

Thermus thermophilus L11 Methyltransferase, PrmA, Is Dispensable for Growth and Preferentially Modifies Free Ribosomal Protein L11 Prior to Ribosome Assembly

Dale M. Cameron,¹† Steven T. Gregory,¹ Jill Thompson,¹ Moo-Jin Suh,² Patrick A. Limbach,² and Albert E. Dahlberg^{1*}

Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, Rhode Island,¹ and Rieveschl Laboratories for Mass Spectrometry, Department of Chemistry, University of Cincinnati, Cincinnati, Ohio²

Received 4 March 2004/Accepted 2 June 2004

The ribosomal protein L11 in bacteria is posttranslationally trimethylated at multiple amino acid positions by the L11 methyltransferase PrmA, the product of the *prmA* gene. The role of L11 methylation in ribosome function or assembly has yet to be determined, although the deletion of *Escherichia coli* *prmA* has no apparent phenotype. We have constructed a mutant of the extreme thermophile *Thermus thermophilus* in which the *prmA* gene has been disrupted with the *htk* gene encoding a heat-stable kanamycin adenyltransferase. This mutant shows no growth defects, indicating that *T. thermophilus* PrmA, like its *E. coli* homolog, is dispensable. Ribosomes prepared from this mutant contain unmethylated L11, as determined by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), and are effective substrates for in vitro methylation by cloned and purified *T. thermophilus* PrmA. MALDI-TOF MS also revealed that *T. thermophilus* L11 contains a total of 12 methyl groups, in contrast to the 9 methyl groups found in *E. coli* L11. Finally, we found that, as with the *E. coli* methyltransferase, the ribosomal protein L11 dissociated from ribosomes is a more efficient substrate for in vitro methylation by PrmA than intact 70S ribosomes, suggesting that methylation in vivo occurs on free L11 prior to its incorporation into ribosomes.

Extensive modification is a feature of many components of the translational apparatus, with the most common being methylation. In addition to methylated ribosomal RNAs, several ribosomal proteins are also methylated or acetylated. The most highly methylated protein component of the bacterial translational apparatus is the 50S ribosomal subunit protein L11 (2), which comprises a major part of the factor-binding region of the 50S subunit. Cryo-electron microscopic reconstructions of ribosome–EF-Tu (22, 25) and ribosome–EF-G (1) complexes demonstrated a dynamic conformation of L11 and a direct contact of the L11 region of the ribosome with both elongation factors. How, or even whether, methylation of L11 influences its function in multiple aspects of protein synthesis is not yet understood.

Escherichia coli L11 is trimethylated at three amino acid positions, specifically Ala1 (the N-terminal methionine is removed posttranslationally), Lys3, and Lys39, acquiring a total of nine methyl groups (11, 17). These methyl groups are added by a single enzyme, the L11 methyltransferase PrmA, encoded by the *prmA* gene (7, 27). This protein is conserved among bacteria but is absent from eukarya and archaea (4). Nevertheless, a *prmA* null mutant of *E. coli* has no detectable phenotype (27), leaving open the question of the function of this modification.

The substrate recognition mechanism of this enzyme is of particular interest because it methylates multiple positions on the same protein. Presumably, the sites of methylation exist in structurally similar contexts, although the N terminus of L11 in the X-ray crystal structure (29) is disordered, preventing a definitive answer to this question. It is also not known if PrmA produces all modifications during one binding event or if it has multiple, distinct binding states, e.g., one for each trimethylation. It is also possible that the flexible N terminus of L11 adopts several distinct conformations within the PrmA active site, facilitating the multiple modifications. The detection of mono- and dimethylated lysine residues in cloned and overproduced protein (24) leads to the speculation that the methyl groups are added sequentially in the reaction. An important step toward answering these questions would be the development of a system for the biochemical characterization of PrmA activity and a system for high-resolution structural analysis of the PrmA-L11 complex.

Here we describe the construction of a *prmA* null mutant of *Thermus thermophilus* HB8 as a source of unmethylated ribosomes to act as substrates for in vitro methylation assays using cloned and purified *T. thermophilus* PrmA methyltransferase. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) of ribosomal proteins from the *prmA* null mutant revealed that, in contrast to the 9 methyl groups on *E. coli* L11, *T. thermophilus* L11 is modified with a total of 12 methyl groups. We also describe a biochemical characterization of *T. thermophilus* L11 methylation in vitro. Our choice of *T. thermophilus* was based on the ease of genetic manipulation of this organism as well as the structural information available regarding *Thermus* ribosomes. Furthermore, the PrmA protein from *T. thermophilus* HB8 is currently being

* Corresponding author. Mailing address: Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, RI 02912. Phone: (401) 863-2223. Fax: (401) 863-1182. E-mail: Albert_Dahlberg@brown.edu.

† Present address: Department of Cellular and Molecular Pharmacology and Howard Hughes Medical Institute, University of California at San Francisco, San Francisco, CA 94143.

examined by X-ray crystallography, and crystals diffracting to 1.9 Å have been reported (14). This raises the possibility that a structural basis for an understanding of the recognition mechanism of this enzyme may be elucidated.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *T. thermophilus* strain HB8 (ATCC 27624) (20) was employed as the wild-type parental strain for construction of the *prmA* null mutant. *T. thermophilus* strains were grown in ATCC medium 1598 (*Thermus* enhanced medium, or TEM), either in liquid form or solidified with 2.8% (wt/vol) Difco agar. Growth rate measurements were performed in *Thermus* minimal medium, which is equivalent to TEM without tryptone or yeast extract and with 0.4% sucrose as a carbon source, 10 mM NH₄Cl, 20 μg of lysine/ml, and 40 ng each of paraminobenzoic acid, nicotinic acid, calcium pantothenate, thiamine, pyridoxine, biotin, and cyanocobalamin/liter. Growth rate measurements were performed by use of a Klett-Summerson photometer. A pUC18 clone of the heat-stable kanamycin-adenyltransferase gene, *htk*, was a kind gift from H. Kagamiyama (Osaka Medical College). A *T. thermophilus* HB8 streptomycin-dependent mutant was a kind gift of A. Carter and V. Ramakrishnan (MRC, Cambridge, United Kingdom). The transformation of *T. thermophilus* was performed according to a standard method (16).

Oligonucleotides. All oligonucleotides were obtained from Qiagen. The oligonucleotides used for cloning *prmA* and for constructing the *prmA* null mutant (TLK90) were as follows: Tth *prmA*-1, 5'-GATATACATATGTGGGTTTACCGCTTAAGGGC-3'; Tth *prmA*-2, 5'-GCTCGAATTCAGTGGTGGTGGTGGTGGTCCCTCCGTAGGCGAGGAGG-3'; Tth *prmA*-3, 5'-CCAGGTACCGTGTGGGTTTACCGGCTTAAGGGC-3'; Tth *prmA*-4, 5'-GGTCTGCAGGCCGGGCTCAATGACCAAGGGG-3'; Tth *prmA*-5, 5'-CCACTGCAGCCCCAGGCGGAGGCGAACGCCAAGCGG-3'; Tth *prmA*-6, 5'-GGTAAGCTTCCAGTCCGTAGGCGAGGAGG-3'; Tth *htk*-1, 5'-CCACTGCAGGGTACCCGTGACGGCGGATATGG-3'; and Tth *htk*-2, 5'-GGTCTGCAGCGTAA CCAACATGATTAAC-3'. Oligonucleotides for cloning *prmA* were based on the published *T. thermophilus* HB8 sequence (GenBank accession number AB103400). Oligonucleotides used for diagnostic PCRs were as follows: primer A, 5'-GTGTGGGTTTACCGGCTTAAGGGC-3'; primer B, 5'-GCGGGGGC GCACCCGTTCCG-3'; primer C, 5'-GGCCCTCGCCCGCCACCTCCGCC CCGGG-3'; primer D, same as Tth *prmA*-6; primer E, 5'-CCCCGGGGCGG AGGTGGCGGCGAGGCGC-3'; primer F, same as Tth *htk*-1; and primer G, same as Tth *htk*-2.

Purification of ribosomes. Ribosomes from wild-type HB8 and strain TLK90 were purified essentially as previously described (5). Ribosomes were stored at -80°C in a buffer containing 10 mM HEPES-KOH (pH 7.6), 10 mM MgCl₂, 50 mM NH₄Cl, 5 mM β-mercaptoethanol, and 10% (vol/vol) glycerol.

MALDI analysis. All MALDI-MS experiments were done on a Reflex IV reflectron MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, Mass.) equipped with a nitrogen laser, as previously described (23). For all protein analyses, saturated sinapinic acid (SA; Fluka, Milwaukee, Wis.) in 33% aqueous acetonitrile plus 0.1% trifluoroacetic acid was used as the matrix. Samples were prepared by mixing 1 μl of acidified sample solution (approximately 1.8 pmol of ribosome) with 9 μl of matrix. Protein mass spectra were obtained in the positive ion mode at an acceleration voltage of 20 kV by accumulating 300 laser shots. All samples were analyzed under identical instrumental parameters. Acids and organic solvents were high-performance liquid chromatography grade or higher.

Overexpression and purification of *T. thermophilus* HB8 PrmA. C-terminally His₆-tagged PrmA was purified from *E. coli* BL21(DE3) containing the plasmid clone pET30bprmA (see Results for a description of the construction of this plasmid). Cultures were grown to mid-logarithmic phase, and expression of the enzyme was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM final concentration). Growth was continued for 2 h, after which the cells were harvested, washed, and lysed by passage through a French press (20,000 lb/in²) in a buffer containing 50 mM Tris-HCl (pH 7.6), 500 mM NaCl, 5 mM imidazole, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, and 10% (vol/vol) glycerol. After a DNase I treatment, the lysates were cleared by centrifugation at 20,000 × g for 20 min. The supernatants were heated to 65°C for 30 min to denature *E. coli* proteins, cleared once again by centrifugation, and then gently mixed with a Ni-nitrilotriacetic acid (Ni-NTA) slurry (Qiagen) for 2 h at 4°C. The Ni-NTA resin bound with PrmA was then collected by centrifugation, washed twice with a buffer containing 50 mM Tris-HCl (pH 7.6), 500 mM NaCl, 20 mM imidazole, and 1 mM DTT, and then mixed gently for 1 h at 4°C to elute the protein in a buffer containing 50 mM Tris-HCl (pH 7.6), 500 mM NaCl, 500 mM imidazole, and 1 mM DTT. The eluate was

concentrated and diluted multiple times by the use of Centricon spin columns into a storage buffer containing 10 mM HEPES-KOH (pH 7.6), 10 mM MgCl₂, 50 mM NH₄Cl, 5 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride. The protein concentration was determined by measuring the absorbance at 280 nm (1 mg of His₆-PrmA/ml gives an absorbance at 280 nm of 2.025).

In vitro methylation assays. Methylation assays were carried out at 50°C in a final volume of 70 μl and contained 50 nM ribosomes (intact or RNase A treated), 100 nM PrmA, and 100 nM S-adenosyl-L-[methyl-³H]methionine (³H-SAM; specific activity, 4.37 × 10¹¹ Bq/mmol) in a buffer containing 10 mM HEPES-KOH (pH 7.6), 10 mM MgCl₂, 50 mM NH₄Cl, and 5 mM β-mercaptoethanol or in buffer A (50 mM Tris-HCl [pH 7.6], 600 mM KCl, 10 mM EDTA, 10 mM β-mercaptoethanol). Reactions were started by the addition of ³H-SAM, and 20-μl samples were removed at each time point and placed into 1 ml of 10% trichloroacetic acid. The samples were heated to 100°C for 10 min and then passed through Whatman GF/A glass microfiber filters. The filters were washed three times with 3 ml of 5% trichloroacetic acid each time and then were dried, and radioactivity was estimated by liquid scintillation spectrometry.

RESULTS

Construction of *T. thermophilus prmA* null mutant indicates that PrmA-directed methylation is dispensable for growth.

The *T. thermophilus* HB8 *prmA* gene has been identified by others based on its homology with the *E. coli* gene (14) (GenBank accession number AB103400). Thus, a null mutant allele of *prmA* was constructed by gene disruption (Fig. 1) in which wild-type *T. thermophilus* HB8 was transformed with a plasmid (pUC19prmA::htk) containing the *T. thermophilus prmA* gene interrupted by a heat-stable kanamycin resistance gene, *htk*. To ensure that any resulting gene product would not be functional, the region of the *prmA* gene that encodes the SAM binding motif was deleted. For the construction of pUC19prmA::htk, the 5' end of the *T. thermophilus prmA* coding region was amplified by a PCR using a forward primer containing a KpnI restriction site (Tth *prmA*-3) and a reverse primer containing a PstI restriction site (Tth *prmA*-4), giving a product of 306 bp. The 3' end of the *T. thermophilus prmA* coding region was amplified by a PCR using a forward primer containing a PstI restriction site (Tth *prmA*-5) and a reverse primer containing a HindIII restriction site (Tth *prmA*-6), giving a product of 319 bp. Both products were appropriately digested and ligated simultaneously into KpnI- and HindIII-digested pUC19 to form plasmid pUC19prmA.

A DNA fragment containing the coding region of the *htk* gene was amplified by PCR from a pUC18 clone (13) by the use of primers containing PstI restriction sites (Tth *htk*-1 and Tth *htk*-2), giving a product of 1,093 bp. This DNA fragment was digested with PstI and inserted into the PstI site of pUC19prmA to form pUC19prmA::htk (Fig. 1A). Plasmid constructs were verified by sequencing, and in order to avoid problems caused by transcriptional polarity, only constructs containing the *htk* gene inserted into the vector in the same orientation as the *prmA* gene were used for transformations. The final plasmid construct was introduced into *T. thermophilus* HB8 by standard transformation, using 5 μg of pUC19prmA::htk and plating on solid TEM containing kanamycin (100 μg/ml). Since pUC19-derived plasmids do not replicate in *Thermus* spp., kanamycin resistance results from integration of the plasmid into the chromosome at a site of homology. Gene replacement occurs upon resolution of the cointegrate. Transformants arose only on plates containing cells that received plasmid DNA. Genomic DNAs from several transformants were prepared, and the absence of the full-

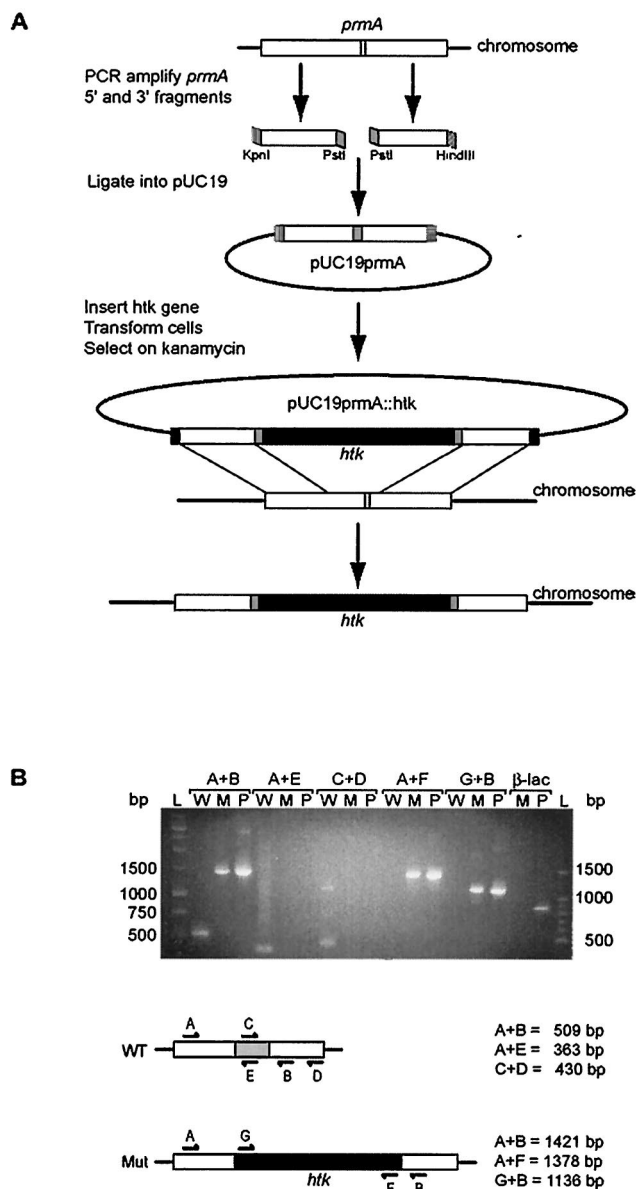


FIG. 1. Construction of *T. thermophilus* *prmA* null mutant. (A) Approximately 165 bp of the wild-type *prmA* gene (white) containing the SAM binding motif (light gray) was replaced by the heat-stable kanamycin resistance gene (*htk*; black). *KpnI* restriction sites are indicated by vertical lines, *HindIII* restriction sites are indicated by horizontal lines, and *PstI* restriction sites are indicated in gray. The resulting *T. thermophilus* strain was designated TLK90. (B) Diagnostic PCRs to confirm disruption of the *prmA* gene in strain TLK90. Genomic DNA from either wild-type HB8 (W), the null mutant (M), or plasmid pUC19prmA::htk (P) was used as a template for PCRs. The primer sets used for each reaction (A to G) are indicated and shown schematically along with the expected product sizes. The region of the *prmA* gene that was deleted from the null mutant is represented schematically in gray, and the *htk* gene is indicated in black. Amplification of the β -lactamase gene (β -lac) from the plasmid template but not from TLK90 genomic DNA template confirmed the absence of this plasmid-derived ampicillin resistance marker gene in the null mutant. Molecular weight DNA ladders are shown (L), and their sizes are indicated in base pairs.

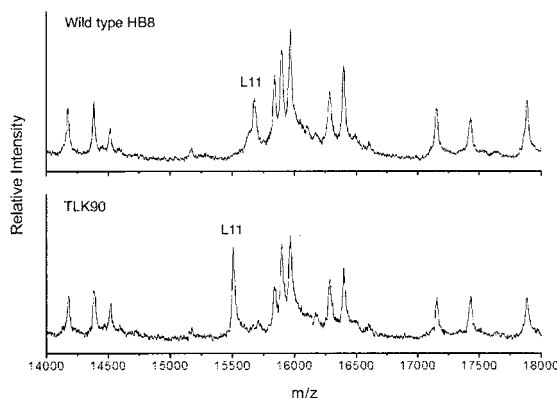


FIG. 2. MALDI-TOF MS of 70S ribosomes from *T. thermophilus* strain HB8 (top) and *prmA*::*htk* null mutant TLK90 (bottom). The portions of the spectra containing proteins in the mass range of 14,000 to 18,000 Da are shown. The peaks corresponding to L11 show masses of 15,675.1 and 15,506.2 Da for the wild type and the mutant, respectively. The mass difference of 169 Da is equivalent to the mass of 12 methyl groups.

length *prmA* gene, the absence of the pUC19 plasmid vector, and the insertion of the *htk* gene were verified by diagnostic PCR and sequencing, as indicated in Fig. 1B. A single transformant, designated TLK90, was used in all subsequent experiments.

The viability of kanamycin-resistant transformants which contained no intact *prmA* gene indicates that this gene is dispensable. Moreover, the *T. thermophilus* *prmA*::*htk* null mutant exhibited no growth defect, with wild-type HB8 and TLK90 having doubling times in *Thermus* minimal medium of 172 ± 10 and 166 ± 11 min, respectively. This result is consistent with the phenotype of a *prmA* null mutant of *E. coli*, which showed no detectable physiological defect (27). We can also conclude, therefore, that L11 methylation by PrmA does not contribute to the thermostability of *T. thermophilus* ribosomes in vivo, as has been suggested for other modifications of the translational apparatus in thermophilic organisms (15).

MALDI-TOF MS of ribosomes. MALDI-TOF MS was performed in order to ascertain the methylation state of the ribosomal protein L11 in ribosomes from wild-type *T. thermophilus* HB8 and the *prmA*::*htk* null mutant (TLK90; Fig. 2). The molecular mass values obtained for unmethylated and methylated L11 were 15,506.2 and 15,675.1 Da, respectively. The mass value for unmodified L11 was precisely the same as that predicted from the gene sequence (GenBank accession number X81375). The mass difference of 169 Da is equivalent to a total of 12 methyl groups, which is 3 more than the 9 methyl groups introduced by the *E. coli* enzyme.

Disruption of the *prmA* gene does not suppress streptomycin dependence caused by a mutation in ribosomal protein S12. The involvement of the ribosomal protein L11 in the interaction of the ribosome with EF-Tu (22, 25), plus the observation that mutations in L11 and in the L11-binding site of 23S rRNA suppress nonsense mutations (18, 26), suggested the possibility that the absence of L11 methylation by PrmA might affect translational accuracy. Such effects can in some instances be manifested as a suppression of streptomycin dependence due to mutant alleles of *rpsL*, encoding the ribosomal protein S12

(3). Indeed, an early study reported that ribosomes from a strain that had spontaneously reverted from streptomycin dependence contained L11 with an altered electrophoretic mobility (10). This is perhaps one of the few aspects of the *E. coli* *prmA* null mutant that has not been previously examined. We therefore introduced the *prmA::htk* null allele into a mutant of strain *T. thermophilus* HB8 carrying a P90L streptomycin dependence mutation in ribosomal protein S12 (5a) by transformation with chromosomal DNA from strain TLK90. Kanamycin-resistant transformants were sequenced to confirm both the presence of mutated *rpsL* and the disruption of *prmA* and were screened for the ability to grow in the absence of streptomycin. All transformants tested retained the streptomycin dependence phenotype, suggesting that a strong error-prone phenotype is not produced by the absence of L11 methylation.

Cloning of *T. thermophilus* HB8 *prmA* gene and purification of PrmA enzyme. The coding region of the *prmA* gene was amplified by PCR from a genomic DNA template by use of a forward primer, Tth prmA-1, containing an NdeI restriction site, and a reverse primer, Tth prmA-2, containing an EcoRI restriction site and the complement of a stop codon followed by a (GTG)₆ repeat encoding a His₆ tag. The resulting DNA fragment was inserted into the NdeI and EcoRI sites of plasmid pET30b to form plasmid pET30bprmA. Plasmid constructions were verified by sequencing.

PrmA expression was induced with 1 mM IPTG. Heat treatment of the crude extract resulted in the denaturation and precipitation of the *E. coli* proteins without affecting the integrity of *T. thermophilus* PrmA, which is heat stable. Further purification was achieved by Ni-NTA chromatography. The fractionation of the PrmA extract is demonstrated in Fig. 3A. From this gel, we estimate that purification was achieved to >95%. The apparent molecular mass of the cloned protein was 29 kDa, which is in good agreement with the 27.6 kDa deduced from its gene sequence (14) plus approximately 1 kDa for the His₆ tag.

Methylation of ribosomes from *prmA::htk* null mutant by cloned and purified PrmA. Ribosomes from the *prmA::htk* null mutant were examined for the ability to act as a substrate for cloned PrmA in vitro. As shown in Fig. 3B, ribosomes from wild-type *T. thermophilus* HB8 did not act as efficient substrates because they contain methylated L11. In contrast, ribosomes from the *prmA::htk* null mutant TLK90 incorporated ³H-radiolabeled methyl groups in a PrmA-dependent manner. Under the conditions used, the reaction proceeded at an initial rate of 2.07×10^{-4} methyl groups incorporated/ribosome/s for approximately 90 min, after which the amount of ³H detected actually began to decrease, probably due to the aggregation and/or precipitation of reaction components after such a long incubation at 50°C. Since *T. thermophilus* L11 is methylated in vivo with 12 methyl groups and since a maximum of only 1.1 methyl groups was incorporated per ribosome, only very partial methylation of fully assembled L11 was observed. The slow time course of methyl group incorporation into wild-type ribosomes (2.3×10^{-5} /ribosome/s) was not significantly different from that observed in the absence of ribosomes and may have been due to the detection of ³H-SAM bound to the PrmA enzyme. Alternatively, this low-level incorporation of methyl groups into wild-type ribosomes may have been due to the presence of under-methylated ribosomes in the reaction. This

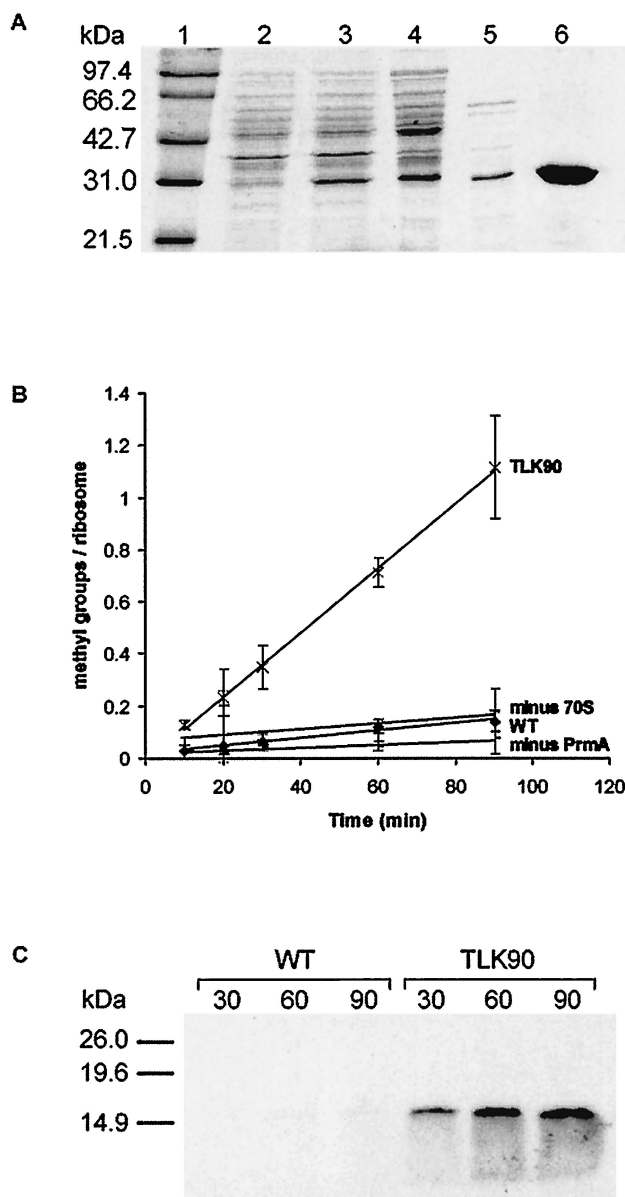


FIG. 3. (A) Purification of cloned His₆-tagged *T. thermophilus* L11 methyltransferase, PrmA, from *E. coli* BL21(DE3) carrying plasmid pET30bprmA. Lane 1, molecular size markers (sizes are indicated); lane 2, whole-cell lysate prior to induction; lane 3, whole-cell lysate after 2-h induction with IPTG; lane 4, cleared lysate after DNase treatment; lane 5, supernatant after 30-min incubation of lysate at 65°C; lane 6, purified PrmA after Ni-NTA purification, desalting, and concentration. (B) In vitro methylation of ribosomes from wild-type HB8 and the *prmA* null mutant, TLK90. Methylation assays were performed over a 90-min time course. ♦, ribosomes from wild-type HB8 (WT); ×, ribosomes from TLK90; +, reaction without ribosomes (minus 70S); −, ribosomes from TLK90 without the enzyme (minus PrmA). The results shown were obtained from at least three independent experiments. (C) ³H-labeled products after in vitro methylation of ribosomes from wild-type HB8 (WT) and the *prmA* null mutant (TLK90). Methylation assays were performed, with samples being removed after 30, 60, and 90 min and electrophoresed through a sodium dodecyl sulfate–15% polyacrylamide gel (1 pmol equivalent of ribosomes per lane). After fixing in a 10% methanol–acetic acid solution, the gels were incubated for 20 min in Enlightening rapid autoradiography enhancer (Perkin-Elmer), dried, and exposed to film for 72 h. The positions of molecular mass markers on the gel are indicated.

^3H incorporation, however, was not due to an exchange of methyl groups on L11 by PrmA, as the addition of a vast excess of unlabeled SAM to the reaction did not lead to a decrease in the amount of ^3H incorporated (data not shown). These results indicate that ribosomes from the null mutant are substantially less methylated than ribosomes from the wild-type parental strain, presumably within L11.

To confirm that L11 is the sole ribosomal component that is methylated by PrmA, we subjected ribosomes to electrophoresis through a sodium dodecyl sulfate–15% polyacrylamide gel after *in vitro* methylation with PrmA (Fig. 3C). Ribosomes from TLK90 contained a single ^3H -methylated protein of a size corresponding to that of L11 (~15.5 kDa). Furthermore, the extent of methylation increased with the reaction time, consistent with the results shown in Fig. 3B. In contrast, ribosomes from wild-type HB8 did not contain significant amounts of ^3H -methylated protein, even after 90 min. Importantly, no other mass differences between ribosomal proteins from wild-type HB8 and TLK90 were detected by MALDI-TOF MS (data not shown), indicating that L11 is the only ribosomal protein target for methylation by PrmA.

Dissociated L11 is methylated more efficiently than ribosome-assembled L11. It has been shown that high salt and low magnesium concentrations stimulate the methylation of *E. coli* L11 in crude extracts, perhaps suggesting that free L11 is methylated more efficiently than ribosome-assembled L11 (8). Consistent with this, an early study found that free L11 obtained by LiCl extraction of 50S subunits prepared from methionine-starved cells was methylated approximately twice as efficiently as intact 50S subunits (6). However, methionine starvation is likely to result in ribosomes that contain a heterogeneous population of L11 with various degrees of methylation, and since the methylation state of L11 could potentially affect its interaction with the ribosome, it is possible that under-methylated L11 was preferentially extracted from the subunits, thereby enriching the pool of methyl group acceptors. More recently, it was proposed that the N-terminal domain of PrmA, which contains the moderately conserved PrmA motif, may be structurally similar to RNA-binding proteins exhibiting the ferredoxin-like fold (4). Based on this, it was suggested that PrmA may interact with a larger ribonucleoprotein complex, presumably an assembly intermediate, rather than free L11. Thus, the issue of whether PrmA acts *in vivo* on fully assembled 50S ribosomal subunits or on free L11 prior to assembly remains unresolved. We therefore compared the PrmA-directed methylation of intact 70S ribosomes with methylation of either ribosomes dissociated in a buffer containing high salt but lacking magnesium (buffer A) or ribosomes treated with RNase A (Fig. 4). The methylation of TLK90 ribosomes in buffer A occurred to a substantially greater extent than that of intact ribosomes (7.3 methyl groups/L11 protein compared to 1.1 methyl groups/L11 protein for intact ribosomes). Similarly, methylation was also stimulated by RNase treatment (5.7 methyl groups/L11 protein), although to a lesser extent than ribosomes dissociated in buffer A. Since L11 protects a 61-nucleotide rRNA fragment from RNase cleavage in *E. coli* ribosomes (21), treatment with RNase A may produce L11-rRNA ribonucleoprotein complexes that are less efficient substrates for methylation than free L11. In both cases, the reactions proceeded for approximately 90 min before the amount

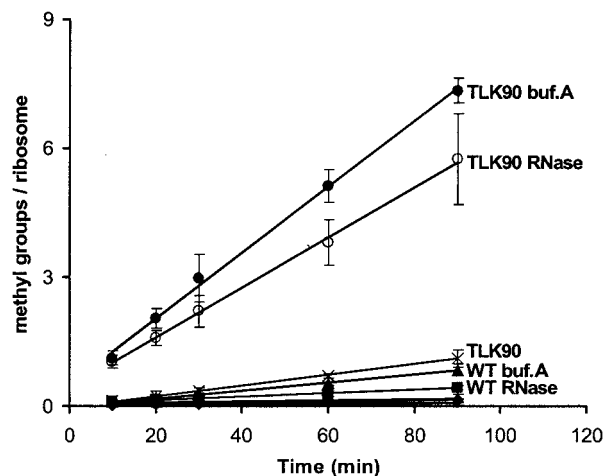


FIG. 4. *In vitro* methylation of ribosomes from wild-type HB8 and the *prmA* null mutant (TLK90). The ribosomes were either intact, dissociated in a buffer containing a high salt concentration but lacking magnesium (buffer A), or dissociated by a treatment with RNase A. Methylation assays were performed for a 90-min time course. ●, ribosomes from TLK90 in buffer A (TLK90 buf.A); ○, ribosomes from TLK90 treated with RNase A (TLK90 RNase); ×, ribosomes from TLK90; ▲, ribosomes from wild-type HB8 in buffer A (WT buf.A); ■, ribosomes from wild-type HB8 treated with RNase A (WT RNase). Clustered near the bottom of the plot are ribosomes from wild-type HB8 (WT; filled diamonds), a reaction without ribosomes (+), and ribosomes from TLK90 without the enzyme (–). The results shown were obtained from at least three independent experiments.

of ^3H detected began to decrease, as noted earlier. However, due to the accelerated reaction rates, the reactions proceeded to >60.0% completion during the 90-min time course, taking into account the 12 methyl groups present in *T. thermophilus* L11. It is noteworthy that the dissociation of wild-type ribosomes in buffer A or by RNase treatment also led to a modest increase in the incorporation of ^3H -labeled methyl groups, further supporting the possibility that the reactions contained a population of under-methylated wild-type ribosomes. Taken together, these results suggest that PrmA preferentially recognizes free L11 prior to its incorporation into 50S subunits *in vivo*.

DISCUSSION

In this study, we have described the construction of a *T. thermophilus prmA* null mutant and the *in vitro* methylation of under-methylated ribosomes and ribosomal protein L11 by cloned and purified *T. thermophilus* PrmA. As previously observed for *E. coli* (27), the *T. thermophilus* PrmA enzyme is dispensable, despite its phylogenetic conservation (4).

The purified PrmA protein is capable of methylating *T. thermophilus* L11 to a stoichiometry in excess of seven methyl groups per protein molecule. Direct evidence that the sites of methylation are the same as those in *E. coli* is lacking, but the high degree of conservation between both the L11 proteins and the methyltransferases is highly suggestive that this may be the case. Indeed, in a clone of *E. coli* carrying overexpressed, plasmid-borne *T. thermophilus* L11, the third lysine appeared to carry a mixture of mono- and dimethylations (24), indicating

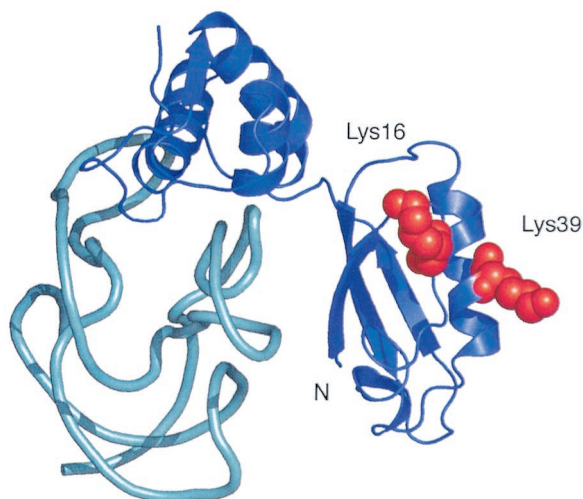


FIG. 5. Three-dimensional structure of *Thermotoga maritima* ribosomal protein L11 (blue) and its associated binding site on 23S rRNA (cyan) as determined by X-ray crystallography (29). Amino acid residues Lys16 and Lys39 are shown with red space-filling diagrams to indicate their mutual proximity. The N-terminal seven amino acid residues of L11, including Ala1 and Lys3, are disordered in the structure and are not shown.

that *E. coli* PrmA was capable of recognizing *T. thermophilus* L11 as a substrate. The lysine at position 39, the most C-terminal of the methylated residues in *E. coli*, is conserved in *T. thermophilus* L11, with a very high level of surrounding amino acid conservation. Finally, as in *E. coli* L11, in which the N-terminal alanine is trimethylated, *T. thermophilus* L11 is also N-terminally blocked (24).

The MALDI-TOF MS data presented here indicate that *T. thermophilus* L11 contains three additional methyl groups, consistent with trimethylation of an additional lysine. An alignment of the *E. coli* and *T. thermophilus* L11 sequences revealed the presence of a lysine residue at position 16 of the *T. thermophilus* protein that is a methionine in the *E. coli* homolog. Lys16 and Lys39 in the *Thermotoga maritima* L11-rRNA cocrystal structure (29) are located on the same side of the N-terminal domain of L11, raising the possibility that Lys16 is methylated by PrmA in *T. thermophilus* (Fig. 5). The N-terminal seven residues of L11 are disordered in the crystal structure, precluding any conclusion about their proximity to Lys16 and Lys39. It is possible, however, that they could reach the same side of the N-terminal domain containing Lys16 and Lys39. This would, in principle, allow PrmA to methylate all four positions during one binding event. Whether the difference in the extent of methylation between the *T. thermophilus* and *E. coli* proteins is biologically relevant or simply fortuitous is unknown at this point.

To date, no convincing role for PrmA has been demonstrated. Previous studies with the *E. coli* null mutant exhaustively examined phenotypic effects, but none were found (27). Thus, the absence of L11 methylation does not appear to affect either growth physiology or ribosome function in any detectable way. Even L11 itself is not absolutely essential, although L11 null mutants of *Bacillus megaterium* (9), *Bacillus subtilis* (28), and several *Streptomyces* species (19) are severely debil-

itated. The facts that PrmA is ubiquitous in the bacterial clade, is highly conserved, and engages in an energetically costly process all suggest an important function, despite two independent findings of its dispensability in phylogenetically divergent genera, *Escherichia* (27) and *Thermus* (this study). Continued investigation of this protein is therefore warranted. The inference that PrmA preferentially recognizes free L11 suggests a role for this interaction during 50S subunit assembly. There are several examples for which ribosome modification is dispensable while the existence of the modifying enzyme is essential for assembly. Most notably, the 23S rRNA pseudouridine synthase RluD is indispensable, even though mutagenesis of the catalytic residue does not create a phenotype (12). Thus, the dispensability of PrmA does not exclude its participation in assembly, as it is feasible that its role is redundant and can be performed by other components of the assembly apparatus. This issue could be resolved genetically by identifying synthetically lethal mutants derived from the *T. thermophilus* *prmA* null mutant described here.

The *T. thermophilus* *prmA* null mutant will provide a source of unmethylated ribosomes for future investigations. Of particular interest are the aspects of L11-ribosome interaction that interfere with PrmA-directed methylation. It is not yet clear whether the preference of PrmA for free L11 is due to conformational differences between the free and ribosome-bound forms of L11 or if there are steric clashes between PrmA and other components of the ribosome. We have previously isolated thiostrepton-resistant L11 mutants of *T. thermophilus* HB8 which exhibit a more open conformation within the ribosome (5). It is possible that the L11 methylation sites of such mutants may be more accessible to PrmA. In order to address this question fully, more information regarding the interaction between PrmA and L11 is needed. Ultimately, such studies would be greatly aided by high-resolution structural information, such as crystal structures of thermophilic PrmA alone or a cocrystal structure of a PrmA-L11 complex.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants GM19756 (to A.E.D.) and GM58843 (to P.A.L.).

We are grateful to members of the Dahlberg laboratory for critical reading of the manuscript, to H. Kagamiyama of Osaka Medical College for providing the *htk* clone, and to A. Carter and V. Ramakrishnan for providing us with the *T. thermophilus* HB8 *rpsL* P90L mutant strain prior to publication.

ADDENDUM IN PROOF

The *T. thermophilus* HB8 PrmA crystal structure has now been solved (PDB entry 1UFK) and released by Kamanishi and colleagues.

REFERENCES

1. Agrawal, R. K., J. Linde, J. Sengupta, K. H. Nierhaus, and J. Frank. 2001. Localization of L11 protein on the ribosome and elucidation of its involvement in EF-G-dependent translocation. *J. Mol. Biol.* **311**:777-787.
2. Alix, J. H. 1992. Extrinsic factors in ribosome assembly, p. 173-184. In K. H. Nierhaus, A. R. Subramanian, V. A. Erdmann, F. Franceschi, and B. Wittmann-Liebold (ed.), *The translational apparatus*. Plenum Publishing, New York, N.Y.
3. Allen, P. N., and H. F. Noller. 1991. A single base substitution in 16S ribosomal RNA suppresses streptomycin dependence and increases the frequency of translational errors. *Cell* **66**:141-148.
4. Bujnicki, J. M. 2000. Sequence, structural, and evolutionary analysis of

- prokaryotic ribosomal protein L11 methyltransferases. *Acta Microbiol. Pol.* **49**:19–28.
5. Cameron, D. M., J. Thompson, S. T. Gregory, P. E. March, and A. E. Dahlberg. 2004. Thiostrepton-resistant mutants of *Thermus thermophilus*. *Nucleic Acids Res.* **32**:3220–3227.
 - 5a. Carter, A. 2002. Ph.D. thesis. Cambridge University, Cambridge, United Kingdom.
 6. Chang, F. N., L. B. Cohen, I. J. Navickas, and C. N. Chang. 1975. Purification and properties of a ribosomal protein methylase from *Escherichia coli* Q13. *Biochemistry* **14**:4994–4998.
 7. Colson, C., J. Lhoest, and C. Urlings. 1979. Genetics of ribosomal protein methylation in *Escherichia coli*. III. Map position of two genes, *prmA* and *prmB*, governing methylation of proteins L11 and L3. *Mol. Gen. Genet.* **169**:245–250.
 8. Colson, C., and H. O. Smith. 1977. Genetics of ribosomal protein methylation in *Escherichia coli*. *Mol. Gen. Genet.* **154**:167–173.
 9. Cundliffe, E., P. Dixon, M. Stark, G. Stoffer, R. Ehrlich, M. Stoffer-Meilicke, and M. Cannon. 1979. Ribosomes in thiostrepton-resistant mutants of *Bacillus megaterium* lacking a single 50S subunit protein. *J. Mol. Biol.* **132**:235–252.
 10. Dabbs, E. R. 1978. Mutational alterations in 50 proteins of the *Escherichia coli* ribosome. *Mol. Gen. Genet.* **165**:73–78.
 11. Dognin, M. J., and B. Wittmann-Liebold. 1980. Purification and primary structure determination of the N-terminal blocked protein, L11, from *Escherichia coli* ribosomes. *Eur. J. Biochem.* **112**:131–151.
 12. Gutsell, N. S., M. D. Del Campo, S. Raychaudhuri, and J. Ofengand. 2001. A second function for pseudouridine synthases: a point mutant of RluD unable to form pseudouridines 1911, 1915, and 1917 in *Escherichia coli* 23S ribosomal RNA restores normal growth to an RluD-minus strain. *RNA* **7**:990–996.
 13. Hashimoto, Y., T. Yano, S. Kuramitsu, and H. Kagamiyama. 2001. Disruption of *Thermus thermophilus* genes by homologous recombination using a thermostable kanamycin-resistant marker. *FEBS Lett.* **506**:231–234.
 14. Kaminishi, T., H. Sakai, C. Takemoto-Hori, T. Terada, N. Nakagawa, N. Maoka, S. Kuramitsu, M. Shirouzu, and S. Yokoyama. 2003. Crystallization and preliminary X-ray diffraction analysis of ribosomal protein L11 methyltransferase from *Thermus thermophilus* HB8. *Acta Crystallogr. D* **59**:930–932.
 15. Kowalak, J. A., J. J. Dalluge, J. A. McCloskey, and K. O. Stetter. 1994. The role of posttranscriptional modification in stabilization of transfer RNA from hyperthermophiles. *Biochemistry* **33**:7869–7876.
 16. Koyama, Y., T. Hoshino, N. Tomizuka, and K. Furukawa. 1986. Genetic transformation of the extreme thermophile *Thermus thermophilus* and of other *Thermus* spp. *J. Bacteriol.* **166**:338–340.
 17. Lederer, F., J. H. Alix, and D. Hayes. 1977. *N*-Trimethylalanine, a novel blocking group, found in *E. coli* ribosomal protein L11. *Biochem. Biophys. Res. Commun.* **77**:470–480.
 18. Murgola, E. J., F. T. Pagel, K. A. Hijazi, A. L. Arkov, W. Xu, and S. W. Zhao. 1995. Variety of nonsense suppressor phenotypes associated with mutational changes at conserved sites in *Escherichia coli* ribosomal RNA. *Biochem. Cell Biol.* **73**:925–931.
 19. Ochi, K. 1990. *Streptomyces relC* mutants with an altered ribosomal protein ST-L11 and genetic analysis of a *Streptomyces griseus relC* mutant. *J. Bacteriol.* **172**:4008–4016.
 20. Oshima, T., and K. Imahori. 1974. Description of *Thermus thermophilus* (Yoshida and Oshima) comb. nov., a nonsporulating thermophilic bacterium from a Japanese thermal spa. *Int. J. Syst. Bacteriol.* **24**:102–112.
 21. Schmidt, F. J., J. Thompson, K. Lee, J. Dijk, and E. Cundliffe. 1981. The binding site for ribosomal protein L11 within 23S ribosomal RNA of *Escherichia coli*. *J. Biol. Chem.* **256**:12301–12305.
 22. Stark, H., M. V. Rodnina, J. Rinke-Appel, R. Brimacombe, W. Wintermeyer, and M. van Heel. 1997. Visualization of elongation factor Tu on the *Escherichia coli* ribosome. *Nature* **389**:403–406.
 23. Suh, M.-J., and P. A. Limbach. 2004. Investigation of methods suitable for the matrix-assisted laser desorption/ionization mass spectrometric analysis of proteins from ribonucleoprotein complexes. *Eur. J. Mass Spectrom.* **10**:89–99.
 24. Triantafyllidou, D., M. Simitopoulou, F. Franceschi, and T. Choli-Papadopoulou. 1999. Structural and functional studies on the overproduced L11 protein from *Thermus thermophilus*. *J. Protein Chem.* **18**:215–223.
 25. Valle, M., J. Sengupta, N. K. Swami, R. A. Grassucci, N. Burkhardt, K. H. Nierhaus, R. K. Agrawal, and J. Frank. 2002. Cryo-EM reveals an active role for aminoacyl-tRNA in the accommodation process. *EMBO J.* **21**:3557–3567.
 26. Van Dyke, N., and E. J. Murgola. 2003. Site of functional interaction of release factor 1 with the ribosome. *J. Mol. Biol.* **330**:9–13.
 27. Vanet, A., J. A. Plumbridge, M. F. Guerin, and J. H. Alix. 1994. Ribosomal protein methylation in *Escherichia coli*: the gene *prmA*, encoding the ribosomal protein L11 methyltransferase, is dispensable. *Mol. Microbiol.* **14**:947–958.
 28. Wienen, B., R. Ehrlich, M. Stoffer-Meilicke, G. Stoffer, I. Smith, D. Weiss, R. Vince, and S. Pestka. 1979. Ribosomal protein alterations in thiostrepton- and micrococin-resistant mutants of *Bacillus subtilis*. *J. Biol. Chem.* **254**:8031–8041.
 29. Wimberly, B. T., R. Guymon, J. P. McCutcheon, S. W. White, and V. Ramakrishnan. 1999. A detailed view of a ribosomal active site: the structure of the L11-RNA complex. *Cell* **97**:491–502.