The PRC-barrel domain of the ribosome maturation protein RimM mediates binding to ribosomal protein S19 in the 30S ribosomal subunits

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ABSTRACT

The RimM protein in *Escherichia coli* is associated with free 30S ribosomal subunits but not with 70S ribosomes. A $\Delta rimM$ mutant is defective in 30S maturation and accumulates 17S rRNA. To study the interaction of RimM with the 30S and its involvement in 30S maturation, RimM amino acid substitution mutants were constructed. A mutant RimM (RimM-YY $\rightarrow$ AA), containing alanine substitutions for two adjacent tyrosines within the PRC β -barrel domain, showed a reduced binding to 30S and an accumulation of 17S rRNA compared to wild-type RimM. The (RimM-YY $\rightarrow$ AA) and $\Delta rimM$ mutants had significantly lower amounts of polysomes and also reduced levels of 30S relative to 50S compared to a wild-type strain. A mutation in *rpsS*, which encodes r-protein S19, suppressed the polysome- and 16S rRNA processing deficiencies of the RimM-YY $\rightarrow$ AA but not that of the $\Delta rimM$ mutant. A mutation in *rpsM*, which encodes r-protein S13, suppressed the polysome deficiency of both *rimM* mutants. Suppressor mutations, found in either helices 31 or 33b of 16S rRNA, improved growth of both the RimM-YY $\rightarrow$ AA and $\Delta rimM$ mutants. However, they suppressed the 16S rRNA processing deficiency of the RimM-YY $\rightarrow$ AA mutant more efficiently than that of the $\Delta rimM$ mutant. Helices 31 and 33b are known to interact with S13 and S19, respectively, and S13 is known to interact with S19. A GST-RimM but not a GST-RimM(YY $\rightarrow$ AA) protein bound strongly to S19 in 30S. Thus, RimM likely facilitates maturation of the region of the head of 30S that contains S13 and S19 as well as helices 31 and 33b.

Keywords: RimM; 30S maturation; 16S rRNA processing; S19; S13; helices 31 and 33b

INTRODUCTION

Up to 40% of the total energy production in *Escherichia coli* is consumed by ribosomal biogenesis emphasizing the importance of a coordinated synthesis and assembly of ribosomal components. The 50S and 30S ribosomal subunits can be reconstituted into fully active ribosomes from the isolated components in vitro. However, nonphysiological

conditions like heat-activation steps and different Mg²⁺ concentrations are required to achieve a reasonable output of mature ribosomal subunits. The maturation of the 50S and 30S subunits in vivo requires auxiliary proteins that are not part of mature ribosomes. Apart from rRNA processing enzymes and rRNA/r-protein modifying enzymes, a number of other proteins assist in the assembly of the 50S and 30S subunits in *E. coli*. The DEAD box RNA helicases (Fuller-Pace 1994), SrmB (Nashimoto et al. 1985; Nishi et al. 1988; Charollais et al. 2003), DbpA (Fuller-Pace et al. 1993; Tsu and Uhlenbeck 1998), and CsdA (formerly DeaD) (Toone et al. 1991; Charollais et al. 2004) are assumed to open up RNA structures and allow proper folding of the ribosome. The DnaK chaperone system (Alix and Guérin 1993; Maki et al. 2003), additional heat-shock proteins including GroEL (El Hage et al. 2001), as well as the essential GTPase Era (Nashimoto et al. 1985; Nashimoto 1993), the cold-shock protein RbfA (Dammel and Noller

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1995; Jones and Inouye 1996), and the YrdC protein (Kaczanowska and Rydén-Aulin 2004) are likely involved in ribosome assembly.

The RimM protein is associated with free 30S subunits but not with 70S ribosomes, suggesting an involvement in maturation of the 30S subunits or in translation initiation (Bylund et al. 1997). RimM is present in most eubacterial species whose genomes have been sequenced; however, it is not found in any species of the domain *Archaea*. In addition, RimM-like proteins are reported from at least four eukaryotic species: the malaria parasites *Plasmodium falciparum* and *Plasmodium yoelii*, the malaria mosquito *Anopheles gambiae*, and the chloroplast of the plant *Arabidopsis thaliana*. An in-frame deletion of *rimM* ($\Delta rimM$), removing all except the first five and last five codons, decreases the growth rate sevenfold and reduces the translational efficiency at 37°C (Bylund et al. 1997). Moreover, the $\Delta rimM$ mutant accumulates 17S rRNA, suggesting a role for RimM in maturation of the 30S subunits (Bylund et al. 1998). Extragenic suppressor mutations to the $\Delta rimM$ mutation increase the growth rate and translational efficiency of the $\Delta rimM$ mutant (Bylund et al. 1997, 1998, 2001). Some of these mutations alter the carboxy-terminal 16S rRNA-binding domain of r-protein S13 (Bylund et al. 1997). The remaining suppressor mutations increase the expression of *rbfA* (Bylund et al. 1998, 2001). The carboxy-terminal half of RimM was proposed to contain a PRC β -barrel domain (Anantharaman and Aravind 2002). Previously, a mutant RimM protein (RimM-YY→AA) containing alanine substitutions for two adjacent tyrosines within the PRC β -barrel domain showed a reduced binding to the 30S ribosomal subunits (Lövgren and Wikström 2001).

In this paper, we show that the RimM-YY→AA mutant accumulated 17S rRNA relative to mature 16S, had lower amounts of 30S relative to 50S subunits, and had reduced levels of polysomes in comparison to a wild-type strain. Mutations in two adjacent helices (31 and 33b) of 16S rRNA and in the genes for r-proteins S13 and S19 were found to suppress the slow growth of the RimM-YY→AA mutant. Furthermore, a GST-RimM wild-type protein bound strongly to S19 in the 30S subunits, whereas a GST-RimM(YY→AA) mutant protein did not. These findings suggest that RimM is important for the maturation of the head region of the 30S subunits in which helices 31 and 33b as well as r-proteins S13 and S19 are located.

RESULTS

Identification of functionally important amino acid residues in RimM

Functionally important amino acids in RimM were identified by using two different mutagenic approaches. First, to obtain random mutations in *rimM*, plasmid pMW279, carrying *rimM* under the control of an IPTG-inducible pro-

motor, was mutagenized with hydroxylamine. Derivatives of pMW279 that contained *rimM* alleles that could not completely complement the slow growth of a $\Delta rimM$ mutant were identified. Two *rimM* alleles complemented the slow growth at 37°C but not at 42°C and expressed RimM proteins with the single amino acid substitutions G17E and G27R, respectively. Another *rimM* allele encoded a RimM-G121D protein that showed less complementation than wild-type RimM, when the expression from the IPTG-inducible promoter was limited. The amounts of RimM-G17E and RimM-G27R were 10-fold higher at both 37°C and 42°C than the amount of RimM in a wild-type strain (data not shown). Thus, the G17E and G27R substitutions severely affected the function of RimM, and the poor complementation by the two RimM mutant proteins at 42°C did not result from a decreased stability at higher temperatures.

Second, to identify conserved amino acids that might be of functional importance, the RimM amino acid sequences from 17 evolutionary distant species were aligned (Fig. 1). Some positions in the amino terminal part of RimM are highly conserved, including G17 and G27, which were identified as important for RimM function in the random mutagenesis screen above. In RimM from *E. coli*, these glycines are part of the sequences GKMG and GIRG, which are similar to the GXXG motif of the KH domain in RNA-binding proteins (Gibson et al. 1993; Liu et al. 1995). To investigate whether these sequences were important for the binding of RimM to the 30S subunits, we replaced the four glycines and the two basic amino acids of the two GXXG motifs with alanines. Strains with these alanine substitutions (G17A, K18A, G20A, G24A, R26A, and G27A) in chromosomally encoded RimM showed growth rates that were similar to that of a wild-type strain at both 37°C and 42°C (data not shown).

In the proposed PRC β -barrel domain of RimM, the conserved aromatic amino acids in positions 106–108, together with the conserved aspartate in position 137 are suggested to bind protein (Anantharaman and Aravind 2002). The two tyrosines in positions 106 and 107 and the aspartate in position 137 were replaced with alanines. The D137A substitution reduced the growth rate at temperatures from 21°C to 44°C, and the effect was more pronounced at the higher temperatures. The growth deficiency was likely due to a decreased stability of the mutant protein, because the RimM levels were more than fivefold lower in the mutant compared to the wild type (data not shown). In contrast, the double substitution Y106A/Y107A reduced the function of RimM, because it lowered the growth rate threefold (Fig. 2) without affecting protein stability (Lövgren and Wikström 2001). The single substitution in position 106 had no effect on the growth rate, whereas that in position 107 slightly reduced the growth rate, especially at 42°C (data not shown).

Charged amino acid side chains are likely exposed on the surface of proteins and might, in the case of RimM, interact

FIGURE 1. Amino acid sequence alignment of RimM from different eubacterial species, the malaria mosquito *Anopheles gambiae*, and *Arabidopsis thaliana*. Eco, *Escherichia coli*; Bsu, *Bacillus subtilis*; Vpa, *Vibrio parahaemolyticus*; Nme, *Neisseria meningitidis*; Ath, *Arabidopsis thaliana*; Syn, *Synechocystis* sp. Strain PCC6803; Cte, *Clostridium tetani*; Aae, *Aquifex aeolicus*; Cht, *Chlorobium tepidum*; Fnu, *Fusobacterium nucleatum*; Mlo, *Mesorhizobium loti*; Mle, *Mycobacterium leprae*; Dra, *Deinococcus radiodurans*; Aga, *Anopheles gambiae*; Bbu, *Borrelia burgdorferi*; Hpy, *Helicobacter pylori*. The numbering to the left of the top row indicates the position in the respective sequence of the first amino acid shown. Amino acid substitutions were isolated for the positions indicated by arrows. The * indicates the position of the N84K substitution suppressing RimM-YY→AA.

with the 30S subunits. Therefore, charged amino acid residues adjacent to each other in RimM (E36-D37, E102-E103, K109-D110) were replaced by alanines. None of the double substitutions E36A/D37A, E102A/E103A, and K109A/D110A affected the growth rate of the cells on minimal medium plates at 25°C–41.5°C or on rich medium plates at 25°C–44°C (data not shown).

FIGURE 2. The effect of S13Δ89–99 and S19H83Y on the growth rate of the $\Delta rimM$ and RimM-YY→AA mutants grown in LB medium at 37°C. The growth rate was determined in four independent experiments and is expressed as k ($= \ln 2/g$, where g is the mass doubling time in h). The variation between the experiments is shown as error bars.

Isolation of suppressor mutations that increase the growth rate of the RimM-YY→AA mutant

The RimM-YY→AA protein binds less well to the 30S subunits than does the wild-type RimM protein (Lövgren and Wikström 2001). The RimM-YY→AA mutant was used to select for suppressor mutations that restored binding of RimM-YY→AA to the 30S subunits resulting in faster growth. One suppressor strain (JML025) contained an N84K substitution in RimM, which increased the growth rate of the RimM-YY→AA mutant twofold (the specific growth rate, $k = \ln 2/g$, where g is the mass doubling time in h, was 1.17 and 0.57, respectively). The growth rate of the wild-type strain MW100 was 1.61 in the same experiment. To examine whether the N84K substitution also restored the binding of RimM to the 30S subunits, a cell extract of the RimM-YY→AA mutant containing the N84K substitution (JML025) was fractionated by sucrose gradient centrifugation and screened for the presence of RimM with an anti-RimM antiserum. No higher amounts of RimM were observed in fractions containing the 30S subunits from the RimM-YY→AA/N84K suppressor strain compared to those from the RimM-YY→AA mutant (data not shown). Thus, the N84K substitution did not restore the binding to the 30S subunits, although it improved the function of the 30S subunits (see below).

Two of the suppressor strains (JML001, JML026) contained extragenic mutations that were similar to previously isolated mutations in the *metY-nusA-infB* operon that in-

crease expression of *rbfA* (Bylund et al. 2001). Another mutation (*spr-8*, strain MW145) changed the histidine codon in position 83 of *rpsS*, for r-protein S19, to a tyrosine codon (data not shown). To investigate whether the S19H83Y protein also suppressed the slow growth of a strain lacking RimM, the *rpsS* mutation was combined with the $\Delta rimM$ mutation. S19H83Y was found to be a weaker suppressor of the $\Delta rimM$ mutant than were mutations that increased expression of *rbfA*, although they all suppressed the slow growth of the RimM-YY $\rightarrow$ AA mutant similarly (data not shown). These results indicated that the H83Y substitution in S19 might have restored a physical interaction between the RimM-YY $\rightarrow$ AA protein and S19. Therefore, a cellular extract of an S19H83Y RimM-YY $\rightarrow$ AA double mutant was fractionated by sucrose gradient centrifugation and screened for the presence of RimM with an anti-RimM antiserum. However, S19H83Y did not increase the amount of the RimM-YY $\rightarrow$ AA protein associated with the 30S subunits (data not shown).

Effect of alterations in r-proteins S13 and S19 on polysome profiles of the RimM-YY $\rightarrow$ AA and $\Delta rimM$ mutants

To estimate the translational defects of the RimM-YY $\rightarrow$ AA and $\Delta rimM$ mutants and to examine the ability of alterations in S13 and S19 to suppress these defects, we analyzed polysome profiles of the different strains by sucrose gradient centrifugation of cellular extracts. The $\Delta rimM$ mutant (MW37) showed a significantly lower amount of polysomes than the wild type (MW100) and, hence, an accumulation of free 30S and 50S subunits (Fig. 3A,E). The RimM-YY $\rightarrow$ AA mutant showed a similar translational defect, although it was not as severe as that for the $\Delta rimM$ mutant (Fig. 3, cf. B and E). Interestingly, both *rimM* mutants had significantly lower amounts of free 30S subunits relative to 50S subunits compared to the wild type, indicating that a deficiency in the maturation of the 30S subunits could explain the low amounts of polysomes. Previously obtained mutations in *rpsM*, which encodes r-protein S13, improved translation in a $\Delta rimM$ mutant (Bylund et al. 1997). The *rpsM873* mutation removed amino acids 89 to 99 of S13. The S13 Δ 89-99 protein partially corrected the imbalance in the amount of 30S to 50S subunits and increased the amount of polysomes of both the RimM-YY $\rightarrow$ AA (Fig. 3C)

FIGURE 3. The effect of S13 Δ 89-99 and S19H83Y on polysome profiles of the $\Delta rimM$ and RimM-YY $\rightarrow$ AA mutants grown in LB medium at 37°C. Different ribosome particles are marked above corresponding peaks in A.

and the $\Delta rimM$ mutant (Fig. 3F). Also, the S19H83Y protein increased the amount of polysomes of both *rimM* mutants (Fig. 3D,G). In the RimM-YY $\rightarrow$ AA mutant, the effect of S19H83Y was greater than that of S13 Δ 89-99 (Fig. 3B–D), whereas in the $\Delta rimM$ mutant, the effect of S19H83Y was much less than that of S13 Δ 89-99 (Fig. 3E–G). For the two *rimM* mutants as well as their S13 Δ 89-99 and S19H83Y derivatives, the relative growth rate (Fig. 2) correlated very well with the amount of polysomes detected, demonstrating that the slow growth of *rimM* mutants is due to poor translation.

Mutations in helices 31 and 33b of 16S rRNA suppress the slow growth of the RimM-YY $\rightarrow$ AA and $\Delta rimM$ mutants

Conceivably, if RimM interacts with 16S rRNA of the 30S subunits, compensatory mutations in 16S rRNA could be isolated that would restore the interaction between the RimM-YY $\rightarrow$ AA protein and 16S rRNA. However, because *E. coli* contains seven chromosomal *rrn* operons (for rRNA), a single mutation may not give any observable suppression. Therefore, we took advantage of an *E. coli* strain in which all chromosomal *rrn* operons have been inactivated (Asai et al. 1999a) and the production of ribosomes is dependent on an *rrn* operon present on plasmid pHK-*rrnC*⁺

(Asai et al. 1999b). The *rimM* mutant gene (*rimM120*) for RimM-YY→AA was introduced into this strain. In this genetic background, RimM-YY→AA reduced the growth rate of the cells twofold (Fig. 4A) compared to a threefold reduction in the growth rate of strains containing all chromosomal *rrn* operons (Fig. 2). Several faster-growing suppressor mutants of the RimM-YY→AA mutant (JML068) were isolated, the fastest of which had growth rates that were close to 90% of the *rimM*⁺ strain (Fig. 4A). Twelve suppressor mutations were localized to plasmid pHK-*rrnC*⁺ and identified by DNA sequencing. Ten of the mutations were in four different positions of helix 31, and two were in the identical position of helix 33b of 16S rRNA (Fig. 5). To investigate whether the 16S rRNA mutations restored the binding of the RimM-YY→AA protein to 30S subunits, total cell extracts of three of the suppressor strains (JML075, JML079, and JML098) and of appropriate control strains were subjected to sucrose gradient centrifugation. Fractions were analyzed for the presence of the RimM-YY→AA pro-

FIGURE 4. The effect of suppressor mutations in helices 31 and 33b of 16S rRNA on the growth rate of the RimM-YY→AA (A) and $\Delta rimM$ (B) mutants in LB medium at 37°C. The growth rate was determined in three independent experiments and is expressed as k ($= \ln 2/g$, where g is the mass doubling time in h). The variation between the experiments is shown as error bars.

FIGURE 5. Mutations in helices 31 and 33b of 16S rRNA that suppress the slow growth of the RimM-YY→AA and $\Delta rimM$ mutants JML068 and JML114.

tein with an anti-RimM antiserum by Western blotting. The 16S rRNA mutations did not restore binding of the RimM-YY→AA protein to the 30S subunits (data not shown). To determine whether the 16S rRNA mutations could also suppress a $\Delta rimM$ mutant, the suppressor plasmids were substituted for the suppressor-free *rrn*-plasmid pKK1192U in strain JML114 ($\Delta rimM102/pKK1192U + p70$). All of the mutant plasmids partially suppressed the slow growth of the $\Delta rimM$ mutant, indicating that the suppressor mutations were not allele-specific (Fig. 4B).

RimM is not directly responsible for the processing of 16S rRNA

The processing of 16S rRNA is initiated by RNase III cleavages in a double-stranded region composed of the sequences flanking mature 16S rRNA (Young and Steitz 1978). The generated 17S rRNA contains 115 extra nucleotides (nt) at the 5' end and 33 at the 3' end compared to mature 16S rRNA. In wild-type cells, RNase E and G remove the extra 115 nt at the 5' end, whereas the removal of the 33 nt at the 3' end occurs in one step by an unknown RNase and is dependent on the processing by RNase E at the 5' end (Li et al. 1999). Because a $\Delta rimM$ mutant accumulates 17S rRNA (Bylund et al. 1998), we investigated whether the RimM-YY→AA mutant was deficient in processing of 16S rRNA. The ability of the RimM-YY→AA mutant to convert 17S to 16S rRNA was determined by primer extension analysis of the 5'-end of 16S rRNA. The RimM-YY→AA mutant showed five-to-sevenfold increased levels of 17S rRNA compared to the wild type; however, the processing deficiency was not as pronounced as for the

$\Delta rimM$ mutant (Fig. 6A; Table 1). To examine whether RimM could be the unknown RNase processing the 3'-end of 17S rRNA, total RNA, from a wild-type strain and from the $\Delta rimM$ and RimM-YY $\rightarrow$ AA mutants, was analyzed by Northern blotting with oligonucleotides specific for either the region just upstream of the 3'-end of mature 16S rRNA or the extra nucleotides present in 17S rRNA. The two *rimM* mutants showed only a partial accumulation of unprocessed 3' ends (data not shown), mimicking the results for the processing of the 5' end in the same mutants. Thus, RimM cannot be the unknown RNase that processes the 3' end of 16S rRNA, and the observed partial processing deficiency was probably an indirect effect of poor processing by RNase E at the 5' end.

The effect of the suppressor mutations on the processing of 16S rRNA

The 16S rRNA processing deficiency of *rimM* mutants is likely the result of a defect in a step in the maturation of the 30S subunits that precedes the final processing of 17S into 16S rRNA (Bylund et al. 1998). To examine whether mutations suppressing the two *rimM* mutations improved the processing of 16S rRNA, we determined the processing ef-

iciency of different suppressor strains by primer extension analysis of the 5'-end of 16S rRNA. Interestingly, S19H83Y suppressed partially the processing deficiency of the RimM-YY $\rightarrow$ AA mutant but not that of the $\Delta rimM$ mutant (Fig. 6A; Table 1). S13 Δ 89-99, on the other hand, did not suppress the processing deficiency of either of the *rimM* mutants (Fig. 6A; Table 1). To our surprise, the N84K substitution in the RimM-YY $\rightarrow$ AA protein improved the processing efficiency threefold (Table 1), although it did not improve the binding of RimM-YY $\rightarrow$ AA to the 30S subunits (see above). The five different suppressor mutations in the plasmid-carried 16S rRNA gene improved the processing of 16S rRNA in the RimM-YY $\rightarrow$ AA mutant 1.5-to-fourfold, whereas they only improved the processing 1.6-fold or less in the $\Delta rimM$ mutant (Fig. 6B; Table 2). Note that the mutation with the least effect (A975U) in the RimM-YY $\rightarrow$ AA mutant (JML098) was the one that had the greatest effect in the $\Delta rimM$ mutant (JML124).

The tyrosines of the PRC-barrel domain of RimM bind specifically to r-protein S19 in the 30S subunits

RimM is found associated with free 30S subunits but not with 70S ribosomes (Bylund et al. 1997). To identify the

FIGURE 6. The effect of different suppressor mutations on the 16S rRNA processing deficiency of *rimM* mutants. The 5'-end of 16S rRNA was determined by primer extension analysis. RIII indicates a primer extension product of 179 nt corresponding to 17S rRNA processed at the RNase III site 115 nt upstream of the 5'-end of mature 16S rRNA. M indicates a product of 64 nt corresponding to the 5'-end of mature 16S rRNA. The sequencing ladder shown is of *rrnC* in plasmid pHK-*rrnC*⁺ and was obtained by using the same primer as for the primer extension reactions. (A) The effect of S13 Δ 89-99 and S19H83Y on the 16S rRNA processing in $\Delta rimM$ and RimM-YY $\rightarrow$ AA mutants. (B) The effect of 16S rRNA mutations on 16S rRNA processing in $\Delta rimM$ and RimM-YY $\rightarrow$ AA mutants.

TABLE 1. The effect of S19H83Y, S13Δ89-99, or RimM-N84K on the processing of 16S rRNA in *rimM* mutants

Strain	Relevant traits	Accumulation of 17S rRNA ^a			
		Exp. I	Exp. II	Exp. III	Exp. IV
MW100	wt	0.074	0.029	0.051	0.061
MW136	RimM-YY→AA	0.39	0.18	0.38	0.39
MW154	RimM-YY→AA S19H83Y	0.26	0.087	n.d.	n.d.
JML031	RimM-YY→AA S13Δ89-99	0.43	0.21	n.d.	n.d.
JML025	RimM-YY→AA/N84K	n.d.	n.d.	0.12	0.14
MW37	Δ <i>rimM</i>	0.54	0.33	n.d.	n.d.
MW158	Δ <i>rimM</i> S19H83Y	0.64	0.32	n.d.	n.d.
PW152	Δ <i>rimM</i> S13Δ89-99	0.52	0.34	n.d.	n.d.

^aThe amount of 17S rRNA to the total amount of 16S rRNA [$R_{III} / (M + R_{III})$] was calculated from the amount of radioactivity in the primer extension products obtained in experiments like that shown in Figure 6A.

binding site for RimM in the 30S subunits, a hybrid protein between glutathione S-transferase (GST) and RimM was used to pull out proteins interacting with RimM. The GST-RimM protein expressed from plasmid pJML005 complemented the slow growth of a Δ*rimM* mutant (GOB191), suggesting that the RimM part of the hybrid protein was functional (data not shown). The GST-RimM protein from total cell extracts of strain GOB191/pJML005 was bound to a glutathione column, which was then washed with increasing concentrations of NaCl. Finally, the GST-RimM protein plus proteins bound to RimM were eluted from the column. When extracts were prepared and analyzed at 4°C, several proteins smaller than 30 kDa in size were found in the stringent wash fractions and in the eluate (Fig. 7A). The eluate contained all small subunit r-proteins except S3, S14, S16, and S21 as determined by electro-spray ionization tandem mass spectrometry (ESI-MS/MS) (data not shown). In addition, r-protein L1 and some proteins not involved in translation were found in the eluate. Further, the eluate was shown by primer extension analysis to contain 16S rRNA (Fig. 7C), indicating that the GST-RimM protein bound to 30S subunits and not to free r-proteins. In contrast, when extracts of a strain expressing a GST-RimM(YY→AA) mutant protein were analyzed, no r-proteins were present in the eluate or in the stringent wash fractions (Fig. 7B). Thus, these results are consistent with the previous findings that the two conserved tyrosines are important for the binding of RimM to the 30S subunits (Lövgren and Wikström 2001).

When extracts that contained the GST-RimM wild-type protein were pre-

pared and analyzed at room temperature (~21°C), most 30S subunit r-proteins were released from the column at washes with up to 0.6 M NaCl (Fig. 8A). All 30S structural proteins except S6, S12, S15, S17, S18, S19, S20, and S21 were identified when total protein in the 0.6 M NaCl wash fraction was analyzed by ESI-MS/MS (data not shown). The most intense protein bands (≤ 30 kDa in Fig. 8A) were identified as r-proteins S2, S3, S4, S5, S7, S9, S10, S11, S13, and S17 by MALDI mass spectrometry. Moreover, 16S rRNA was present in the 0.6 M NaCl wash fraction as shown by primer extension analysis (data not shown), suggesting that intact 30S subunits were initially bound to the GST-

RimM protein not only at 4°C but also at room temperature. No r-proteins were found in the 0.6 M NaCl wash fraction when an extract of the strain expressing the GST-RimM(YY→AA) mutant protein was analyzed by ESI-MS/MS (Fig. 8B; data not shown). Interestingly, r-protein S19, as identified by MALDI mass spectrometry (data not shown), copurified with the GST-RimM wild-type protein but not with the GST-RimM(YY→AA) mutant protein (Fig. 8, cf. A and B). These findings demonstrate that the tyrosines present in positions 106 and 107 of wild-type RimM but absent in the RimM-YY→AA protein are crucial for the binding of RimM to S19 in the 30S subunits. Note also that S19 was not present among those r-proteins that were identified in the 0.6 M NaCl wash fraction when the GST-RimM wild-type protein was purified, suggesting that S19 bound strongly to the GST-RimM protein. Thus, the

TABLE 2. The effect of suppressor mutations in 16S rRNA on the processing of 16S rRNA in *rimM* mutants

Strain	Relevant traits	Accumulation of 17S rRNA ^a			
		Exp. I	Exp. II	Exp. III	Exp. IV
JML105	RimM-wt 16S rRNA-wt	0.066	0.016	0.039	0.075
JML068	RimM-YY→AA 16S rRNA-wt	0.32	0.14	0.23	n.d.
JML075	RimM-YY→AA 16S rRNA-C970G	0.12	0.047	0.058	n.d.
JML079	RimM-YY→AA 16S rRNA-ΔA974	0.18	0.051	0.052	n.d.
JML089	RimM-YY→AA 16S rRNA-C962U	0.13	0.046	0.092	n.d.
JML090	RimM-YY→AA 16S rRNA-G1015A	0.12	0.037	0.098	n.d.
JML098	RimM-YY→AA 16S rRNA-A975U	0.23	0.075	0.15	n.d.
JML113	Δ <i>rimM</i> 16S rRNA-wt	0.46	n.d.	n.d.	0.54
JML120	Δ <i>rimM</i> 16S rRNA-C970G	0.40	n.d.	n.d.	0.45
JML121	Δ <i>rimM</i> 16S rRNA-ΔA974	0.38	n.d.	n.d.	0.32
JML122	Δ <i>rimM</i> 16S rRNA-C962U	0.42	n.d.	n.d.	0.36
JML123	Δ <i>rimM</i> 16S rRNA-G1015A	0.45	n.d.	n.d.	0.40
JML124	Δ <i>rimM</i> 16S rRNA-A975U	0.30	n.d.	n.d.	0.32

^aThe amount of 17S rRNA to the total amount of 16S rRNA [$R_{III} / (M + R_{III})$] was calculated from the amount of radioactivity in the primer extension products obtained in experiments like that shown in Figure 6B.

FIGURE 7. Analysis of cellular components copurifying with GST-RimM hybrid proteins at 4°C. (A) A cell extract of strain GOB191/pJML005 ($\Delta rimM$ /GST-RimM) was prepared by freeze-thawing, and the GST-RimM hybrid protein in the extract was adsorbed to Glutathione Sepharose 4B, washed at the indicated molar concentrations of NaCl and finally eluted by reduced glutathione, all at 4°C. FT, flow-through; E, eluate. (B) As in A, but the strain used was GOB191/pJML051 [$\Delta rimM$ /GST-RimM(YY→AA)]. (C) Primer extension analysis of 16S rRNA prepared from the flow-through and eluate from A, and from 30S subunits obtained after sucrose gradient centrifugation of a total cell extract of the wild-type strain MW100.

interaction between RimM and S19 at room temperature seems to be stronger than that between S19 and other components of the 30S subunits, at least at higher NaCl concentrations.

The 30S subunits bound to the GST-RimM wild-type protein both at 4°C and at room temperature contained mature 16S rRNA and almost no 17S rRNA. This was unexpected, because a $\Delta rimM$ mutant accumulates 17S rRNA and therefore RimM has been suggested to bind 30S subunits that contain 17S rRNA rather than fully processed 16S rRNA.

To investigate whether S19H83Y, which improved the processing of 16S rRNA of the RimM-YY→AA mutant but not that of the $\Delta rimM$ mutant, could restore the binding of the RimM-YY→AA protein to the 30S subunits, the GST-RimM and GST-RimM(YY→AA) proteins were purified from strains expressing the S19H83Y protein. The S19H83Y protein copurified with the GST-RimM protein only (Fig. 8C) and not with the GST-RimM(YY→AA) protein (Fig. 8D) when extracts were prepared and analyzed at room

temperature. Further, no larger amounts of r-proteins were found in the wash fractions of the S19H83Y GST-RimM(YY→AA) strain than in those of the S19-wt GST-RimM(YY→AA) strain. Thus, S19H83Y did not seem to restore the binding of the RimM-YY→AA protein to the 30S subunits.

DISCUSSION

Identification of functionally important positions in RimM

The introduction of charged amino acids in two of the most conserved positions of RimM (G17E and G27R) dramatically reduced the stability of the protein (data not shown), but also reduced its function at high temperature without a further decrease in stability, implying a structural role of the conserved glycines. More subtle substitutions in the same positions (G17A and G27A) had no significant effects on the function of RimM. Because alanine for glycine substitutions are believed to affect the function of a protein mainly when glycines are important for the flexibility of a polypeptide chain, our results suggest other structural roles for these glycines in RimM. The double substitution Y106A/Y107A reduced the affinity of RimM for the 30S subunits, implying that the two tyrosines are important for the binding of RimM to the 30S subunits (this paper; Lövgren and Wikström 2001). These tyrosines together with the highly conserved aspartate in position 137 of a proposed PRC β -barrel domain are suggested to line a cleft into which a peptide of an interacting protein could be accommodated (Anantharaman and Aravind 2002). The D137A substitution severely reduced the stability of RimM, which precluded the possibility to assess whether or not this position in RimM interacts with the 30S subunits. However, the effect on the stability suggests an important role of this aspartate for the structure and stability of the PRC domain. Another substitution (G121D) in the PRC domain also affected the structure and/or function of RimM, as plasmid-encoded RimM variants with this substitution showed a reduced ability to complement chromosomal deletions of *rimM*.

Identification of the target region for RimM in the 30S subunits

Alterations in r-proteins S13 and S19 as well as mutations in helices 31 and 33b of 16S rRNA suppress the slow growth of a RimM-YY→AA mutant and to different degrees that of a $\Delta rimM$ mutant. These alterations are all located in a distinct region of the head of the 30S subunit (Fig. 9) in which helix 33b interacts with S19, helix 31 contacts S13, and S13 and S19 interact with each other (Wimberly et al. 2000). In addition, wild-type RimM, in contrast to the RimM-YY→AA

FIGURE 8. Analysis of cellular components copurifying with GST-RimM hybrid proteins at 21°C. Cellular extracts were prepared by sonication and the GST-RimM hybrid proteins were purified as described in the Figure 7 legend. Similar results were obtained when extracts were prepared by freeze-thawing. (A) Strain GOB191/pJML005 ($\Delta rimM$ S19-wt/GST-RimM). The proteins indicated in the lane with the 0.6 M NaCl wash fraction were identified as 30S subunit r-proteins by MALDI mass spectrometry: 1, S2; 2, S3; 3, S4; 4, S7; 5, S5; 6, S9; 7, S11 and S13; 8, S10; and 9, S17. In addition, one weak band above that containing S2 was identified as r-protein L2. (B) Strain GOB191/pJML051 [$\Delta rimM$ S19-wt/GST-RimM(YY→AA)]. (C) Strain MW158/pJML005 ($\Delta rimM$ S19H83Y/GST-RimM). (D) Strain MW158/pJML051 [$\Delta rimM$ S19H83Y/GST-RimM(YY→AA)].

protein, bound strongly to S19 in the 30S subunits, suggesting that the tyrosines in positions 106–107 of RimM mediate the binding to S19. One possible role for RimM in ribosome maturation might be to facilitate the interaction of S19 with helix 33b, and perhaps also the interaction between S13 and helix 31. In the maturation of the 30S subunits, the 5'-end processing of 16S rRNA has been suggested to be one of the last steps, which would also activate the 30S subunits (see Nierhaus 1991). Conceivably, a defect in any step in the 30S maturation process that precedes the processing of the 5'-end of 16S rRNA might result in a 16S rRNA processing deficiency and also a translational deficiency. Thus, the 16S rRNA processing deficiency observed for the $\Delta rimM$ and RimM-YY→AA mutants (this paper; Bylund et al. 1998) could stem from poor or inappropriate interactions in the S13-S19 region of the 30S subunits. Interestingly, S19 plays a key role in r-protein-dependent conformational rearrangements of 16S rRNA during 30S maturation in vitro (Holmes and Culver 2004).

RimM-YY→AA-dependent and RimM-YY→AA-independent suppression

The S19H83Y protein partially restored the ability of the RimM-YY→AA mutant to process 16S rRNA and to form polysomes. However, S19H83Y had less effect on the growth and polysome deficiencies and no effect on the 16S

rRNA processing deficiency of the $\Delta rimM$ mutant. Thus, to mediate efficient suppression, S19H83Y is dependent on the presence of the RimM-YY→AA protein, especially for the

FIGURE 9. Alterations in the *E. coli* 30S subunit (indicated in the model for the 30S subunit of *Thermus thermophilus*) that suppress *rimM* mutations. The structure of the 30S subunit is from Wimberly et al. (2000) and was retrieved from the Protein Data Bank (PDB no. 1FJF). Only part of the structure is shown. Helices 31 and 33b of 16S rRNA are presented as stick models in green except for the positions with suppressor mutations, which are in space-fill models. R-protein S13 is shown in a blue ribbon model except for a region in gray, which contains the alterations that suppress *rimM* mutations. S19 is shown in a yellow ribbon model with the most carboxy terminal amino acid, R81, of the determined structure, colored red. Thus, the alteration, H83Y, in S19 that suppressed RimM-YY→AA is in a position just outside of the determined structure.

processing of 16S rRNA. Four of the five different mutations in helices 31 and 33b of 16S rRNA were more efficient suppressors of the 16S rRNA processing deficiency of the strain containing the RimM-YY→AA protein compared to that of a mutant lacking RimM, although the 16S rRNA mutations had similar effects on the growth rate of the two *rimM* mutants. The fifth 16S rRNA mutation (A975U) suppressed the processing deficiency similarly in the two *rimM* mutants; however, it was the weakest of the five suppressor mutations in the RimM-YY→AA mutant. Interestingly, the effect of the 16S rRNA suppressor mutations on the growth rate did not correlate with their effect on the processing of 16S rRNA. Further, S13Δ89-99 increased the growth rate of and amount of polysomes in the Δ*rimM* and RimM-YY→AA mutants, although it did not correct the 16S rRNA processing deficiency. Taken together, these findings suggest that for the suppression of the translational defects of the *rimM* mutants, there are two different mechanisms. One mechanism improves the function of 30S subunits containing fully processed 16S rRNA. This is corroborated by earlier results showing that mature ribosomes in a strain lacking RimM are not as efficient as wild-type ribosomes in translating the *lacZ* mRNA (Bylund et al. 1997). A second mechanism improves the maturation of the 30S subunits, resulting in more efficient processing of 16S rRNA. Evidently, the ability of the different ribosomal suppressor mutations to mediate efficient processing of 16S rRNA requires the presence of the RimM-YY→AA (or wild-type RimM) protein. This suggests that the poor interaction of the RimM-YY→AA protein with the 30S subunits in combination with structural changes in the 30S subunits brought about by the suppressor mutations can promote efficient processing of 16S rRNA. Alternatively, the ribosomal suppressor mutations might partially restore the interaction of the RimM-YY→AA protein with the 30S subunits, despite the fact that we have been unable to demonstrate any such effects. We have assumed that the interaction detected between RimM and 30S subunits after sucrose gradient centrifugation of cellular extracts involves immature 30S subunits that contain partially processed 16S rRNA, because the Δ*rimM* and RimM-YY→AA mutants accumulate 17S rRNA, and because RimM is not associated with 30S subunits in 70S ribosomes (containing only fully processed 16S rRNA). However, the analysis of 30S subunits copurifying with a GST-RimM protein suggests that the binding detected is with 30S subunits containing fully processed 16S rRNA. Possibly, two types of interactions between RimM and the 30S subunits take place: a weak interaction, which we have been unable to detect, occurs initially between RimM and the 30S subunits that contain 17S rRNA, followed by a stronger binding, which we have observed in this study, once the 30S subunits have matured further and contain fully processed 16S rRNA. Interestingly, the N84K substitution in RimM-YY→AA improved processing of 16S rRNA, suggesting that it did improve binding of this RimM protein to the 30S subunits

although it did not affect binding in our assay. The simplest explanation of these contradictory results, assuming that RimM is not required for the synthesis of a protein involved in 16S rRNA processing, is that the N84K substitution, and possibly some of the ribosomal suppressors, such as S19H83Y, improve the proposed weak interaction between the RimM-YY→AA protein and the 30S subunits.

Are RimM and RbfA involved in 30S maturation steps both before and after processing of 16S rRNA?

RimM and RbfA bind to free 30S subunits in vivo (Dammel and Noller 1995; Bylund et al. 1997), and the GTPase Era binds to 16S rRNA and 30S subunits in vitro (Sayed et al. 1999). Cells lacking RimM or RbfA or cells depleted for Era accumulate 17S rRNA (Bylund et al. 1998; Inoue et al. 2003; Xia et al. 2003), and the levels of polysomes decrease with a concomitant increase in free 30S and 50S subunits (this paper; Dammel and Noller 1995; Inoue et al. 2003). Thus, these three proteins seem to take part in late steps of the maturation of the 30S subunits that precede the conversion of 17S to 16S rRNA. In addition, increased expression of RbfA suppresses a cold-sensitive mutation in the 5'-terminal helix of 16S rRNA, and RbfA was proposed to bind to this helix (Dammel and Noller 1995). The 5'-terminal helix is found only in mature 16S rRNA and not in 17S rRNA (Srivastava and Schlessinger 1989), suggesting that RbfA is involved in maturation after formation of 16S rRNA. Interestingly, a mutant RbfA protein missing the 25 most carboxy-terminal amino acids does not associate stably with 30S subunits and does not suppress the cold sensitivity of the helix mutation. However, the mutant RbfA protein complemented the 16S rRNA processing defect of the *rbfA* null mutant (Xia et al. 2003). Thus, RbfA may be involved in steps of 30S maturation both before and after the conversion of 17S to 16S rRNA. Increased expression of RbfA partially suppresses translational defects of the Δ*rimM* mutant (Bylund et al. 1998). Previously, we suggested that RimM participates before RbfA in 30S maturation and that RbfA cannot bind efficiently to the 30S subunits in the absence of RimM as the 30S subunits have not matured correctly. Conceivably, an increased level of RbfA would suppress a weak binding. However, in a Δ*rimM* mutant grown at 37°C, most RbfA molecules are associated with the 30S subunits and almost no free RbfA is found (J.M. Lövgren and P.M. Wikström, unpubl.). Thus, the 30S maturation deficiency of the Δ*rimM* mutant might be partly explained by limiting levels of free RbfA. This explanation would also account for why an increased amount of RbfA partially suppresses the defects of *rimM* mutants. Thus, if RimM acts before RbfA, then the reason for RbfA not being released from the 30S subunits in the Δ*rimM* mutant could be that there is a block in the next step of the maturation process. Such a step could be the binding of Era, because increased levels of Era suppress an *rbfA* mutant (Inoue et al.

2003). However, increased levels of Era do not suppress the slow growth of the $\Delta rimM$ mutant, as would have been expected. Alternatively, RbfA might act before RimM and will normally only be released from the 30S subunits when RimM has participated in the maturation process, which would explain the out-titration of RbfA in the $\Delta rimM$ mutant. Consequently, RbfA and RimM might bind, in that order, to immature 30S subunits and facilitate maturation steps that precede and are required for efficient conversion of 17S to 16S rRNA. Then, RbfA could also participate in the formation of the 5' terminal helix of mature 16S rRNA before being released from the 30S subunits. Possibly, RimM could be involved in maturation steps after the final processing of 16S rRNA, as indicated by the presence of mature 16S in the 30S subunits that copurified with the GST-RimM hybrid protein.

Here we have shown that the role of RimM in ribosome maturation involves the binding to S19 in the head of the 30S subunits. Our future strategies will aim at revealing any interplay between RimM and RbfA, and it will be interesting to determine whether RbfA takes part in maturation of the S19 region of the 30S subunits.

MATERIALS AND METHODS

Strains, phages, plasmids, and growth conditions

Relevant strains, phages, and plasmids used are listed in Table 3. Strain JML068 ($\Delta 7rrn rimM120/pHK-rrnC^+$ p70) was constructed in several steps: First, *pheA18::Tn10* from CAG12158 (Singer et al. 1989) was introduced into strain MW136 (*rimM120*) by P1 transduction selecting for Tc^R . From the resulting strain, MW148 (*rimM120 pheA18::Tn10*), the *rimM120* mutation was transferred to strain TA527 ($\Delta 7rrn/pHK-rrnC^+$ pTRNA66) by selecting for the linked *pheA18::Tn10*, yielding strain JML042 ($\Delta 6rrn rrnG^+$ *rimM120 pheA18::Tn10/pHK-rrnC^+ pTRNA66). Then, $\Delta(rrsG-gltW-rrlG)30::lacZ^+$ from strain TA527 was reintroduced by P1 transduction of strain JML042 selecting for Phe^+ , yielding strain JML067 ($\Delta 7rrn rimM120/pHK-rrnC^+$ pTRNA66). Finally, pTRNA66 (Sp^R) of strain JML067 was replaced by the similar p70 (Tc^R) plasmid from strain AVS69009 (Vila-Sanjurjo et al. 1999) by transformation, selecting for Tc^R and screening for Sp^S . A derivative of strain JML068, JML071 ($\Delta 7rrn rimM120/pKK1192U$ p70), was constructed by transformation of JML068 with plasmid pKK1192U (Cb^R) (Makosky and Dahlberg 1987), which carries an *rrnB* operon with a mutation in 16S rRNA (C1192U) conferring Sp^R , selecting for Sp^R and Cb^R , and screening for Km^S (i.e., the loss of $pHK-rrnC^+$). Strains JML113 and JML114 containing $\Delta rimM102$ instead of *rimM120* were constructed in a similar way as JML068 and JML071, respectively.*

Plasmid pJML005 that carries a fusion between the glutathione S-transferase gene (*gst*) and *rimM* (codons 2–182) was constructed by PCR-amplifying the wild-type *rimM* with oligonucleotides 5'-TTTGGATCCAAACAACCTACCGCGCAAG-3' and 5'-TTTGAATTCTTAAAAACAGGGTCCCAATC-3', digesting the PCR fragment with BamHI and EcoRI, and inserting it into similarly

digested pGEX-4T-2. The latter oligonucleotide destroyed the BamHI site in the 3'-end of *rimM* without changing the encoded amino acid sequence. Plasmid pJML051 expressing a GST-RimM(YY→AA) hybrid protein was constructed in the same way using chromosomal DNA from the RimM-YY→AA mutant MW136 as the template for PCR amplification.

Rich medium used was LB (Bertani 1951). Cultures were grown at indicated temperatures, and the growth was monitored at 600 nm using a Shimadzu UV-1601 spectrophotometer.

Isolation of rimM mutants

Random mutagenesis of *rimM* was done using hydroxylamine. Ten μ g of plasmid pMW279, which carries the *rimM* gene, was mutagenized at 75°C with 1 M of hydroxylamine for 90 min, and then dialyzed against water. The wild-type strain MW100 was transformed with the mutagenized pMW279, and transformants were transduced to kanamycin resistance using $\lambda 439\Delta rimM-2$ (Persson et al. 1995). Two *rimM* alleles complemented the slow growth at 37°C but not at 42°C; however, they contained two mutations each. One of these alleles, in plasmid pMW316, encoded a RimM protein with two amino acid substitutions, G17E and G121D, while the other allele, in plasmid pMW317, encoded a RimM protein with a single substitution, G27R, and contained a silent mutation in codon 13. The two mutations in pMW316 were cloned apart, yielding plasmids pMW324 (RimM-G17E) and pMW334 (RimM-G121D). The silent mutation in pMW317 was removed by PCR, yielding plasmid pMW325 (RimM-G27R).

Oligonucleotide-directed mutagenesis by using either single-stranded M13 DNA templates (Kunkel 1985; Kunkel et al. 1987) or overlap extension PCR (Ho et al. 1989) was done to create specific amino acid substitutions in RimM. The mutations were cloned into plasmid pMW296 carrying a 4.6-kb chromosomal fragment that contains the *rimM* region. This fragment was then transferred to plasmid pMAK705, which was used to introduce the mutant alleles into the *E. coli* chromosome as described (Hamilton et al. 1989).

Isolation of suppressor mutations

To isolate faster-growing derivatives of the RimM-YY→AA mutants MW136 and JML068, several cultures were inoculated from independent colonies and grown overnight in rich liquid medium. An aliquot of each overnight culture was streaked out on rich medium plates and subinoculated into fresh liquid medium. This procedure was repeated until revertants appeared on the plates. Only one revertant from each original culture was saved for further analyses. To investigate whether the suppressor mutations in strain JML068 were plasmid-carried, plasmid DNA from the suppressor mutants was used to transform strain JML071 selecting for Km^R ($pHK-rrnC$) and screening for Sp^S . Plasmid preparations that conferred fast growth were used to transform strain DH5 α (Hanahan 1983) selecting for Km^R and screening for Tc^S , to obtain clones that only contained the $pHK-rrnC$ plasmid and not the p70 tRNA plasmid. Plasmid DNA from such $Km^R Tc^S$ DH5 α derivatives was used to transform strain JML071, selecting for Km^R , to investigate whether the suppressor mutations were on the *rrn*-plasmid.

TABLE 3. Bacterial strains, bacteriophages, and plasmids

Strain, phage, or plasmid	Genotype	Origin or reference ^a
Strains		
CAG12158	<i>pheA18::Tn10</i>	(Singer et al. 1989)
GOB191	Hfr P4X $\Delta rimM102$ <i>pheA18::Tn10</i>	P1(CAG12158) X MW37
JML001	MW136 <i>spr-9</i> (Δ A in codon 412 of <i>nusA</i>)	
JML025	Hfr P4X <i>rimM131</i> (AAC to AAA in codon 84, TAC to GCT in codon 106, and TAC to GCG in codon 107)	
JML026	MW136 <i>spr-19</i> ($\Delta T_{in\beta}$)	
JML031	MW136 <i>rpsM873 zhc-2421::Tn10</i>	
JML037	Hfr P4X <i>rimM121</i> (GAA to GCA and GAC to GCG in codons 36 and 37, respectively)	
JML039	Hfr P4X <i>rimM122</i> (GAA to GCA and GAG to GCG in codons 102 and 103, respectively)	
JML040	Hfr P4X <i>rimM123</i> (AAA to GCA and GAC to GCG in codons 109 and 110, respectively)	
JML068	TA527 <i>rimM120/pHK-rrnC⁺ p70</i> (substituted for pTRNA66)	
JML071	JML068 pKK1192U (substituted for pHK-rrnC ⁺)	
JML075	JML068 <i>spr-41</i> (C970G in <i>rrsC</i>)	
JML079	JML068 <i>spr-45</i> (Δ A975 in <i>rrsC</i>)	
JML089	JML068 <i>spr-53</i> (C962U in <i>rrsC</i>)	
JML090	JML068 <i>spr-54</i> (G1015A in <i>rrsC</i>)	
JML098	JML068 <i>spr-62</i> (A975U in <i>rrsC</i>)	
JML105	TA527 p70 (substituted for pTRNA66)	
JML113	TA527 $\Delta rimM102/pHK-rrnC+ p70$ (substituted for pTRNA66)	
JML114	TA527 $\Delta rimM102/pKK1192U$ and p70 (substituted for pHK-rrnC ⁺ and pTRNA66, respectively)	
JML120	JML113 <i>spr-41</i> (C970G in <i>rrsC</i>)	
JML121	JML113 <i>spr-45</i> (Δ A975 in <i>rrsC</i>)	
JML122	JML113 <i>spr-53</i> (C962U in <i>rrsC</i>)	
JML123	JML113 <i>spr-54</i> (G1015A in <i>rrsC</i>)	
JML124	JML113 <i>spr-62</i> (A975U in <i>rrsC</i>)	
JML145	Hfr P4X <i>rimM134</i> (GAC to GCA in codon 137)	
MW37	Hfr P4X $\Delta rimM102$ <i>yfiB::nptI</i>	(Persson et al. 1995)
MW100	Hfr P4X	(Wikström et al. 1988)
MW122	Hfr P4X <i>rimM114</i> (GGT to GCT in codon 20)	
MW124	Hfr P4X <i>rimM115</i> (GGT to GCT in codon 24)	
MW130	Hfr P4X <i>rimM113</i> (AAA to GCA in codon 18)	
MW134	Hfr P4X <i>rimM116</i> (CGT to GCT in codon 26)	
MW136	Hfr P4X <i>rimM120</i> (TAC to GCT in codon 106 and TAC to GCG in codon 107)	
MW138	Hfr P4X <i>rimM112</i> (GGA to GCA in codon 17)	
MW139	Hfr P4X <i>rimM117</i> (GGG to GCG in codon 27)	
MW145	MW136 <i>rpsS876</i> (<i>spr-8</i>)	
MW154	MW145 <i>zhc-2421::Tn10</i>	
MW155	MW136 <i>zhc-2421::Tn10</i>	
MW158	MW37 <i>rpsS876 zhc-2421::Tn10</i>	
MW160	Hfr P4X <i>yfiB::nptI rpsS876 zhc-2421::Tn10</i>	
MW162	Hfr P4X <i>rimM118</i> (TAC to GCT in codon 106)	
MW163	Hfr P4X <i>rimM119</i> (TAC to GCG in codon 107)	
PW151	MW37 <i>zhc-2421::Tn10</i>	
PW152	MW37 <i>rpsM873</i> (deletion of codons 89 to 99) <i>zhc-2421::Tn10</i>	
TA527	$\Delta(rsa-ileT-alaT-rrlA)34 \Delta(rrsB-gltT-rrlB)101$ $\Delta(rrsC-gltU-rrlC)15::cat \Delta(rrsD-ileU-alaU-rrlD)25::cat$ $\Delta(purDH-rrnE-metA) \Delta(rrsG-gltW-rrlG)30::lacZ^+$ $\Delta(rrsH-ileV-alaV-rrlH)103 ara \Delta lac thi$ pTRNA66 pHK-rrnC ⁺	(Asai et al. 1999a)

(continued)

Ribosome profiles after sucrose gradient centrifugation

Cell cultures were grown in LB at 37°C to 70 Klett units. Polyosomes were stabilized by addition of chloramphenicol as described (Brow and Noller 1983). Cells were harvested by centrifugation,

and polysome extracts were prepared by a previously described freeze-thaw and lysozyme method (Ron et al. 1966) and fractionated by sucrose gradient centrifugation according to Powers and Noller (1990), with the exception that the sucrose was prepared in 10 mM Tris-HCl, pH 7.5, 50 mM KCl, 6 mM β -mercaptoethanol, 10 MgCl₂. For ribosome profiles under conditions that dissociate

TABLE 3. Continued

Strain, phage, or plasmid	Genotype	Origin or reference ^a
Bacteriophages		
λ439ΔrimM-2	ΔrimM102 yfiB::nptI	(Persson et al. 1995)
P1 vir		Laboratory stock
Plasmids		
pGEX-4T-2	bla lacI ⁺ P _{tac} -gst	Amersham Biosciences
pJML005	bla lacI ⁺ P _{tac} -gst-rimM ⁺	
pJML051	bla lacI ⁺ P _{tac} -gst-rimM120	
pMAK705	cat	
pMW279	P _{lac} -rimM ⁺ -cat-rnnBT ₁ bla	(Hamilton et al. 1989)
pMW296	ffh ⁺ rpsP ⁺ -rimM ⁺ -trmD ⁺ -rplS ⁺ yfiB ⁺ bla	(Persson et al. 1995)
pMW316	pMW279 rimM107 (GGA to GAA in codon 17 and GGC to GAC in codon 121)	
pMW317	pMW279 rimM108 (CCC to CCT in codon 13 and GGG to AGG in codon 27)	
pMW324	pMW279 rimM109 (GGA to GAA in codon 17)	
pMW325	pMW279 rimM111 (GGG to AGG in codon 27)	
pMW334	pMW279 rimM110 (GGC to GAC in codon 121)	

^aUnless otherwise noted, the origin was this study.

the ribosomal subunits, S30 extracts were prepared and fractionated by sucrose gradient centrifugation as previously described (Bylund et al. 1997).

Primer extension on rRNA

Total RNA was prepared using the Qiagen RNA/DNA and Qiagen RNeasy Kits (QIAGEN). RNA from ribosomal particles purified by sucrose gradient centrifugation was prepared by three successive extractions with phenol/chloroform/isoamyl alcohol (25:24:1). The RNA was then precipitated by ethanol and dissolved in 50 μL of water. Primer extension was performed as described earlier (Bylund et al. 1998).

Preparation of cell extracts and purification of GST-RimM proteins

One-hundred-mL cultures were grown in LB medium to 70 Klett Units, harvested by centrifugation, and resuspended in 0.5 mL of 20 mM Tris-HCl pH 7.0, 150 mM NaCl. Cells were disrupted either by 10–15 cycles of 5-sec sonication at setting 20% on a Vibra cell VCX400 (Sonics and Materials), or by freeze-thawing three times in the presence of lysozyme (1 μg/mL). RNase-free DNase I (10 U) was added, cell debris was removed by centrifugation, and the cell extracts were adsorbed to 0.1 mL of Glutathione Sepharose 4B (Amersham Biosciences). The Sepharose was packed into MicroSpin columns (Amersham Biosciences) and washed with 0.5 mL volumes of 20 mM Tris-HCl, pH 7.0, containing increasing concentrations of NaCl. Finally, the GST-RimM fusions were eluted from the columns with 0.5 mL of 10 mM reduced glutathione (Sigma-Aldrich) in 50 mM Tris-HCl, pH 8.0.

Mass spectrometric analyses

Total protein in fractions from purification of GST-RimM proteins was precipitated with acetone, dissolved, digested with

trypsin according to the manufacturer's directions (Trypsin Gold; Promega), and purified on a ZipTip (Millipore). Peptide separation and ESI-MS/MS (Q-TOF Ultima Waters-Micromass, MS Technologies) were performed as described (Szkanderova et al. 2003) by using a reverse-phase C18 column (Atlantis, NanoEase 75 μm × 150 mm) and analyzed with ProteinLynx GlobalSERVER version 2.05 (Waters-Micromass, MS Technologies). Peptides were identified using the Swiss-Prot database (Swiss Institute of Bioinformatics, Geneva, Switzerland).

Protein bands were cut out from colloidal Coomassie-stained (Neuhoff et al. 1990) polyacrylamide gels and digested according to Shevchenko et al. (1996). The extracted peptide solution was purified on a ZipTip and analyzed by MALDI mass spectrometry (Voyager-DE STR, Applied Biosystems) according to Szkanderova et al. (2003). Proteins were identified by using the Swiss-Prot database and the Mascot search engine (www.matrixscience.com).

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