The \textit{rlmB} Gene Is Essential for Formation of Gm2251 in 23S rRNA but Not for Ribosome Maturation in \textit{Escherichia coli}

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In \textit{Saccharomyces cerevisiae}, the rRNA Gm2270 methyltransferase, Pet56p, has an essential role in the maturation of the mitochondrial large ribosomal subunit that is independent of its methyltransferase activity. Here we show that the proposed \textit{Escherichia coli} ortholog, RlmB (formerly YjfH), indeed is essential for the formation of Gm in position 2251 of 23S rRNA. However, a \textit{ΔrlmB} mutant did not show any ribosome assembly defects and was not outgrown by a wild-type strain even after 120 cell mass doublings. Thus, RlmB has no important role in ribosome assembly or function in \textit{E. coli}.

The assembly of the 50S and 30S ribosomal subunits has been proposed to be a self-assembly process as demonstrated by the ability to reconstitute in vitro fully active ribosomes from the isolated components (36). However, recent observations indicate that proteins that assist in the formation of ribosomes, besides the ribosomal components, rRNA-processing enzymes, and tRNA-modifying and ribosomal-protein-modifying enzymes, have to be present in vivo. Indeed, there are at least seven candidates in \textit{Escherichia coli}: SrmB and DbpA, two DEAD box RNA helicases (14, 15, 26, 33, 37, 46); Era, an essential GTPase (30, 31–34, 41); RlmM and RbfA, two proteins associated with free 30S subunits but not with 50S subunits or 70S ribosomes (5, 6, 10, 27); and DnaK and GroEL, two molecular chaperones that at least at high temperature seem important for ribosome maturation (1, 12, 42). Whether DnaK and GroEL are directly or indirectly involved in the assembly of ribosomes in \textit{E. coli} is not known.

In \textit{Saccharomyces cerevisiae}, the Pet56p protein catalyzes 2'-O-methylation at the universally conserved G at position 2270 of 23S rRNA (43), corresponding to position 2251 of 23S rRNA of \textit{E. coli}. Further, pet56 null mutants lack functional mitochondrial ribosomes, indicating that Pet56p is essential for the in vivo assembly of the mitochondrial large ribosomal subunits (43). Recently it was demonstrated that Pet56p variants with amino acid substitutions in the SAM binding site, which abolished methyltransferase activity, could support the in vivo assembly of functional mitochondrial ribosomes, suggesting that Pet56p has a role in ribosome assembly that is independent of its methyltransferase activity (T. L. Mason, personal communication). In \textit{E. coli}, the protein encoded by the \textit{yjfH} gene is a putative 2'-O-methyltransferase, based on its similarities to other 2'-O-methyltransferases, especially Pet56p (21). The \textit{yjfH} gene in \textit{E. coli} encodes a hypothetical protein 243 amino acids in length and is downstream from the \textit{mr} gene, encoding RNase R (9). Just upstream from \textit{yjfH} there are sequences that match those for promoters dependent on \textit{σ}70 for transcription initiation, and downstream from \textit{yjfH} there is a sequence characteristic of rho-independent transcriptional terminators. To investigate whether YjfH was the \textit{E. coli} rRNA Gm2251 methyltransferase, a chromosomal deletion of \textit{yjfH} was constructed. The 835-bp region upstream from \textit{yjfH} was amplified by PCR using the oligonucleotides \textit{mr}-R1 and \textit{ΔyjfH}-R1, trimmed with \textit{Hind}III and \textit{SalI}, and cloned into the temperature-sensitive plasmid vector pMAK705, yielding plasmid pMW458 (Table 1). The 917-bp region downstream from \textit{yjfH} was amplified by PCR using the oligonucleotides \textit{ΔyjfH}-F1 and \textit{yjfH}-R1, trimmed with \textit{SalI} and \textit{BanH}I, and inserted into plasmid pMW458. The resulting plasmid, pMW465, which contains an in-frame deletion that covers all except the first two and last three codons of \textit{yjfH} replaced by a \textit{SalI} site, was used to delete the \textit{yjfH} gene on the chromosome of strain MW100 following the procedure described by Hamilton et al. (23). One of the resulting strains, MW244, was confirmed by PCR analyses to contain the \textit{yjfH} deletion on the chromosome. A \textit{yjfH} strain, MW245, was isolated together with MW244 and used as a control. Further, the \textit{yjfH} gene was amplified by PCR using the oligonucleotides \textit{yjfH}-F2 and \textit{yjfH}-R2, trimmed with \textit{EcoR}I and \textit{Hind}III, and cloned into pBAD30, yielding plasmid pMW467. rRNA and tRNA from the \textit{yjfH} strain MW245, the \textit{ΔyjfH} mutant MW244, and the mutant harboring the \textit{yjfH} plasmid pMW467 were prepared (13), degraded with nuclease P1 and alkaline phosphatase to nucleosides (17), and analyzed by high-pressure liquid chromatography (HPLC) (16). The \textit{ΔyjfH} mutant MW244 did not show any deficiency in the modification of tRNA (data not shown); however, it was found to lack Gm in 23S rRNA (Fig. 1B). This modification deficiency was fully complemented by plasmid pMW467 (Fig. 1C). These findings and the similarity of YjfH to Pet56p in \textit{S. cerevisiae}, together with the fact that in \textit{E. coli} Gm is found in only one position in rRNA, suggest that YjfH is indeed the \textit{E. coli} rRNA Gm2251 methyltransferase. Therefore, we rename YjfH RlmB (for “rRNA large-subunit methylation”).

RlmB is dispensable for fast growth. To examine whether RlmB is important for efficient growth, the growth rate at 37°C in Luria-Bertani (LB) medium (2) was determined for the \textit{ΔrlmB} mutant MW244 and the wild-type strain MW245. The specific growth rate, \(k = \ln 2/g\), where \(g\) is the mass doubling time (in hours), of the \textit{ΔrlmB} mutant was identical to that of the \textit{rlmB} strain (1.34; standard deviations of 0.021 and 0.031,
respective). Further, no difference between the two strains was observed in their ability to grow at 21, 30, 37, and 44°C on rich medium plates or at 30, 37, and 42.5°C on rich medium plates containing glucose. Also, stationery-phase cell culture density, measured as optical density at 600 nm, and survival in stationary phase, monitored by viable count determinations, after 24 and 48 h of incubation at 37°C in LB medium did not differ between the two strains (data not shown). To test the ability of the ΔlmB mutant to grow in competition with the rlmB+ strain over the whole range of the growth cycle, the two strains were grown separately in LB medium at 37°C with shaking to stationary phase, at which the optical density at 600 nm was 5.1 for both strains. Equal amounts of stationary-phase cells from each strain were inoculated into LB medium with shaking at 30°C and incubation steps of the mixed culture were repeated five times, and samples were taken at the start and after each cycle and plated on rich medium. To determine the ratio of cell doublings, approximately 50% of the cells in the mixed culture were still more than 120 cell doublings, approximately 50% of the cells in the mixed culture of the ΔlmB mutant to grow in competition with the rlmB+/H9004 mutant (data not shown). Since pet56 mutations of S. cerevisiae that lack the rRNA Gm2270 methyltransferase are deficient in the maturation of mitochondrial large ribosomal subunits, we examined whether RlmB was essential for assembly of ribosomes in E. coli. Polysome extracts of strains MW244 (ΔlmB) and MW245 (rlmB+) were prepared (40) and fractionated by sucrose gradient centrifugation (39). No differences between the two strains were observed with respect to the amounts of ribosomal subunits, 70S ribosomes, or polysomes (data not shown). To detect any subtle deficiency in ribosome maturation of the ΔlmB mutant, the kinetics of ribosome assembly were analyzed using pulse-labeling techniques. Log-phase cultures of the two strains grown in rich MOPS medium (35) lacking uracil were labeled with [3H]uridine for 1 and 2 min. Cellular extracts were prepared and analyzed by sucrose gradient centrifugation under conditions that dissociated the 70S ribosomes into 50S and 30S subunits (29). The amounts of 50S subunit assembly intermediates in the two strains were indistinguishable at both 1 and 2 min of labeling (data not shown). Furthermore, when the maturation of the 50S subunits was probed by primer extension analysis of the 5′ end of 23S rRNA (6), no increased accumulation of precursors to 23S rRNA was observed in the ΔlmB mutant (data not shown). These findings strongly suggest that RlmB is not important for ribosome maturation in E. coli.

In E. coli, 16S rRNA contains 11 modified nucleosides, of which 10 are methylated, while 23S rRNA contains 23 modified nucleosides, of which 14 are methylated (3). Some of the methylated nucleosides have ribose methylations (2′-O-methylations), whereas others have base methylations. Modified nucleosides are clustered at the functional domains of rRNA, such as the A and P sites of 16S rRNA and the peptidyltransferase center of 23S rRNA (3). It has been proposed that the modifications could affect rRNA maturation and structure, ribosomal subunit association, tRNA binding, and peptidyltransferase activity; however, little is known about their exact functions. In E. coli, three 16S rRNA methyltransferases (RsmA [KsgA], RsmB [RrmB], and RsmC) and two 23S rRNA methyltransferases (RrmA and RrmI [PtsI]) have been identified. A mutation in the E. coli ksgA (rsmA) gene, encoding the 16S rRNA m5A1518,1519 methyltransferase (24), leads to a reduced growth rate in some media and a reduced polypeptide-synthetic activity in vitro (25), whereas a disruption of the gene for RsmB, the 16S rRNA m2G1207 methyltransferase (19, 44), has no discernible effect on the growth rate of the cells (19). No mutation in rsmC, encoding the 16S rRNA m7G1207 methyltransferase (45), is available; however, RsmC cannot be essential for ribosome function, since functional 30S subunits can be prepared using 16S rRNA lacking all modifications (11, 28). A strain that lacks RrmA, the 23S rRNA m7G745 methyltransferase, shows a 1.4-fold-increased mass doubling time, respectively. Further, no difference between the two strains was observed in their ability to grow at 21, 30, 37, and 44°C on rich medium plates or at 30, 37, and 42.5°C on medium E plates containing glucose. Also, stationery-phase cell culture density, measured as optical density at 600 nm, and survival in stationary phase, monitored by viable count determinations, after 24 and 48 h of incubation at 37°C in LB medium did not differ between the two strains (data not shown). To test the ability of the ΔlmB mutant to grow in competition with the rlmB+ strain over the whole range of the growth cycle, the two strains were grown separately in LB medium at 37°C with shaking to stationary phase, at which the optical density at 600 nm was 5.1 for both strains. Equal amounts of stationary-phase cultures of mutant and wild-type cells were mixed, diluted 1010-fold in LB medium, and incubated for 24 h. The dilution and incubation steps of the mixed culture were repeated five times, and samples were taken at the start and after each cycle and plated on rich medium. To determine the ratio of ΔlmB cells to rlmB+ cells in the mixed culture, 48 colonies from each sampling time were subjected to PCR with oligonucleotides rnr-F1 and yjfH-R2. Even after six cycles, corresponding to more than 120 cell doublings, approximately 50% of the cells in the mixed culture were still ΔlmB mutants (data not shown). Thus, the lack of RlmB did not confer any disadvantage to the mutant cells in their competition with rlmB+ cells.

RlmB is not important for ribosome maturation. Since pet56 mutants of S. cerevisiae that lack the rRNA Gm2270 methyltransferase are deficient in the maturation of mitochondrial large ribosomal subunits, we examined whether RlmB was essential for assembly of ribosomes in E. coli. Polysome extracts of strains MW244 (ΔlmB) and MW245 (rlmB+) were prepared (40) and fractionated by sucrose gradient centrifugation (39). No differences between the two strains were observed with respect to the amounts of ribosomal subunits, 70S ribosomes, or polysomes (data not shown). To detect any subtle deficiency in ribosome maturation of the ΔlmB mutant, the kinetics of ribosome assembly were studied using pulse-labeling techniques. Log-phase cultures of the two strains grown in rich

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<th>Strain, plasmid, or oligonucleotide</th>
<th>Description</th>
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<tr>
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**FIG. 1.** HPLC analysis of modified nucleosides in rRNA. Only the part of the chromatograms that showed a difference between the strains is shown. (A) Strain MW245 (rlmB)/H11001 (trmH); (B) strain MW244 (ΔrlmB); (C) strain MW244 (ΔrlmB)/pMW467 (rlmB).**

...an increased amount of free ribosomal subunits, and a decreased polypeptide chain elongation rate (20). Similarly, a mutant deficient in RmJ, the 23S rRNA Um2552 methyltransferase (4, 7), has a severe growth disadvantage and a dramatically altered polysome profile (4, 8). Since the ΔrlmB mutant studied here showed no growth or ribosome assembly defects, Gm2251 cannot play any essential role in ribosome assembly or function, which is also supported by measurements of the peptideyltransferase activity of reconstituted ribosomes containing in vitro-transcribed 23S rRNA lacking Gm2251 (18). However, we cannot exclude the possibility that Gm2251 has some importance for ribosome function under conditions which we have not tested. Our results also suggest that RlmB itself has no important function in ribosome assembly. This contrasts with the situation in *S. cerevisiae*, where the RlmB counterpart, Pet56p, has an essential function in the maturation of the mitochondrial large ribosomal subunit that is independent of its methyltransferase activity (43; Mason, personal communication). In comparison to RlmB, Pet56p has an N-terminal extension of 143 amino acids. Conceivably, the maturation function of Pet56p might reside in this part of the protein, explaining why the ΔrlmB mutant was not deficient in ribosome maturation. This maturation function might be completely absent in *E. coli* or might be performed by another protein or by 23S rRNA.

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