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Decreased requirement for 4.5S RNA in 16S and 23S rRNA mutants of *Escherichia coli*

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Abstract 4.5S RNA is the bacterial homolog of the mammalian signal recognition particle (SRP) RNA that targets ribosome-bound nascent peptides to the endoplasmic reticulum. To explore the interaction of bacterial SRP with the ribosome, we have isolated rRNA suppressor mutations in *Escherichia coli* that decrease the requirement for 4.5S RNA. Mutations at C732 in 16S rRNA and at A1668 and G1423 in 23S rRNA altered the cellular responses to decreases in both Ffh (the bacterial homolog of SRP54) and 4.5S RNA levels, while the C1066U mutation in 16S rRNA and G424A mutation in 23S rRNA affected the requirement for 4.5S RNA only. These data are consistent with a dual role for 4.5S RNA, one involving co-translational protein secretion by a 4.5S–Ffh complex, the other involving free 4.5S RNA.

Key words: Secretion; Signal recognition particle; 4.5S RNA; rRNA; Ffh

1. Introduction

4.5S RNA is a 114 nucleotide long RNA and is the bacterial homolog of signal recognition particle (SRP) RNA involved in protein export [1,2]. In *Escherichia coli*, 4.5S RNA is encoded by the essential *ffs* gene and can form a complex with the Ffh protein, a 48 kDa homolog of SRP54 [3]. In addition, the *E. coli* homolog of the α subunit of the SRP receptor, the FtsY protein, has been shown to bind to the Ffh–4.5S RNP complex in a reaction that requires GTP and results in the stimulation of GTP hydrolysis by Ffh [4]. Thus, it appears that elements of the protein secretory machinery have been conserved throughout evolution and that the *E. coli* 4.5S RNA–Ffh RNP complex, along with the FtsY protein, represents a minimal SRP and SRP receptor, respectively.

Despite the abundance of phylogenetic and biochemical data suggesting the involvement of 4.5S RNA together with Ffh and FtsY polypeptides in protein secretion, only recently have genetic analyses established a link between the bacterial secretory pathway and the presumptive SRP components [5,6]. However, genetic analysis has also suggested the involvement of 4.5S RNA in several steps of protein synthesis apparently unrelated to protein export. Mutant forms of elongation factor G and of several aminoacyl tRNA synthetases have been isolated in selections for suppressors that reduce, but do not eliminate, the requirement for 4.5S RNA [7–9]. In addition, elongation factor G has been shown to bind to 4.5S RNA in vitro, thus linking 4.5S RNA to EF-G function [10,11], while early biochemical analyses have suggested a role for 4.5S RNA in initiation of protein synthesis [12].

Analysis of SRP function in eukaryotic systems has shown that interaction of the SRP with nascent peptides causes a transient arrest of translation [13,14]. However, the nature of the SRP–ribosome association and the existence of similar interactions in bacterial systems are currently unknown. In order to begin to address the interaction of 4.5S RNA with ribosomes, we have isolated a series of ribosomal mutations that decreased the requirement for 4.5S RNA. Based on the belief that most ribosomal functions are RNA-based, we have targeted ribosomal RNA (rRNA) in our genetic selections for sites of functional interaction between the ribosome and 4.5S RNA. Two 16S and three 23S rRNA mutations were isolated by this means. Based on the ability of the rRNA mutants to sustain cell growth after depletion of Ffh protein, the suppressors can be subdivided into two subclasses that we interpret to reflect altered functional interactions between the ribosome and free 4.5S RNA or 4.5S RNA complexed with Ffh. It is proposed that these mutations decrease the requirement for 4.5S RNA by (i) altering a direct or functional interaction of free 4.5S RNA with the ribosome or ribosome-bound EF-G, or (ii) altering the interaction of the 4.5S–Ffh complex with the ribosome during co-translational export of proteins.

2. Materials and methods

2.1. Bacterial strains and plasmids

Strain S1693 (*F⁻ lacIQ relA1 recA1 rpsL lacY::Tn10 mini-tet ffs::kan591 (λimm434 nin5 XhoI::Ptac-ffs)*) contains an engineered copy of the *ffs* gene under the control of the P_{Tac} promoter and an interruption of the native *ffs* gene [7,8]. Growth of this strain requires at least 10 μM IPTG for sufficient 4.5S RNA synthesis. WAM113 (*F⁻ lacU169 araD139 rpsL thi ffh1::kan8 (Para-ffh⁺* Amp^R)) contains an interruption of the *ffh* gene and a plasmid with the *ffh* gene under the control of the *araBAD* promoter [15]. This strain requires arabinose for growth. *E. coli* strain XL-1 was used to manipulate M13 phages and strain BW313, (*dut⁻ ung⁻*) was use to produce uracil-containing DNA for site-directed mutagenesis [16]. CSH116 (*F⁻ ara Δ(gpt-lac)5 rpsL mutD5 zae-502::*Tn*10*) was used for random mutagenesis of plasmid DNA [17]. The ampicillin-resistant plasmid pKK1192U contains the intact *rrnB* operon transcribed constitutively from the P1 P2 promoter [18]. This plasmid also carries the C1192U mutation in 16S rRNA that confers spectinomycin resistance. pKK1192U is considered the wild type control in these experiments. To express the rRNA suppressor mutants in WAM113, the rRNA mutants were

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cloned from pKK1192U-derived plasmids into pACYC184 which is compatible with the pBR322-derived *ffh* plasmid and confers chloramphenicol resistance. LB was used as the growth medium throughout.

2.2. Mutagenesis

Random mutagenesis of pKK1192U was accomplished by repeated cycles of growth and dilution of CSH116 pKK1192U in LB plus ampicillin, after which the plasmids were isolated by standard procedures [17]. Site-directed mutagenesis was carried out as described by Kunkel et al. [16] using as templates, M13 clones containing the relevant regions of 16S or 23S rRNA.

2.3. Selection and identification of mutants

S1693 was transformed with randomly mutagenized pKK1192U and plated on LB plates containing kanamycin, ampicillin and 100 μM IPTG. At this level of inducer, there is sufficient 4.5S RNA to support growth of wild type cells. Suppressors were isolated by re-streaking the transformants on plates containing 2 μM IPTG. Plasmid DNA from clones exhibiting suppressor activity was isolated and S1693 was re-transformed with these plasmid DNAs to ensure that the suppressor activity was plasmid encoded. Restriction fragments of the mutant *rrnB* operons were subcloned into M13 and the mutations were identified by nucleotide sequencing using Sequenase (USB Corporation, Cleveland, OH, USA).

2.4. Growth rate determinations

Overnight cultures of S1693, containing either a wild type or mutant derivative of pKK1192 grown in LB supplemented with ampicillin, kanamycin and 100μM IPTG, were used to inoculate flasks containing 10 ml of LB containing ampicillin (200 mg/l), kanamycin (50 mg/l) and IPTG (present either at 2 μM or 100 μM). Cultures were grown at 37°C with shaking and growth was monitored in a Klett–Summerson colorimeter. The ability of the rRNA mutations to suppress the requirement for the Ffh protein was tested by transforming strain WAM113 with either wild type or mutant derivatives of pACYC1192U and assaying the ability of these strains to grow in the absence of arabinose. Cultures were grown overnight in the presence of arabinose, washed twice with LB and used to inoculate 10 ml of LB containing kanamycin (50 mg/l), chloramphenicol (33 mg/l), with or without arabinose, and monitoring the growth thereafter.

2.5. Polysome preparation and primer extension analysis of rRNA and 4.5S RNA

Overnight cultures of S1693 harboring rRNA suppressor mutations were grown in LB containing kanamycin, ampicillin and 2 μM IPTG and were diluted to an OD_{600} of 0.075 into fresh, antibiotic-containing medium supplemented with either 2 μM or 100 μM IPTG. Cells were grown at 37°C to an OD_{600} of 0.60 and disrupted by the freeze–thaw lysozyme method [19]. Lysates were fractionated on sucrose gradients as described [20]. RNAs were extracted from the various gradient fractions and analyzed by primer extension analysis, using a ^{32}P-labeled primer as described previously [21]. Extension products were analyzed using a Fuji–MacBas phosphorimaging system.

3. Results and discussion

3.1. Isolation of rRNA mutations that reduce the requirement for 4.5S RNA

In an effort to identify cellular components that interact with 4.5S RNA, suppressor mutations were isolated by Brown that permitted cell growth at reduced 4.5S RNA levels [7–9]. This approach utilized a strain (S1693) in which transcription of the 4.5S RNA from the Ptac promoter was dependent upon the presence of the inducer IPTG at a concentration of at least 10 μM in the growth medium. IPTG concentrations below this were insufficient to sustain growth. Suppressor mutations in the genes encoding EF-G, valyl and glutamyl tRNA synthetases and the 1067 region of 23S rRNA that interacts with EF-G permitted growth on low levels (1–2 μM) of IPTG. These studies suggested EF-G and/or its binding site on 23S rRNA might interact with 4.5S RNA during translation. As a means of identifying other potential ribosomal sites that impinge upon 4.5S function, we have used the same approach devised by Brown to isolate rRNA mutations that permit cell growth at low levels of 4.5S RNA.

Strain S1693 was transformed with randomly mutagenized plasmid pKK1192U carrying a copy of the *rrnB* operon. Cells able to grow at reduced levels of 4.5S RNA were selected by streaking transformants on medium containing ampicillin and 2 μM IPTG and shown by re-transformation to be plasmid-dependent. The sites of the mutations were identified by DNA sequencing and each of the mutations was re-constructed by site-directed mutagenesis. The location of the suppressor mutations on the secondary structure maps of 16S and 23S rRNA is shown in Fig. 1. In 16S rRNA, mutations at C732U in helix 23 and C1066U in helix 34 were isolated. The three 23S rRNA mutations were G424A in domain I, G1423A in domain III and A1668G in domain IV.

3.2. Growth characteristics of rRNA mutants

When grown in the presence of 100 μM of IPTG to induce synthesis of high levels of 4.5S RNA, the doubling times of S1693 strains containing C732U, C1066U, G424A and A1668G mutants ranged from 52 to 61 min, compared with a doubling time of 46 min for the wild type pKK1192U-containing strain (Table 1). When the same strains were grown in the presence of 2 μM IPTG (where only low levels of 4.5S RNA were synthesized), there was a substantial increase in the

Table 1
Growth rates of rRNA mutants and distribution of plasmid-encoded rRNA in sucrose gradients

rRNA mutant	Doubling time (min)		% of mutant rRNA in 30S, 50S and 70S ribosomes and polysomes							
			30S		50S		70S		Polysomes	
	2 μM IPTG	100 μM IPTG	2 μM IPTG	100 μM IPTG	2 μM IPTG	100 μM IPTG	2 μM IPTG	100 μM IPTG	2 μM IPTG	100 μM IPTG
WT	NA	46 ± 2	NA	84 ± 8	NA	NA	NA	77 ± 2	NA	80 ± 4
16S mutants										
C732U	69 ± 1	56 ± 5	89 ± 2	88 ± 7	NA	NA	70 ± 4	59 ± 7	77 ± 2	57 ± 3
C1066U	126 ± 7	56 ± 2	88 ± 2	66 ± 4	NA	NA	86 ± 1	42 ± 3	88 ± 1	58 ± 9
23S mutants										
G424A	66 ± 4	52 ± 2	NA	NA	74 ± 2	72 ± 2	69 ± 1	68 ± 4	69 ± 2	71 ± 3
G1423A	151 ± 4	ND	NA	NA	76 ± 9	77 ± 3	78 ± 12	72 ± 5	85 ± 6	77 ± 15
A1668G	75 ± 4	61 ± 4	NA	NA	66 ± 4	70 ± 1	52 ± 3	58 ± 4	57 ± 3	47 ± 4

Strain S1693 expressing each of the indicated rRNA mutants was grown in LB medium containing ampicillin and IPTG. The distribution of mutant rRNA in ribosome fractions was assayed by primer extension. Each primer extension assay and each growth rate presented represents the mean ± S.E.M. of at least three independent determinations. WT = wild type; NA = not applicable; ND = not determined.

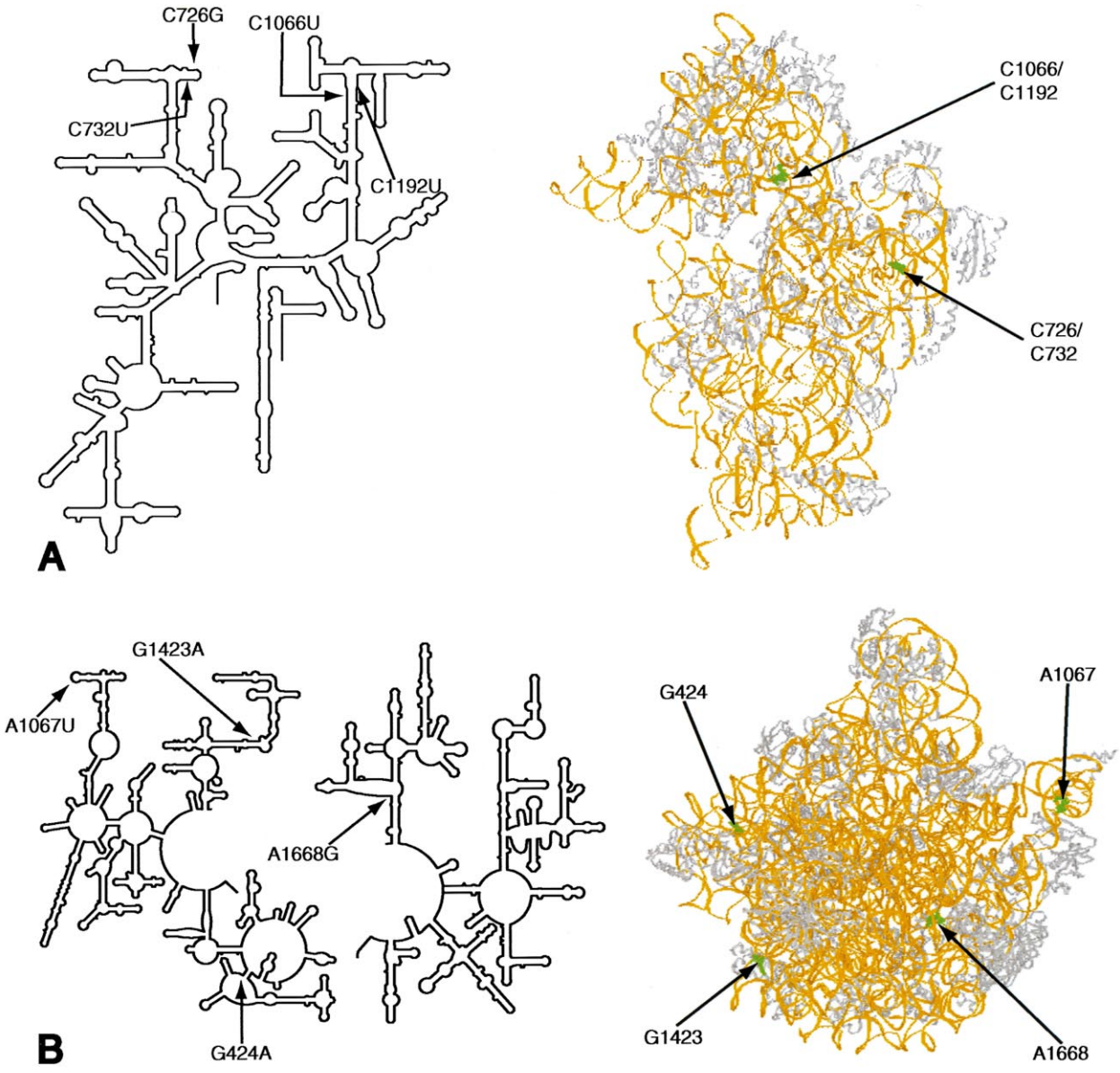

Fig. 1. Location of 16S (A) and 23S (B) rRNA mutations on the corresponding rRNA secondary structure (left panels) and crystal structure (right hand panels) of 30S and 50S subunits, respectively.

doubling time for cells harboring the C1066U 16S rRNA mutation, but the increase for the other mutants was about 25%. The increased doubling times observed at low levels of IPTG reflect both the ability of the mutant ribosomes to function at low concentrations of 4.5S RNA and the effects of the rRNA mutations on other steps of translation. The mutation at position G1423 had the most deleterious phenotype. Strains harboring this mutation grew slowly and rapidly accumulated faster growing clones in the absence of selective pressure for function at low levels of 4.5S RNA. This prevented a reliable measurement of doubling time at 100 μM IPTG. However, this mutant displayed the strongest suppressor activity, since the cells were able to grow in the complete absence of IPTG, while the other four mutants still required a low level (2 μM) of IPTG. This suggests that while the G1423A mutation reduced the need for 4.5S RNA, it had deleterious effect(s) on other steps of protein synthesis.

Additional rRNA mutations that had previously been characterized by our laboratory were also tested for their ability to suppress the requirement for 4.5S RNA. These included the G2661C and C2666U mutations in the α-sarcin loop and the A1067U mutation in the GTPase center of 23S rRNA [22,23]. Both of these regions of 23S rRNA have been implicated in ribosome–EF-Tu and EF-G interactions, and the A1067U had previously been shown to suppress the 4.5S RNA requirement [8]. The G1491U and C726G mutations in 16S which alter the fidelity of decoding were also tested [22,24]. Of these mutations, only the A1067U mutation in 23S and C726G in 16S rRNA were able to suppress the 4.5S RNA requirement. The ability of the C726G mutation is perhaps not unexpected, given its proximity to the C732U mutation isolated genetically in this study.

3.3. *Distribution of mutant rRNA and 4.5S RNA in ribosome fractions*

The ability of the mutant rRNAs to become assembled into

ribosomal subunits and engage in protein synthesis under conditions of high and low levels of 4.5S RNA was assayed by primer extension. The data presented in Table 1 illustrate a differential behavior of the 16S and 23S rRNA mutants: whereas all three 23S mutants were well represented in subunit, 70S and polysome pools when grown in the presence of limiting or high levels of 4.5S RNA, both 16S mutants were selectively incorporated into the 70S and polysome pool only when grown with limiting amounts of 4.5S RNA. The differential behavior of the 16S and 23S rRNA suppressors suggests distinct mechanisms of action for the small and large subunit rRNA mutants.

With the exception of the C1066U mutant, all rRNA mutants showed abnormal sucrose gradient profiles, irrespective of the intracellular 4.5S RNA concentration. These profiles were characterized by the presence of large amounts of free subunits and low levels of 70S ribosomes. When the C1066U mutant was grown in the presence of high levels of 4.5S RNA, it displayed an essentially wild type profile, however, when the same strain was grown in the presence of low levels of 4.5S RNA, the ribosome profile resembled that of the other rRNA mutants. These data, combined with the decreased levels of C1066U rRNA in all fractions when mutants cells were grown in the presence of high levels of IPTG, suggest that the mutant ribosomes may be unstable when grown in the absence of selection for ribosome function.

The pKK1192U plasmid used here as the wild type rRNA plasmid contains the C1192U mutation conferring resistance to spectinomycin. Mutations at C1066 also confer resistance to spectinomycin [25] and thus plasmid-encoded rRNA from strains containing the C1066U substitution contains two mutations, each conferring resistance to spectinomycin. To examine the contribution of each individual mutation to the decreased requirement for 4.5S RNA, plasmids expressing either the C1192U or C1066U were introduced into strain S1693. None of the transformants grew on reduced levels of IPTG, demonstrating that the C1066U mutation alone did not permit growth at low levels of 4.5S RNA. Thus the combination of C1066U and C1192U mutations was necessary for suppression of the requirement for high intracellular levels of 4.5S RNA. Both the C1066U and C1192U mutations disrupt pairing in the upper part of helix 34 where spectinomycin binds and it is likely that the combination of both mutations leads to a gross destabilization of this region on the helix. Other rRNA mutations that decrease the requirement for 4.5S RNA appear to be unaffected by the presence of the C1192U mutation: the C726G and A1067U mutations 16S and 23S rRNAs, respectively were each constructed in plasmid pKK3535 in the absence on the C1192U mutation [23,24] and it appears that the C1192U mutation only affects the suppressor activity of the adjacent C1066U mutation.

The EF-G and aminoacyl tRNA synthetase mutants isolated by Brown as suppressors of the requirement for 4.5S RNA all altered the sedimentation position of 4.5S RNA from the top of the sucrose gradients (where most of the small RNAs are typically located) to a position that co-sedimented with the 70S and polysome fractions [8]. The altered sedimentation pattern of 4.5S derives, presumably, from an elongation of the time frame during translation when 4.5S RNA is associated with the ribosome. Analysis of the sedimentation pattern of 4.5S RNA in the rRNA mutants isolated here showed that in all five of the extracts examined, the 4.5S RNA was consistently found at the top of the gradient and not in the 70S ribosome or polysome fractions (data not shown). Growth of each of the strains in the presence of either 2 μM or 100 μM IPTG did not influence the distribution of 4.5S RNA in the gradients. Thus the rRNA mutants isolated in this study appear to reduce the requirement for 4.5S RNA by a mechanism distinct from that observed with the EF-G and aminoacyl tRNA synthetase mutants.

3.4. Interaction of mutant rRNAs with P48/Ffh

SRP-mediated protein export involves the action of SRP RNA bound to SRP54 protein (the eukaryotic homolog of Ffh). If the rRNA mutations described above that support cell growth at lower 4.5S RNA levels exert this effect by altering ribosome–SRP interactions, it follows that these mutations should also reduce the requirement for Ffh protein. This prediction was tested using strain WAM113 where the levels of Ffh protein can be controlled by manipulating the concentration of arabinose in the growth medium [15]. WAM113 was transformed with the mutant rRNA plasmids and growth of the transformants was examined after withdrawal of arabinose. WAM113 carrying the wild type rRNA plasmid pACYC1192U ceased growth after two to three cell doublings following removal of arabinose. A similar pattern of cell growth was observed in transformants expressing the C1066U and G424A mutants in 16S and 23S rRNAs, respectively. However, expression of the C732U mutant in 16S rRNA or the A1668G and G1423A mutants in 23S rRNA allowed continued growth for approximately twice as many doublings before cell growth slowed and eventually ceased. The differential behavior of the two groups of rRNA mutants indicates that suppression occurs by distinct mechanisms, only one of which involves 4.5S RNA bound to Ffh.

3.5. Localization of rRNA mutations within the ribosome structure and the mechanism of suppression

The rRNA mutations described here can be divided into two classes of suppressors based on their ability to support cell growth upon depletion of Ffh. The first class, composed of the C1066U and G424A mutants, permits cell growth at lowered concentrations of 4.5S RNA but does not reduce the need for Ffh. Measurements of the intracellular concentrations of 4.5S RNA and Ffh indicate that there are some 400 molecules of 4.5S RNA per 10 000 ribosomes while Ffh is at one fourth the concentration of 4.5S RNA [26]. In addition, while the EF-G mutants isolated by Brown as suppressors of the 4.5S requirement permit some growth upon depletion of Ffh, the aminoacyl tRNA synthetase mutants isolated in the same selection do not [8,26]. These data have led to the proposal that 4.5S has two separate (or sequential) functions in translation, one involving 4.5S bound to Ffh and one involving free 4.5S RNA. In agreement with this proposal, both EF-G and Ffh have each been shown to bind to 4.5S RNA in vitro [10,11]. It is significant, therefore, that two of the rRNA mutants decreasing the requirement for 4.5S RNA (C1066U in 16S rRNA isolated here and the A1067U/C substitutions in 23S rRNA described by Brown [8]) both lie in regions of the ribosome known to be involved in EF-G-promoted translocation [27]. G424, the other mutation leading to a decreased requirement for 4.5S RNA but not affecting Ffh, is located close to the binding site for ribosomal protein L1 (Fig. 1). However, the role of G424 in translation is not known. In

the context of the model proposing a role for free 4.5S RNA, these rRNA mutations could have a direct effect on the interaction of 4.5S RNA with the ribosome-bound EF-G. Alternatively, suppression may be indirect, and occur by affecting the kinetics of this interaction. If indeed 4.5S RNA does interact with the translocating ribosome, it can only affect a subset of translocations, since the intracellular 4.5S RNA concentration is far less than the ribosome concentration [26].

The second class of suppressors, comprising G726C and C732U in 16S rRNA and the A1668G and G1423A mutations in 23S rRNA, alters the requirement for both 4.5S RNA and Ffh protein. This suggests that these mutations affect the interaction of the 4.5S–Ffh complex with the ribosome during the co-translational secretion of proteins. The sites of interaction of SRP with the ribosome are currently unknown, but the distant location of each of these mutations from one another on the ribosome (Fig. 1) renders it unlikely that all three bases constitute part of the SRP binding site. G726 and C732 are positioned between the functionally important 690 and 790 loops that are involved in interactions with tRNAs, 50S subunits and initiation factors, and mutations at position G726 have dramatic effects on multiple steps of translation [24,28]. The G726/C732 region of 16S rRNA forms part of the binding site for ribosomal protein S15. Mutations in ribosomal protein S15 have previously been isolated as suppressors of the temperature-sensitive, secretion-defective *secA51* mutant [29], thus linking secretion to this region of the ribosome. However, suppression by mutant S15 could be indirect and occur by decreasing the rate of translation to a level that is compatible with the residual activities of the *secA51* mutant in secretion.

Nucleotide A1668 is located close to two major intersubunit bridges (B5 and B6) connecting the 1700 region of 23S rRNA and helix 44 of 16S rRNA [27]. G1423 is located on the lower periphery of the 50S subunit, beneath ribosomal protein L9, and its function in translation is unknown. While alterations to the SRP binding site cannot be excluded as one possible mechanism of suppression, the levels of SRP required for cell growth could also be decreased by rendering steps prior to SRP entry rate limiting, thus prolonging the time during translation when SRP can interact with the ribosome. Consistent with this interpretation, we found that strain S1693 (or a derivative) was able to grow in the absence of induction of 4.5S synthesis upon addition of sublethal concentrations of the antibiotics chloramphenicol or fusidic acid or by inhibition of tryptophanyl tRNA synthetase by indoleacrylic acid [9] or starvation for isoleucine by addition of valine to the growth medium ([9] and our unpublished data).

The explosion of high resolution crystal structures of ribosomes has allowed localization of all of the rRNA mutations described here on the three-dimensional structure of the 70S ribosome. Ongoing X-ray crystallographic and cryo-electron microscopic analyses will undoubtedly also reveal the site(s) of interaction of SRP with the ribosome. The genetic analyses described here indicate, however, that functional interactions of 4.5S RNA with the ribosome are complex and modulated by many factors including the rate of translation and the physiological state of the cell as well as the structure of the ribosome.

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