Identification of the 16S rRNA m^{5}C967 Methyltransferase from *Escherichia coli*†

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ABSTRACT: The *fmu* gene product has been proposed to be an RNA methyltransferase [Koonin, E. V. (1994) *Nucleic Acids Res. 22*, 2476−2478]. *Fmu* has been cloned and expressed, and the encoded 47 kDa protein has been purified and characterized. The enzyme catalyzed specific methylation of C967 of unmodified 16S rRNA transcripts. A 16mer stem−loop structure containing C967 (nt 960−975) was also a good substrate for the enzyme in vitro. Methylation of C967 was confirmed by several methods including analysis of RNase T1 digests and nearest-neighbor analysis. Fmu did not catalyze methylation of transcripts of 23S rRNA. *E. coli* cells that contained kan^r-disrupted *fmu* produced 16S rRNA that could be specifically methylated by Fmu in vitro at C967 but not C1407. Further, *fmu* disruption did not significantly alter the growth rate of *E. coli* in rich or minimal media. We propose renaming this ORF "rrmB" and the enzyme "RrmB" for rRNA methyltransferase.

RNA modifications have been well characterized in *E. coli (1)*. Modified nucleosides are most copious and diverse in tRNA and are also abundant in ribosomal RNA. Eleven modified nucleosides are found in *E. coli* 16S rRNA of which 10 are methylated nucleosides and 1 is pseudouridine (Ψ).[1] 23S rRNA contains 23 modified nucleosides of which 14 are methylated and 9 are Ψ, 1 of which is also methylated. Methylated nucleosides found in rRNA include some containing 2′-*O*-methyl nucleosides and also a number containing methylated bases. Among others, the methylated residues include m^{5}C at positions 967 and 1407 of 16S rRNA *(2)*, and 1962 of 23S rRNA *(1)*.

Although the modified nucleosides of *E. coli* rRNA are well characterized, relatively little is known about the modification enzymes or the functions of modified residues. Thus far, only one 16S rRNA modifying enzyme *(3, 4)* and four 23S rRNA modifying enzymes of *E. coli* have been characterized *(4−7)* (Huang et al., *Biochemistry*, accepted). The only work thus far reported on RNA-m^{5}C MTases involves studies of the 16S rRNA m^{5}C967 activity in partially purified extracts *(8)*.

We have undertaken a program directed at identifying the enzymes responsible for formation of the modified nucleotides in *E. coli* RNAs. Our approach is to (i) utilize sequences of known modification enzymes to identify homologous sequences in genomic databases, (ii) clone and express the genes, (iii) determine whether the gene products indeed possess the predicted enzyme activities, and (iv), if so, determine the location of the catalyzed RNA modification. Hopefully, this approach will enable systematic studies of the functions of specific modified residues of RNA as analyzed by directed gene disruption studies.

The *fmu* gene product was identified as a putative RNA MTase because it contained an AdoMet binding motif and was homologous to a putative RNA binding protein in the nucleolus *(9)*. Conflicting sequence data suggested that this region contained two ORFs, *fmu* and *fmv*, encoding two proteins of 238 and 191 amino acids *(10)*, one ORF encoding a protein of 386 amino acids (Blattner et al., submission to GenBank), or one ORF encoding a protein of 429 amino acids *(11)*.

In this paper we describe the cloning and expression of *fmu* of *E. coli*. We show that it encodes a protein of 429 amino acids that catalyzes methylation at the 5-position of C967 of 16S rRNA both in vitro and in vivo. As such, *fmu* represents the first RNA-m^{5}C MTase gene thus far identified.

MATERIALS AND METHODS

Nucleoside standards were purchased from Sigma. RNase T1, nuclease P1, calf intestinal alkaline phosphatase, and RNase inhibitor were obtained from Boehringer Mannheim, and RNase T2 was from BRL. Vent DNA polymerase was from New England Biolabs, and formamide was from EM Science. All other reagents were the highest grade available. Molecular biology procedures not outlined or specifically referenced were as described *(12)*. Plasmid pWK1, which contains the *E. coli* 16S rRNA gene fused to a T7 promoter *(13)*, and plasmid pCW1, which contains the *E. coli* 23S rRNA gene fused to a T7 promoter *(14)*, were gifts from J. Ofengand (Department of Biochemistry and Molecular Biology, University of Miami School of Medicine, Miami, FL). Plasmid p67YF0 *(15)* used for preparation of yeast tRNA^{Phe} was a gift from O. C. Uhlenbeck (Department of

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[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; Ψ, pseudouridine; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; 2d-TLC, two-dimensional thin-layer chromatography; nt, nucleotide(s); ORF, open reading frame; MTase, methyltransferase; IPTG, isopropylthiogalactosidase; kan, kanamycin; superscript r, resistance.

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Chemistry and Biochemistry, University of Colorado, Boulder, CO). Plasmid pWK1.3, which contains a truncated 16S rRNA gene, and ON-Fb, which is complementary to nt 1398−1417 of 16S rRNA, have been described *(16)*. Plasmid pUC4K containing the kan^r gene was purchased from Pharmacia. Plasmid pMAK705 *(17)*, which contains a temperature-sensitive origin of replication, was provided by Dr. S. R. Kushner (University of Georgia, Athens, GA). T7 RNA polymerase was isolated from *E. coli* BL21(DE3) (Novagen) harboring the plasmid pAR1219 *(18)*. The enzyme was purified as described *(19)*, except that S−Sepharose (Pharmacia) was used instead of Trisacryl SP. [5′-α-^{32}P]ATP (3000 Ci/mmol), [5′-α-^{32}P]CTP (3000 Ci/mmol), and [^{3}H-Me]AdoMet (73 Ci/mmol) were purchased from Amersham.

Cloning Fmu and High-Level Expression of Its Gene Product. *Fmu* was amplified by PCR using genomic DNA from *E. coli* C600 as a template and Vent DNA polymerase. The N-terminal primer 5′-TACTTGCCATGGAAAAA-CAACGTAATTTACGT-3′ extends from +1 to +24 within the *fmu* coding region with an A to G mutation at +4 to create an *Nco*I restriction site (underlined) at the start codon. The C-terminal primer 5′-CGTACGCGGATCCTCAC-TTTTTGATTAGCTTAGC-3′ extends from +1270 to +1290 and has a downstream *Bam*HI restriction site (underlined). The 100 μL PCR reaction contained 5 ng of template plus 250 μM dNTPs and 1 μM each PCR primer. Twenty-five cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s were performed. The amplified 1.3 kb DNA fragment was digested with *Bam*HI and *Nco*I, isolated on a low-melt SeaPlaque agarose gel (FMC), and ligated with *Bam*HI/*Nco*I-digested vector pET15b (Novagen). The ligated product was transformed into *E. coli* DH5α to give pET-fmu.

For expression, pET-fmu was transformed into B21(DE3) cells (Novagen). Cultures of pET-fmu/BL21(DE3) were grown in 1 L of LB medium at 37 °C until the A_{600} reached 0.8−1.0. IPTG was added to a concentration of 0.5 mM, and the cultures were further incubated for 3 h. Cells were harvested, resuspended in 50 mL of buffer A (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM DTT, and 10% glycerol), and disrupted by three passes through a French press at 18K psi. Cellular debris was removed by centrifugation at 15000*g*. The supernatant was dialyzed against buffer A and applied on a DEAE-Sepharose column (1.5 × 6 cm) equilibrated with buffer A. The column was washed with buffer A and eluted with a 100 mL linear gradient of 0.05−0.5 M NaCl in buffer A at 2 mL/min. Fractions containing Fmu were pooled, concentrated, and desalted using a Centriprep concentrator.

RNA Synthesis. Unmodified RNA substrates were synthesized by runoff transcription of appropriate templates *(16)*. Linearized plasmids used for *E. coli* 16S rRNA, 23S rRNA, yeast tRNA^{Phe}, and 3′-truncated 16S rRNAs (D1, D5) have been described *(16)*. pWK1.3 digested with *Bst*NI was the template for internal fragment F8 (nt 927−982 of 16S rRNA); ON-T7Fc (5′-TTCGCGTTGCATCGAACTATAGT-GAGTCGATTTA-3′) was the template for fragment Fc (nt 960−975 of 16S rRNA). Purification of substrates and determination of RNA concentrations have been described *(16)*.

Methylation Assays. Typically, reaction mixtures (20 μL) contained 1 μM RNA, 50 μM [^{3}H-Me]AdoMet (1.0 Ci/mmol), 400 units/mL RNase inhibitor, and 1 μM enzyme in methylation buffer [100 mM Tris-HCl, pH 7.5, 40 mM NH_4Cl, 2 mM $Mg(OAc)_2$, and 6 mM DTT] *(20)*. Assays were performed at 37 °C, and incorporation of radiolabeled methyl groups was determined by adsorption on DEAE paper *(21)* or, when specified, by HPLC nucleoside analysis or 2d-TLC nucleotide analysis.

Nucleoside Analysis. [^{3}H-Me]Methyl-RNA (16S rRNA, 40 pmol, 6 × 10^4 dpm; or Fc, 250 pmol, 8 × 10^4 dpm) was extracted with phenol, and twice precipitated with ethanol. For Fc, 10 μL of single-stranded calf thymus DNA (1 μg/μL) was added as a carrier prior to ethanol precipitation. Nuclease P1 digestion and HPLC conditions were as described *(16)*. The retention time of m^{5}C was 10.3 min.

RNase T1 Analysis. Reactions (30 μL) containing 2.7 μM [^{3}H-Me]16S RNA (1.7 × 10^5) or 2.7 μM [^{3}H-Me]Fc (1 × 10^4 dpm) in 50 mM Tris-HCl, pH 8.0, were incubated at 90 °C for 2 min and quick-cooled on ice. RNase T1 (40 units) was added, and the mixtures were incubated at 37 °C for 3 h, mixed with 30 μL of formamide dye, and applied to 7 M urea−20% PAGE.

Nuclease Hybridization−Protection. ON-T7 Fc and ON-Fb were used in nuclease protection assays as described *(16)*.

Nearest-Neighbor Analysis. Fragment Fc (20 pmol) prepared with [5′-α-^{32}P]ATP was methylated with 500 μM AdoMet and 1 μM enzyme in a 20 μL reaction mixture containing methylation buffer. RNase T2 digestion and 2d-TLC of [3′-^{32}P]NMPs were as described *(16)*.

Disruption of Fmu. pUC4K was digested with *Pst*I, and the 1.2 kb fragment containing the kan^r gene was gel-isolated and ligated with pET-fmu which had been previously digested with *Nsi*I (695 bases into the *fmu*) and dephosphorylated. The resulting plasmid (pET-fmuK) was digested with *Xba* I and *Hin*dIII, and the 2.7 kb fragment containing kan^r-disrupted *fmu* was gel-purified and ligated with *Xba*I/*Hin*dIII-digested pMAK705 to yield pMAK-fmuK. *E. coli* JM101 was transformed with plasmid pMAK-fmuK, and gene replacement was performed as described *(17)*. The resulting *fmu*$^-$ strain was kan resistant and chloramphenicol-sensitive.

Methylation of 16S rRNA from Fmu-Disrupted E. coli. 16S rRNA was purified from wild-type and *fmu*-disrupted *E. coli* JM101 as described *(22)*. Methylation of rRNA was carried out in a 20 μL reaction containing 0.4 μM RNA, 30 μM [^{3}H-Me]AdoMet (1.5 Ci/mmol), and 5 μM Fmu. The extent of the reaction was determined as described above. RNase T1 analysis was carried out as described above in a reaction containing ^{3}H-Me-methylated 16S rRNA (6 pmol, 2 × 10^5 dpm) and 300 units of RNase T1.

RESULTS

Cloning and High-Level Expression of Fmu. *Fmu* was amplified by PCR and cloned into pET15b. The 5′ PCR primer contained an *Nco*I site for cloning resulting in a K2E mutation. Since the N-terminal end of the protein is not conserved among *fmu* homologues in other species (D. V. Santi and R. Reid, unpublished observations), we assumed that the mutation would not effect enzymatic activity. Three independent nucleotide sequence submissions to GenBank covering the *fmu* region have annotated the proposed open reading frame(s) differently [*(10, 11)* and Blattner et al., direct submission of the *E. coli* genome to GenBank]. The sequence of the cloned *fmu* region confirms an ORF which encodes

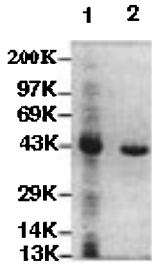

FIGURE 1: SDS−PAGE of Fmu purification. Lane 1, crude extract after IPTG induction; lane 2, after DEAE-Sepharose chromatography. The positions of protein standards are indicated.

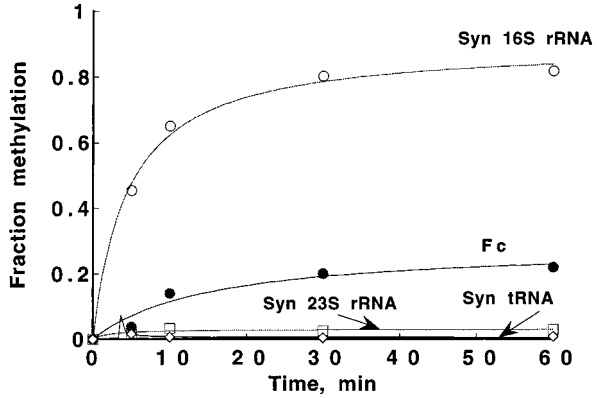

FIGURE 2: Methylation of in vitro synthesized 16S rRNA by Fmu. Reaction mixtures (100 μL) containing 1 μM RNA (16S rRNA, 23S rRNA, tRNA^{Phe}, or 16S rRNA fragment Fc), 50 μM [^{3}H-Me]-AdoMet (1 Ci/mmol), 400 units/mL RNase inhibitor, and 1 μM Fmu in methylation buffer were incubated at 37 °C. Aliquots (18 μL) were withdrawn as specified for DEAE-filter disk assay.

429 amino acids and exactly matches the nucleotide sequence submitted by Mazel et al. *(11)*.

Further evidence for a single ORF was obtained upon expression of *fmu*. As shown in Figure 1, induced cells containing pET-fmu produced a large amount of a 47 kDa protein. The soluble extract from induced pET-fmu showed methylation activity 100-fold higher on 16S rRNA than the control extract. The predicted molecular mass of 47.14 kDa for the recombinant protein is in agreement with the 47 kDa protein observed on SDS−PAGE. Following induction, Fmu was produced at a level approaching 30−50% of total soluble protein. Purification by DEAE-Sepharose chromatography provided enzyme which appears homogeneous on SDS−PAGE (>90%), with yields of 20−30 mg of enzyme/L of *E. coli* culture.

In Vitro Methylation of 16S rRNA. In the presence of [^{3}H-Me]AdoMet, Fmu catalyzed methylation of in vitro synthesized 16S rRNA. The enzyme did not methylate 23S rRNA or yeast tRNA^{Phe} (Figure 2). The methylated product was identified as [^{3}H-Me]m^{5}Cyd (0.8 pmol of m^{5}C/pmol of 16S rRNA) by HPLC analysis of nucleosides or as [^{3}H-Me]m^{5}CMP by 2d-TLC. Thus, the enzyme methylates the 5-position of a single C residue in 16S rRNA.

K_m and k_{cat} values for 16S rRNA were determined at pH 7.5, 37 °C, in the presence of 50 μM [^{3}H-Me]AdoMet. Values of 0.67 μM and 0.012 s^{-1} were obtained for K_m and k_{cat}, respectively.

There are two m^{5}C residues present in the native 1542 nt 16S rRNA, m^{5}C967 and m^{5}C1407. The following experiments were performed to establish which position serves as a substrate for Fmu.

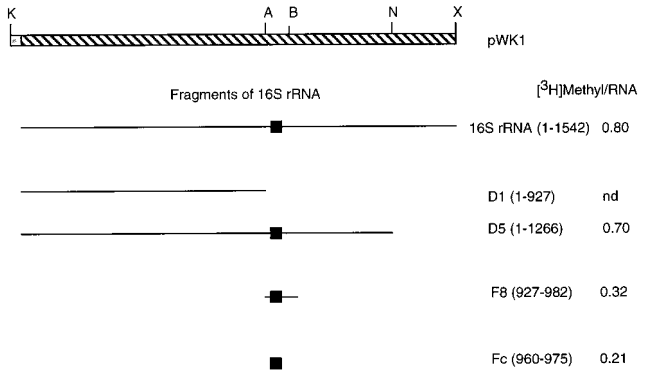

FIGURE 3: Methylation of 16S rRNA fragments. Methylation assays (80 μL) contained 1 μM RNA, 50 μM [^{3}H-Me]AdoMet (1 Ci/mmol), and 1 μM enzyme in methylation buffer. Aliquots (18 μL) were removed after 2, 10, 30, and 60 min incubation at 37 °C for assay; data show the moles of methyl group per mole of RNA after 60 min incubation. (stippled bar) T7 promoter; (hatched bar) 16S rRNA; (black bar) nt 960−975 of 16S rRNA; nd, not detectable.

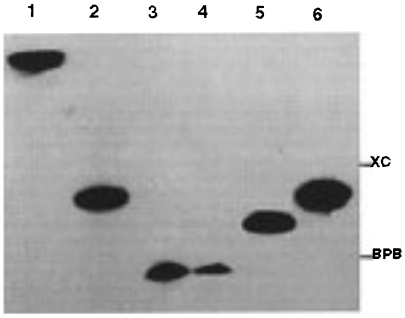

FIGURE 4: 7 M urea−20% PAGE analysis of RNase T1 digests of Fmu-methylated 16S rRNA and Fc (nt 960−975) with [^{3}H-Me]-AdoMet. The gel was fixed with HOAc−methanol−H_2O (1/3/6, v/v/v) for 30 min, soaked in Amplify (Amersham) with shaking for 30 min, and dried for autoradiography. Lane 1, 16S rRNA methylated with [^{3}H-Me]AdoMet by Fmu; lane 2, Fc (nt 960−975) methylated with [^{3}H-Me]AdoMet by Fmu; lane 3, complete RNase T1 digest of [^{3}H-Me]16S rRNA; lane 4, complete RNase T1 digest of [^{3}H-Me]Fc; lanes 5 and 6, 11mer and 17mer standards [T-arms of tRNA methylated with [^{3}H-Me]AdoMet by RUMT *(27)*]. Positions of bromphenol blue (BPB) and Xylene cyanol FF (XC) are marked. Bromphenol blue comigrates with octanucleotides.

RNA Fragments as Substrates for Fmu. The 16S rRNA fragments, D1(nt 1−927), D5 (nt 1−1266), and F8 (nt 927−982), were tested as substrates for Fmu-catalyzed methylation using [^{3}H-Me]AdoMet. [^{3}H-Me] was not found in treated D1 but was observed in D5 and F8 (Figure 3), indicating that the residue modified by Fmu was between nt 927 and 982. We then prepared a small 16mer stem−loop, Fc (nt 960−975), and demonstrated that it was methylated by Fmu (Figure 3).

Analysis of RNase T1 Fragments of 16S rRNA Containing m^{5}C. The Fmu substrates, 16S rRNA and fragment Fc (nt 960−975), were methylated with [^{3}H-Me]AdoMet and digested with RNase T1, and the products were analyzed using 7 M urea−PAGE. RNase T1 digestion of [^{3}H-Me]-16S rRNA gives C967 in a pentanucleotide and C1407 in a decanucleotide; the only radioactive fragment in either T1-hydrolyzed 16S rRNA or T1-hydrolyzed Fc migrated as a pentanucleotide (Figure 4) indicating that C967, but not C1407, is methylated by Fmu.

Nuclease Protection. In vitro transcribed 16S rRNA was methylated by Fmu and [^{3}H-Me]AdoMet, and then hybrid-

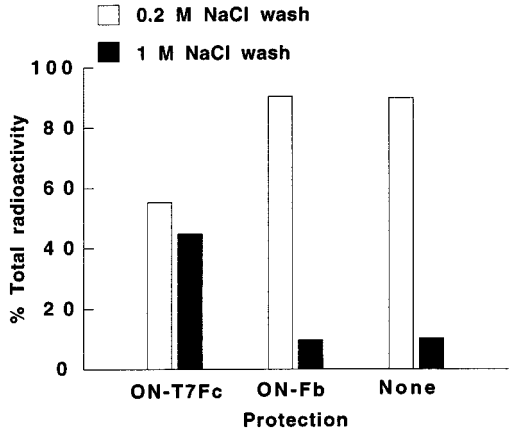

FIGURE 5: Hybridization−nuclease protection of methylated 16S rRNA. Individual mixtures (50 μL) containing 1 pmol of in vitro prepared [^{3}H-Me]16S rRNA [(5−6) × 10^3 dpm] and 50 pmol of oligonucleotide (ON-T7Fc or ON-Fb) in hybridization buffer [40 mM MES, pH 6.4, 0.4 M NaCl, 1 mM EDTA and 80% (v/v) formamide] were incubated at 90 °C for 10 min and slowly cooled to room temperature. To this was added 450 μL of 10 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 5 mM EDTA, and 100 units of RNase T1, and digestion was allowed to proceed for 30 min at 30 °C. The digests were applied to DEAE-cellulose columns (100 μL bed volume) previously equilibrated with 50 mM Tris-HCl, pH 7.5. The columns were washed with 5 × 1 mL of 50 mM Tris-HCl, pH 7.5, and eluted with 5 × 1 mL of 0.2 M NaCl, 50 mM Tris-HCl, pH 7.5, followed by 5 × 1 mL of 1 M NaCl, 50 mM Tris-HCl, pH 7.5. The radioactivity eluting with (*a*) 0.2 M NaCl−50 mM Tris-HCl, pH 7.5, and (*b*) 1 M NaCl−50 mM Tris-HCl, pH 7.5, was used to calculate the ratio of protected to total methylation sites [$b/(a + b)$].

ized to DNA fragments ON-T7Fc (complementary to nt 960−975 of 16S rRNA) or ON-Fb (complementary to nt 1398−1417 of 16S rRNA). The hybridization products were digested with RNase T1, and the protected hybrids were separated from digestion products using DEAE-cellulose columns. In control experiments, 0.2 M NaCl eluted more than 90% of the ^{3}H from complete RNase T1 digests of [^{3}H-Me]16S rRNA, while [5′-^{32}P]ON-T7Fc or [5′-^{32}P]ON-Fb remained bound under these conditions. The [5′-^{32}P]-labeled oligonucleotides and hybrid-protected RNase T1-digested fragments eluted with 1 M NaCl. When T1 digested hybridization products of [^{3}H-Me]16S RNA were treated similarly, about 35% of the [^{3}H-Me] was protected by fragment ON-T7Fc, whereas ON-Fb did not provide protection (Figure 5).

Nearest-Neighbor Analysis. Fc (nt 960−975), containing the putative C967 methylation site, was prepared using [5′-α-^{32}P]ATP, purified by 7 M urea−PAGE, and methylated with AdoMet and Fmu. Nearest-neighbor analysis (RNase T2 digestion followed by 2d-TLC separation of the nucleotides) of methylated Fc showed [3′-^{32}P]m^{5}CMP (Figure 6). Since the 3′-^{32}P of the [3′-^{32}P]m^{5}CMP product was donated by [5′-α-^{32}P]ATP used in RNA synthesis, methylation must have occurred at the Cyd of a 5′-CA sequence. Since C967A968 is the only such sequence in fragment Fc, this analysis confirmed that C967 was the site of methylation.

Disruption of Fmu. Fmu of *E. coli* JM101 was disrupted with a kan^r gene to give a kan^r Fmu-deficient strain. In LB, the doubling time for wild-type JM101 was 30 min, compared with 31 min for the *fmu*$^-$ strain. In M9 medium, the doubling time for JM101 was 75 min, compared with 70 min for the *fmu*$^-$ strain.

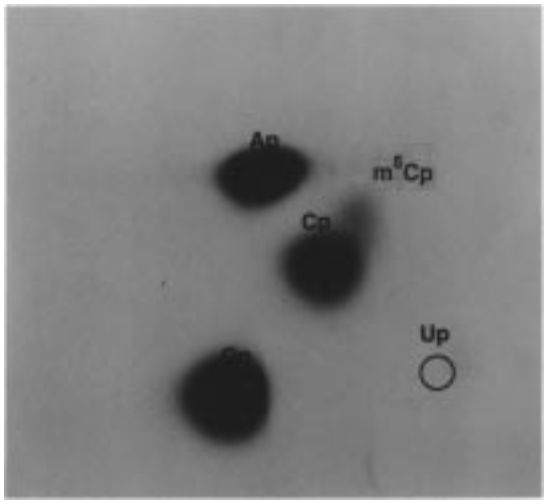

FIGURE 6: Nearest-neighbor analysis of ^{32}P-Fc. Fc containing [5′-α-^{32}P]AMP was prepared by in vitro transcription using the appropriate template, [5′-α-^{32}P]ATP, and NTPs. The reactions (20 μL) containing 1 μM [5′-α-^{32}P]AMP-Fc [(1−5) × 10^4 cpm], 500 μM AdoMet, and 1 μM enzyme in methylation buffer were incubated at 37 °C for 60 min, diluted to 50 μL with H_2O, extracted with phenol, and precipitated with ethanol (5 μg of carrier DNA was added). The methylated [5′-^{32}P]AMP-Fc was digested with RNase T2 (2 units) in 50 mM NaOAc, pH 4.5, 2 mM EDTA at 37 °C for 5 h, and analyzed by 2d-TLC on cellulose plates. Shown is the 3′-^{32}P-nucleotide analysis of RNase T2 digests of [5′-α-^{32}P]-AMP-Fc.

In the presence of [^{3}H-Me]AdoMet and Fmu, 16S rRNA purified from the *fmu*$^-$ strain was modified with 0.9 mol of [^{3}H-Me] incorporated per mole of RNA. No incorporation of radioisotope was detected when 16S rRNA purified from the wild-type strain was used as substrate. RNase T1 digestion of the tritium-labeled 16S rRNA from the *fmu*$^-$ strain revealed only one radioactive band corresponding to a pentanucleotide, consistent with specific methylation at C967. Thus, 16S RNA from *fmu*-disrupted cells clearly lacks methylation at C967.

DISCUSSION

Fmu Is 16S rRNA m^{5}C967 MTase. In the present work, we amplified, cloned, and expressed the *fmu* gene from *E. coli* and purified recombinant Fmu to homogeneity. The DNA sequence and the size of the expressed protein confirmed that *fmu* is an ORF of 1287 bp, encoding a 429 amino acid protein of 47 kDa. We conclude that the sequence of *fmu* reported by Mazel et al. *(11)* is the correct one, and that other reported sequences contained erroneous stop codons or were obtained on strain variants.

We have shown that Fmu methylates the 5-position of a single C residue of in vitro synthesized 16S rRNA and that it does not methylate 23S rRNA. The site of Fmu methylation of 16S rRNA was determined to be C967 as follows. First, overlapping fragments of 16S rRNA were tested as substrates for Fmu, and only those containing C967 yielded m^{5}C. An oligoribonucleotide as small as the 16mer stem−loop at nt 960−975 (Fc) of 16S rRNA was a substrate for enzyme. Second, hybridization of [^{3}H]Me-16S rRNA with an oligonucleotide complementary to nt 960−975 protected the methylated sequence against nuclease cleavage, whereas hybridization with an oligonucleotide complementary to nt 1398−1417 did not. Third, nearest-neighbor analysis of

methylated Fc labeled during synthesis with [5′-α-^{32}P]ATP gave [^{32}P]3′-m^{5}CMP. Since the 3′-^{32}P of 3′-m^{5}CMP must have arisen from an adjacent 5′- [^{32}P]AMP in the RNA, and since the only CA sequence in Fc is C967-A968, C967 was identified as the methylated nucleotide of Fc. Finally, RNase T1 digestion of either Fmu-methylated 16S rRNA or Fmu-methylated Fc yielded a pentanucleotide as the only methylated product. Since RNase T1 digestion of 16S rRNA yields C1407 in a decanucleotide, and since C967 has been shown by nearest-neighbor analysis to be the site of methylation in Fc, C967 must be the target base in 16S RNA. Taken in concert, these experiments demonstrate convincingly that Fmu specifically methylates C967 of 16S rRNA in vitro.

Fmu Gene Disruption. To demonstrate that Fmu also methylates C967 in vivo, we disrupted *fmu* and asked whether C967 of 16S rRNA isolated from the *fmu*$^-$ strain lacked methylation. Here, 16S rRNA was isolated from wild-type and *fmu*-disrupted cells and treated with Fmu and [^{3}H-Me]AdoMet. The RNA from the *fmu*-disrupted cells could be methylated by Fmu whereas RNA from wild-type *E. coli* could not. Further, digestion of the methylated 16S rRNA with RNase T1 gave the methylated base in a pentanucleotide, the T1 fragment size for C967, and not in a decanucleotide, the T1 fragment size for C1407. Thus, C967 of 16S rRNA is methylated by Fmu in vivo as well as in vitro. We propose renaming *fmu "rrmB"* and the encoded protein "RrmB" for rRNA Mtase [rrmA is the previously described 23S rRNA m^{1}G Mtase *(23)*].

Interestingly, there was no significant difference in the growth rate in wild-type versus *fmu*-disrupted JM101 cells in minimal or complete media, demonstrating that Fmu and m^{5}C967 are not essential for growth. This is in accord with the observation that C967A and C967U mutants of 16S rRNA have no obvious phenotype in *E. coli (24)*.

It is noted that Fmu possesses a Pro-Cys motif at positions 324−325 which corresponds to the invariant Pro-Cys consensus found in thymidylate synthases *(25)* and in Motif IV of all known DNA m^{5}C MTases *(26)*. It is reasonable to propose that, as in the latter enzymes, Cys 325 of Fmu serves as a catalytic nucleophile to attack C6 of the target C residue to activate the 5-position for methylation.

In summary, we have cloned and expressed the *fmu* ORF of *E. coli*, and identified Fmu as a 47 kDa protein that methylates the 5-carbon of C967 of 16S rRNA in vitro and in vivo. We have further shown that the essential information for substrate recognition in vitro is contained within the small stem−loop structure of 16S rRNA containing the target Cyt and that Fmu contains the Pro-Cys consensus found in other enzymes which methylate the 5-position of pyrimidines. The function of Fmu or the m^{5}C product remains unknown, since disruption of the *fmu* gene or substitution of the target C in 16S rRNA *(24)* shows no identifiable phenotype. To our knowledge, Fmu represents the first RNA-cytosine MTase identified and characterized. Using *fmu* as a probe to search the currently available genomic databases, we have identified an entire new family of nucleic acid MTases comprised of well over 40 members; the family will be described in a subsequent report.

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