/protein ratio, Promega, USA) for 12 h at room temperature. Peptides corresponding to approximately 0.2 $A_{600}$ units of ribosomes were purified on C18 StageTips (Rappsilber et al., 2007) and analysed using nano-LC-coupled mass spectrometry.
Peak lists for database searches were produced with raw2msm (Olsen et al., 2005), and proteins were identified using the Mascot search engine 2.2 (Matrix Science, UK) run against a custom-made *E. coli* protein database (4978 entries) that included the most commonly observed contaminant sequences, such as proteases, keratins, etc. The search criteria were as follows: full tryptic specificity was required (Trypsin/P); two missed cleavages were allowed; carbamidomethylation was set as a fixed modification; oxidation (M), N-acetylation (protein) and N-formylation (protein) were set as variable modifications; [15N] metabolic labelling was selected as quantification method; precursor ion mass tolerance was 0.8 Da; and the Mascot built-in decoy database option was used to estimate the false discovery rate (FDR) for peptides. The minimum ions score was set to 15 and the significance threshold was adjusted to keep FDR < 1% before saving the results for the subsequent quantitative analysis. Mascot result files were processed with MultiRawPrepare and N15 helper scripts before quantitative data analysis was performed with MSQuant v1.5 (http://www.ncbi.nlm.nih.gov/pubmed/19888749). Only bold red peptides were included in the analysis, classified by Mascot scores as Category A ≥ 33, Category B ≥ 25, Category C ≥ 15; protein was validated with a total ABC score of more than 40. N15 labelling was selected for quantification and only r-proteins were selected for quantifications, all quantified spectra were manually validated to remove borderline hits and erroneous quantifications; relative errors of protein quantifications were ≤ 20% as determined by MSQuant.

**pSILAC labelling**

*Escherichia coli* strain AT713 was grown at 25°C in 75 ml of ‘light’ MOPS minimal medium supplemented with 0.1% glucose and 100 μg ml⁻¹ of each amino acid (including ‘light’ arginine and lysine, Arg0 and Lys0) until the A₆₀₀ reached 0.2. Exponentially growing cells were divided into two parts and treated as described below.

Shortly, for antibiotic treatment one part of cells was pelleted and resuspended in pre-warmed MOPS ‘heavy’-labelled medium (Arg10:Lys8, CNLM-539 and CNLM-291, Cambridge Isotope Laboratories) supplemented with either chloramphenicol or erythromycin (7 and 100 μg ml⁻¹ respectively). Treated cells were grown for further 4 h at 25°C and collected. Control cells not getting antibiotic treatment were initially diluted with equal amount of fresh pre-warmed ‘light’ medium, after 2 h of growth cells were pelleted and resuspended in pre-warmed MOPS minimal ‘medium’-labelled medium (Arg6:Lys4, CLM-2265 and DLM-2640, Cambridge Isotope Laboratories). Control cells were grown for further 2 h at 25°C and collected. The different labelling period in the presence (4 h) or absence of the antibiotics (2 h) was important to have similar label incorporation in both samples, as the antibiotics suppress protein synthesis and thereby label incorporation. Equal amounts of cells, as estimated by optical density, were mixed together and processed as described below.

Cells were lysed in SDS/DTT/TRIS buffer according to FASP protocol (http://www.ncbi.nlm.nih.gov/pubmed/19377485) and total protein concentration was measured using tryptophan fluorescence (excitation 295 nm, emission 350 nm, with an assumption that 1 μg of tryptophan equals to 91 μg of lysate). Next, 100 μg of lysate was LysC and trypsin double-digested according to FASP protocol (http://www.ncbi.nlm.nih.gov/pubmed/19377485), and resulting peptides were fractionated using SAX-C18 StageTip-based protocol (http://www.ncbi.nlm.nih.gov/pubmed/19848406) and analysed using nano-LC-coupled mass spectrometry.

Alternatively, 100 μg of lysate was methanol/chloroform precipitated (http://www.ncbi.nlm.nih.gov/pubmed/6731838) and subjected to in-solution digestion as described above. Digested peptides were fractionated into 12 fractions on an OFFGEL 3100 instrument (Agilent) according to manufacturer’s instructions and modified protocol from http://dx.doi.org/10.1002/pmic.200800351, using 13-cm-long IPG 3-10 DryStrips (GE Healthcare). Peptide-containing fractions were acidified, purified on C18 StageTips (http://www.ncbi.nlm.nih.gov/pubmed/17703201) and analysed using nano-LC-coupled mass spectrometry.

**Nano-LC-MS/MS**

Peptides were separated by reversed-phase chromatography using an Agilent 1200 series nanoflow system (Agilent Technologies) connected to a LTQ Orbitrap classic mass spectrometer (Thermo Electron, Bremen, Germany) equipped with a nanoelectrospray ion source (Proxeon, Odense, Denmark). Purified peptides were dissolved in 0.5% formic acid and loaded on a fused silica emitter (75 μm × 150 mm, Proxeon) packed in-house with RepropurSil C18-AQ 3 μm particles (Dr Maisch, Germany) using a flow rate of 700 n1 min⁻¹. SAX-fractionated SILAC-labelled peptides were separated with 240 min gradients as follows: pH 3–5 fraction – 8–36% B gradient, pH 6 fraction – 8–35% B gradient, pH 8 fraction – 5–33% B gradient, pH 11 fraction – 2–30% B gradient (A: 0.5% acetic acid, B: 0.5% acetic acid/80% acetonitrile) at a flow rate of 200 nl min⁻¹; IEF-fractionated SILAC-labelled peptides were separated with 120 min 3–40% B (A: 0.5% acetic acid, B: 0.5% acetic acid/80% acetonitrile) gradients at a flow rate of 200 nl min⁻¹. Eluted peptides were sprayed directly into LTQ Orbitrap mass spectrometer operated at 180°C capillary temperature and 2.4 kV spray voltage. The LTQ Orbitrap was operated in the data-dependent mode with up to five MS/MS scans being recorded for each precursor ion scan. Precursor ion spectra were recorded in profile in the Orbitrap (m/z 300–1900, R = 60 000, max injection time 500 ms, max 1 000 000 charges); data-dependent MS/MS spectra were acquired in centroid in the LTQ (max injection time 150 ms, max 5 000 charges, normalized CE 35%, wideband activation enabled). Mono-isotopic precursor selection was enabled, singly charged ions and ions with an unassigned charge state were rejected, and each fragmented ion was dynamically excluded for 120 s. All measurements in the Orbitrap mass analyser were performed with lock-mass option enabled (lock masses were m/z 445.12003 and 519.13882).

**Data analysis**

Combined raw data files from SAX and IEF fractionations were analysed with the MaxQuant software package, version 2011 Blackwell Publishing Ltd, *Molecular Microbiology*, 80, 54–67
Antibiotic-induced ribosome assembly defects 65

1.0.13.13 (http://www.ncbi.nlm.nih.gov/pubmed/19029910, http://www.ncbi.nlm.nih.gov/pubmed/19373234). Generated peak lists were searched with the Mascot search engine 2.2 against an *E. coli* protein sequence database (downloaded from http://www.ecogene.org on 22 September 2009) supplemented with common contaminants (e.g. human keratins, trypsin) and reversed sequences of all entries in order to estimate false-positive rates. Mascot searches were performed with full tryptic specificity (Trypsin/P), a maximum of two missed cleavages and a mass tolerance of 0.5 Da for fragment ions. Carbamidomethylation of cysteine was set as fixed and methionine oxidation was set as variable modification. A maximum of three missed cleavages were allowed. In MaxQuant, FDR thresholds were set to 1% at both peptide and protein level, minimum required peptide length was set to six amino acids, maximum peptide PEP was set to 0.005 and at least three peptides and two ratio counts were required for protein identification and quantification.

Acknowledgements

We wish to thank Dr A. Liiv (University of Tartu) for help and advice and Dr K. Vanatalu (Tallinn University) for [3H]-labelled *E. coli* MRE600 biomass. This work was supported by the Human Frontiers of Science Organization (RGY0088/2008 to D.N.W.), by Estonian Science Foundation Grants No. 8197 (T.T.) and 7509 (J.R.), and by SF0180166s08 (T.T.) and SF0180164s08 (J.R.) from the Estonian Ministry of Education and Research. Mass-spectrometric analyses were in part supported by the European Regional Development Fund through the Center of Excellence in Chemical Biology (Institute of Technology, University of Tartu). Electronic data processing in Fig. 6 was supported by SF0180026s09 from the Estonian Ministry of Education and Research (M.R.) and by the EU through the European Regional Development Fund through the Estonian Centre of Excellence in Genomics.

References


Starosta, A., Karpenko, V., Shishkina, A., Mikolajka, A., Sum-


**Supporting information**

Additional supporting information may be found in the online version of this article.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.