Intrinsic resistance to aminoglycosides in *Enterococcus faecium* is conferred by the 16S rRNA m⁵C1404-specific methyltransferase EfmM

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ABSTRACT

Aminoglycosides are ribosome-targeting antibiotics and a major drug group of choice in the treatment of serious enterococcal infections. Here we show that aminoglycoside resistance in *Enterococcus faecium* strain CIP 54-32 is conferred by the chromosomal gene *efmM*, encoding the *E. faecium* methyltransferase, as well as by the previously characterized *aac(6′)-II* that encodes a 6′-N-aminoglycoside acetyltransferase. Inactivation of *efmM* in *E. faecium* increases susceptibility to the aminoglycosides kanamycin and tobramycin, and, conversely, expression of a recombinant version of *efmM* in *Escherichia coli* confers resistance to these drugs. The EfmM protein shows significant sequence similarity to *E. coli* RsmF (previously called YebU), which is a 5-methylcytidine (m⁵C) methyltransferase modifying 16S rRNA nucleotide C1407. The target for EfmM is shown by mass spectrometry to be a neighboring 16S rRNA nucleotide at C1404. EfmM uses the methyl group donor S-adenosyl-L-methionine to catalyze formation of m⁵C1404 on the 30S ribosomal subunit, whereas naked 16S rRNA and the 70S ribosome are not substrates. Addition of the 5-methyl to C1404 sterically hinders aminoglycoside binding. Crystallographic structure determination of EfmM at 2.28 Å resolution reveals an N-terminal domain connected to a central methyltransferase domain that is linked by a flexible lysine-rich region to two C-terminal subdomains. Mutagenesis of the methyltransferase domain established that two cysteines at specific tertiary locations are required for catalysis. The tertiary structure of EfmM is highly similar to that of RsmF, consistent with m⁵C formation at adjacent sites on the 30S subunit, while distinctive structural features account for the enzymes' respective specificities for nucleotides C1404 and C1407.

Keywords: RNA m⁵C methyltransferase; aminoglycoside resistance; mass spectrometry; crystal structure; *Enterococcus faecium*

INTRODUCTION

Aminoglycosides act by binding to the conserved A-site of 16S rRNA on the bacterial 30S small ribosomal subunit and thereby disrupt mRNA decoding (Davies and Davis 1968; Ogle and Ramakrishnan 2005). Several mechanisms of aminoglycoside resistance have been observed in bacterial human pathogens, the most frequent of which involves drug

deactivation by N-acetylation, O-adenylylation, or O-phosphorylation (Magnet and Blanchard 2005). This form of resistance is encountered in *Enterococcus faecium*, where all strains of this species possess a chromosomally encoded 6′-N-aminoglycoside acetyltransferase that confers low-level drug resistance (Moellering et al. 1979; Costa et al. 1993). Other resistance mechanisms reduce the intracellular concentration of free drug by changing outer membrane permeability, decreasing inner membrane transport, increasing excretion by efflux, or by sequestering the drug (Magnet and Blanchard 2005). However, when viewed in terms of molecular interactions at the drug rRNA target, the most interesting forms of resistance involve ribosome binding-site alterations, and these occur via r-protein or 16S rRNA mutations or 16S rRNA methylation.

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The latter mechanism utilizing rRNA methylation has been observed in a wide range of Gram-positive and Gram-negative pathogens, and confers high-level resistance to all clinically available aminoglycosides, with the exception of the structurally distinct compound streptomycin. Resistance via rRNA methylation was initially observed in actinomycetes, and particularly streptomycetes, that are producers of aminoglycosides (Beauclerk and Cundliffe 1987). In drug producers and bacterial pathogens, rRNA methylation occurs post-transcriptionally using S-adenosyl-L-methionine (SAM) as the methyl donor cofactor (Galimand et al. 2003). Two nucleotides within the 16S rRNA decoding region have previously been identified as the key targets: methylation at the N1 position of A1408 confers resistance to 4,5-disubstituted 2-deoxystreptamines (2-DOS) and 4,6-disubstituted 2-DOS aminoglycosides (Skeggs et al. 1985; Cundliffe 1989); whereas methylation at the N7 of G1405 confers resistance only to the 4,6-disubstituted 2-DOS aminoglycosides (Thompson et al. 1985; Cundliffe 1989). In human pathogens, these types of enzyme are generally mediated by plasmids, and include NpmA, which methylates the N1 of A1408 (Wachino et al. 2007), and ArmA and RmtB, which methylate the N7 of G1405 (Liou et al. 2006; Périchon et al. 2007).

In this study, we report a previously uncharacterized aminoglycoside resistance mechanism that involves 16S rRNA methylation. This mechanism occurs naturally in the clinical pathogen *E. faecium* CIP 54-32 together with the other chromosome-coded resistance determinant (the 6'-N-aminoglycoside acetyltransferase) and contributes substantially toward the intrinsic low-level resistance against the aminoglycosides dibekacin, kanamycin, and tobramycin. We demonstrate here that the product of *efmM* (*E. faecium* methyltransferase) is an SAM-dependent m⁵C methyltransferase (MTase) that specifically targets nucleotide 1404 in 16S rRNA. The EfmM enzyme structure has been determined at 2.28 Å resolution by X-ray crystallography, and its methyltransferase domain has been probed by site-directed mutagenesis to define the catalytic amino acid residues at the active site. EfmM and RsmF show high similarity in their primary and high-order structures. RsmF is an endogenous housekeeping methyltransferase found in *Escherichia coli*, and methylates the C5-position of C1407 in the ribosomal A-site (Andersen and Douthwaite 2006) without affecting aminoglycoside binding. Distinctive features in the structures of *E. faecium* EfmM, *E. coli* RsmF, and the most recently reported *Thermus thermophilus* RsmF (Demirci et al. 2010) indicate the molecular basis for their respective nucleotide specificities.

RESULTS AND DISCUSSION

EfmM is a methyltransferase

Genomic DNA from the *E. faecium* strain CIP 54-32 was fragmented and expressed from recombinant plasmids in *E.*

coli. Two fragments from the *E. faecium* chromosome were found to confer kanamycin resistance in *E. coli*. The first fragment, in the recombinant plasmid pAT431, contained the *aac(6')-II* gene that has previously been shown to confer resistance to amikacin, kanamycin, netilmicin, sisomicin, and tobramycin (Costa et al. 1993). The second, in plasmid pAT853, was a HindIII fragment of ~4.8 kb that confers resistance to kanamycin and tobramycin only. Sequencing of the 4.8-kb fragment showed that it is present in the chromosomes of other *E. faecium* strains in the databases, and corresponds to four full open reading frames (ORFs) with the N and C termini of two truncated ORFs at the ends. Three of the complete ORFs correspond to part of a hypothetical transcriptional anti-terminator, a putative transcription regulator (GenPept accession number EAN10349), and a hypothetical protein of unknown function (GenPept accession number EAN10352). None of these seemed likely candidates for conferring drug resistance.

The remaining ORF, at positions 2437 to 3807 in the fragment, was identical (100% identity over 456 amino acids) to a sequence previously annotated as an NOL1/NOP2/sun-type protein (GenPept accession number EAN10351) that belongs to the family of RNA m⁵C MTases. Given the previously characterized cases of rRNA modifications that result in aminoglycoside resistance, this gene was seen as the most likely candidate. Thus, the gene (now designated *efmM*) was amplified by PCR on a 1368-bp fragment (Supplemental Table S1) and cloned into pBAD/His, generating pAT854 (pBADΩ*efmM*) (Supplemental Table S2). The recombinant plasmid conferred on *E. coli* a fourfold or greater increase in resistance to kanamycin and tobramycin; the MICs of amikacin, gentamicin, and netilmicin remained unchanged (Table 1).

The 456-amino-acid sequence of the EfmM protein shows ~48% identity and 65% similarity with various putative RNA m⁵C MTases from Gram-positive bacteria (e.g., *Lactobacillus*, *Pediococcus*, *Streptococcus*, and *Leuconostoc* species). These enzymes were identified using bioinformatics tools, and none has yet been functionally characterized. Two *E. coli* 16S rRNA m⁵C MTases have been characterized: RsmF (GenPept accession number P76273) and Fmu (now RsmB; GenPept accession number P36929), and are ~30% identical to EfmM. Most recently, the RsmF ortholog in *T. thermophilus* has been described (Demirci et al. 2010) and shows a comparable level of identity (Fig. 1). Similarity between EfmM and the RsmF orthologs extends over the full length of the proteins, whereas EfmM and Fmu differ in their respective C- and N-terminal extensions.

Contribution of EfmM to aminoglycoside resistance

The relative contributions of the *aac(6')-II* and *efmM* genes to intrinsic aminoglycoside resistance in *E. faecium* CIP 54-32 were determined by insertion of a chloramphenicol acetyltransferase (*cat*) cassette to inactivate both structural

TABLE 1. Susceptibility of strains to aminoglycosides

Strain	MIC (μg/mL)				
	Kanamycin	Tobramycin	Amikacin	Gentamicin	Netilmicin
<i>E. faecium</i> CIP54-32					
Wild type	128	64	32	4	24
BM4681 ($\Delta aac(6')-Ii$)	12	8	24	4	2
BM4682 ($\Delta efmM$)	64	32	32	4	24
BM4683 ($\Delta aac(6')-Ii/efmM$)	6	6	24	4	2
<i>E. coli</i> TOP10					
pBAD/His	2	0.25	1.5	0.5	0.5
pAT855 (pBAD/His $\Omega aac(6')-Ii$)	256	128	128	1	128
pAT854 (pBAD/His $\Omega efmM$)	24	4	2	0.5	0.5

genes. In comparison with the parental strain (Table 1), a large decrease in the MICs of aminoglycosides that are substrates for AAC(6')Ii was observed for the $\Delta aac(6')-Ii$ strain (BM4681), whereas smaller decreases in the MICs of kanamycin and tobramycin were observed for the $\Delta efmM$ strain (BM4682). The lowest kanamycin and tobramycin MICs were observed for the double mutant *E. faecium* BM4683 [$\Delta aac(6')-Ii/efmM$]. Taken together, these data indicate that both genes make different but nonetheless significant contributions to the intrinsic aminoglycoside resistance of *E. faecium*.

EfmM methylates the 30S ribosomal subunit

Full-length EfmM with an N-terminal histidine-tag was expressed in *E. coli* and purified by Ni-affinity chromatography. The protein eluted as a single peak and, judging from the SDS-PAGE analysis, was homogeneous and conformed to the predicted molecular mass of ~56 kDa (data not shown).

Ribosomal particles were isolated from *E. coli* and used for in vitro methylation assays with the recombinant EfmM (Fig. 2). Efficient methylation was observed when 30S subunits were used as the substrate. In contrast, there was no, or only weak, methylation in reactions with 70S ribosomes, 50S subunits, or 16S rRNA. This demonstrated a clear substrate preference of the EfmM enzyme for the assembled 30S ribosomal subunit.

EfmM methylates nucleotide C1404

The sequence similarity of EfmM to m⁵C RNA MTases, taken together with its ability to confer aminoglycoside resistance and methylate the 30S subunit, strongly indicated that the enzyme targets a cytidine within the aminoglycoside-binding A-site. The 30S component of the ribosomal A-site is located between the 16S rRNA nucleotides 1404–1412 and 1488–1497 (Recht et al. 1996). As a first step toward identifying the methylated nucleotide, nucleotides 1392–1421 and 1478–1507 were isolated in a hybridization protection study with complementary DNA oligonucleotides (Supplemental Fig.

S1; Supplemental Table S1). Isolation of the 1392–1421 region from [*methyl*-³H]-labeled 16S rRNA led to retention of TCA precipitable radioactivity after RNase T1 digestion, whereas oligonucleotide 1478–1507 was unable to protect an rRNA region containing a radiolabeled methyl group (Supplemental Fig. S2); it was thus concluded that EfmM targets a nucleotide in the 1392–1421 region.

No primer extension stops were detected in this region after EfmM methylation (data not shown), and this is consistent with the modification being at m⁵C, which would not interfere with Watson-Crick base-pairing or impede the progress of reverse transcriptase. Localization of the methylated nucleotide was achieved by analyzing the 16S rRNA sequence from C1378 to G1432 using matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. This sequence was isolated from *E. coli* MRE600 and CP79yebU-1 ($\Delta rsmF$) strains, with and without recombinant EfmM, and from *E. faecium* by hybridization to complementary deoxy-oligonucleotides (Supplemental Fig. S1; Supplemental Table S1). After removal of the rest of the rRNAs with nucleases, the protected sequences were purified by gel electrophoresis and digested with RNase T1.

Expression of EfmM in *E. coli* resulted in a single difference in the MS spectra indicating that the mass of the RNase T1 peak at *m/z* 1289 (Fig. 3A,F) was increased by 14 Da to *m/z* 1303 (Fig. 3B,G). The *m/z* 1289 peak corresponds to the CCCGp tetranucleotide from C1402 to G1405 with two methyl groups at nucleotide C1402, where one is attached at the N⁴ position by RsmH and on the other at the 2'-O-ribose by RsmI (Kimura and Suzuki 2010). The absence of unmethylated, or singly methylated, peaks (that would have run at *m/z* 1261 and 1275, respectively) showed that 16S rRNA is stoichiometrically dimethylated at nucleotide C1402. The 14-Da mass increase in the corresponding tetranucleotide (at *m/z* 1303) after EfmM expression indicated the presence of a third methyl group. The lack of any residual peak at *m/z* 1289 (Fig. 3G) showed that methylation by EfmM had been complete. When expressed in the *E. coli* $\Delta rsmF$ strain, EfmM methylated C1404 in exactly the same manner. This strain lacks the natural modification at m⁵C1407 due to loss of

FIGURE 2. Methylation assays. EfmM methylation of the 30S (■) and 50S (▲) ribosomal subunits, 70S ribosome (◇), and 16S rRNA (○) plotted as a function of time. The reactions were performed at 30°C. Error bars were estimated from three repeated measurements.

of the *E. coli* and *E. faecium* *m/z* 1289 peaks by tandem MS fragmentation confirmed that both species contain two methyl groups on nucleotide m⁴Cm1402, and the *m/z* 1303 peak contained an additional methyl group on C1404 (Supplemental Fig. S3). The MS analyses indicate that EfmM adds the 16S rRNA m⁵C1404 modification in its original *E. faecium* host and that, in contrast to the *E. coli* recombinant (Fig. 3G), this modification is slightly less than stoichiometric (Fig. 3H).

Structural organization of EfmM

Four 16S rRNA m⁵C MTase structures have been solved: Fmu from *E. coli* (Foster et al. 2003; PDB ID code 1SQF); the Fmu homolog from *Pyrococcus horikoshii* PH0851 (PDB ID code 2YXL); RsmF from *E. coli* (Hallberg et al. 2006; PDB ID code 2FRX); and the RsmF from *T. thermophilus* (Demirci et al. 2010; PDB ID code 3M6X). An additional crystal structure is available for the human p120 homolog protein from *P. horikoshii*, which has also been proposed to be an m⁵C MTase (Ishikawa et al. 2004). No structure of an m⁵C MTase has yet been solved in complex with its RNA substrate, so a clear picture of how the highly conserved methyltransferase domains recognize and modify different sites in bacterial rRNA is still lacking.

We have determined the structure of the selenomethionylated form of EfmM (456 residues) using single-wavelength anomalous dispersion (SAD) methods and have refined the model at 2.28 Å resolution (*R*_{free} 25.2%) (Table 2). The model starts at T7 and ends at F455, and all residues within the structure, with the exception of 310 to 318, have been

determined precisely. The structure of EfmM is composed of four domains. The N-terminal region (N1 domain) is followed by a central MTase domain and a C-terminal domain containing two modules (C1 and C2 subdomains). This organization is characteristic of subfamily III of RNA m⁵C MTases (Reid et al. 1999).

The N-terminal part of the protein (N1 domain; residues 1 to 94) (cyan in Figs. 1, 4) contains two small modules: one is made of two antiparallel α-helices (α1–α2), and the other is formed by β1α3β2β3α4β4. This N1 domain is characteristic of RNA m⁵C MTases (Reid et al. 1999), and its structure has been described in two *E. coli* ribosomal MTases, RsmF (Hallberg et al. 2006) and Fmu (Foster et al. 2003). Superimposition of the EfmM N1 domain on the corresponding region of RsmF and Fmu gave RMSD values of 1.6 Å and 1.9 Å, respectively, for 66 C_α atoms compared. The topology of the N1 domain is reminiscent of the RNA

FIGURE 3. MALDI-MS spectra of the RNase T1 oligonucleotides from the 16S rRNA sequence C1378–G1432. (A) Spectra of rRNA from wild-type *E. coli* without and (B) with the *efmM* gene. The empirical *m/z* values are given above the peaks and match the theoretical masses (see below) to within 0.3 Da. The CCCG>p fragment (boxed) is shifted from *m/z* 1289 to *m/z* 1303 after EfmM methylation. (C) Higher mass region of rRNA from the *E. coli* Δ*rsmF* strain expressing EfmM; the m⁵C1407 modification is not recovered. (D) Same higher mass region from wild-type *Enterococcus faecium* spectrum showing that the sequence 1393–1401 flies at *m/z* 2859, as in *E. coli*, and thus C1400 remains unmethylated. The *E. faecium* fragment at *m/z* 3178 contains the RsmF-methylated nucleotide m⁵C1407, and this fragment is 1 Da smaller than in *E. coli* due to the U1414C substitution (see Supplemental Fig. S1). (E) Theoretical masses of the monoisotopic *E. coli* fragments with 2'-3'-cyclic phosphates (>p). Fragments 18 Da larger with linear 3'-phosphates are evident in some of the spectra. Fragments smaller than trinucleotides are not shown. (F) Enlargement of the spectrum around the *E. coli* C1402–G1405 fragment without and (G) after EfmM expression. Here, the complete shift of the *m/z* 1289 peak to *m/z* 1303 indicates stoichiometric modification at m⁵C1404. (H) Same spectral region from wild-type *E. faecium* shows that C1404 is incompletely methylated.

TABLE 2. Crystallographic and refinement data

Crystal	EfmM Se-Met
X-ray source	Soleil-Proxima-1
Data collection wavelength (Å)	0.98
Space group	P4 ₁ 2 ₁ 2
Cell parameters: <i>a</i> , <i>b</i> , <i>c</i> (Å)	98.56, 98.56, 106.6
Unique reflections	24,384
Resolution (Å)	2.28
Completeness (%) / redundancy	99.7/6.94
<i>R</i> _{sym} (I) (%) ^a	8.2 (39.7)
Figure of merit before density modification	0.35 at 3.2 Å
<i>R</i> / <i>R</i> _{free} ^b (%)	0.198/0.252
RMSD bonds (Å) / angles (°)	0.007/1.124
Residues in model	440 amino acids 83 water molecules
Mean <i>B</i> -value	
Protein	47.9
Water	40.1

^aValues in parentheses are *R*_{sym} (I) in the highest shell of resolution.

^b*R*_{free} is calculated with 6% of the reflections.

recognition motif, RRM, observed in many RNA binding proteins. In the three methyltransferases compared (Figs. 1, 4B,C), the N1 domain is tightly packed against the MTase domain. In EfmM, the surface binding area of the N1 domain onto the MTase domain is 1949 Å². Two conserved residues, R41 and N43, participate in the anchoring of the N1 domain onto the MTase domain. The side chain of R41 interacts with the G118 CO group and the side chain of Q90, whereas the side chain of N43 is tightly packed against that of W153 and interacts with its CO group. In agreement with earlier descriptions of RNA m⁵C MTases subfamilies (Reid et al. 1999; Foster et al. 2003), the N1 and MTase domains form a common structural core domain.

Following the N1 domain, EfmM contains the characteristic fold of the class I methyltransferases corresponding to a seven-stranded β-sheet (strands A to G) with helices on each side (Z, A, B, and C, D, E; MTase domain; residues 95 to 303) (red and yellow in Fig. 4A). Within the MTase domain, the region corresponding to residues 260 to 282 adopts a structure different from those of the corresponding regions of RsmF and Fmu. Attempts to study SAM binding by soaking and co-crystallization experiments were unsuccessful. Therefore, the position of SAM was modeled from superimposition with the Fmu:SAM structure (PDB ID code 1SQF). SAM fits in a pocket at the C-terminal ends of strands βA to βD where the conserved residues of motifs I to IV are located (Fig. 4A).

The MTase domain is linked to the C-terminal region by a lysine-rich flexible linker (residues 305 to 323). Residues 310 to 318 are disordered in the structure, but, according to the distance between residues 309 and 319 (25.4 Å), the linker can make an elongated connection overhanging the MTase domain. This flexibility contrasts with the structure of the

E. coli RsmF in which a proline-rich linker makes a rigid belt over the MTase domain (Hallberg et al. 2006).

The C-terminal region contains two subdomains, named C1 and C2, packed one on the other. The C1 domain (residues 322 to 390) (orange in Figs. 1, 4A) is built around an antiparallel β-sheet containing four strands flanked by two α-helices. An elongated peptide, containing strand β8, joins β7 to β9. Connection between the C1 and C2 domains involves a small 3₁₀ helix. The C2 domain contains a mixed β-sheet formed by five strands and capped by an α-helix (residues 391 to 456) (pale orange in Figs. 1, 4A). This domain shows structural similarity with the PUA (PseudoUridine synthase and Archaeosine transglycosylase) domains found in some RNA binding proteins (Aravind and Koonin 1999; Perez-Arellano et al. 2007). The PUA domain has been shown to be involved in tRNA binding of archeosine tRNA-guanine transglycosylase complexed to tRNA (Ishitani et al. 2003) and in the co-crystal structure of the tRNA Ψ55 pseudouridine synthase (Hoang and Ferre-D'Amare 2001). More significantly, the PUA domain was found to be crucial for the precise location of the tRNA molecule on the enzymes.

Implications for catalysis and RNA recognition

Active-site cysteine residues are required for resistance

Typical RNA m⁵C MTases possess two characteristic motifs (IV and VI), each of which contains a conserved cysteine residue. Catalysis involves both cysteines and has been studied in detail for *E. coli* Fmu (Liu and Santi 2000; Foster et al. 2003) and for yeast Trm4 (Walbott et al. 2007); a similar mechanism is thought to be employed in m⁵U RNA MTases (Lee et al. 2005; Alian et al. 2008). These studies demonstrated that the cysteine in motif VI is the catalytic nucleophile, while the motif IV cysteine plays a secondary role in catalysis.

The two equivalent residues in EfmM, C185 and C235, are located at the base of the active-site cleft where the two sulfhydryl groups are 3.7 Å apart and close to the activated methyl group of SAM (5.3 and 4.7 Å, respectively). After substitution of these cysteines with alanine or serine, EfmM with single C185A, C185S, C235A, or C235S mutations was no longer able to confer resistance; and, predictably, EfmM with the C185A–C235A and C185S–C235S double mutations was also inactive. Thus, the structural and mutagenesis data indicate that the catalytic pocket of EfmM functions in a manner similar to other RNA m⁵C MTases.

The position of the cytosine substrate was modeled within the catalytic pocket using the structure of the m⁵U RNA MTase RumA complexed with RNA and the SAM cofactor (Lee et al. 2005). The MTase domains of RumA and EfmM were superimposed, and the position of the RumA target U1939 was used to fit C1404 in the active cleft of EfmM (Fig. 4A). This positioning is consistent with a model in which

FIGURE 4. (A) Overall structure of EfmM. (Cyan) The N1 domain; (red) methyltransferase domain α -helices; (yellow) methyltransferase domain β -strands; (green) methyltransferase domain loops; (orange) the C1 domain; (pale orange) the C2 domain. Secondary structures as described in Figure 1 are labeled. The positions of motifs I, II, III, IV, and VI are indicated. The models of AdoMet and C1404 are drawn as sticks. The same color code is used for EfmM in all three views. (B) Superimposition of EfmM with *E. coli* RsmF (PDB ID code 2FRX). (Ribbons) The two proteins; (gray) RsmF. (C) Superimposition of EfmM on *E. coli* Fmu (PDB ID code 1SQF). (Ribbons) The two proteins; (gray) Fmu. (D) Superimposition of EfmM with *T. thermophilus* RsmF (PDB ID code 3M6X). (Ribbons) The two proteins; (gray) RsmF. The figure was drawn using PyMol (DeLano Scientific LLC).

C235 is the catalytic nucleophile leading to the formation of a covalent bond at C6 of C1404. According to the currently accepted reaction mechanism (Foster et al. 2003), after transfer of the methyl group of SAM at C5 of C1404, a proton would then be abstracted from this base position by cysteine C185 to resolve the covalent complex.

Differences in the EfmM and RsmF structures correlate with their target specificities

The data on the structure and function of *E. faecium* EfmM and *E. coli* RsmF invite the question of how these m⁵C MTases specifically recognize their adjacent 16S rRNA targets on the 30S ribosomal subunit. The EfmM target at C1404 engages in a Watson-Crick pair (C1404–G1497) that makes a tertiary interaction with A1519 (Fig. 5A) and is thus more buried than the RsmF target at C1407. Structural data have shown that similar MTases flip out the target base from a stacked structure within the RNA substrate to position it within the catalytic center (Lee et al. 2005; Alian et al. 2008), and it seems likely that EfmM uses a similar mechanism to modify C1404.

RNA m⁵C MTases are divided into eight subfamilies that can be distinguished by their N-terminal and/or C-terminal extensions. These extensions were proposed to serve as RNA-

binding modules, although it remained uncertain how this could be achieved (Reid et al. 1999). The electrostatic potential on the surface of EfmM suggests that the N1 domain and C-terminal region offer the main areas of contact with the rRNA (Fig. 5B). As observed in other m⁵C MTases, the basic character of the molecular surface of the N1 domain and its position close to the carboxyl moiety of SAM at the MTase domain argue in favor of its participation in catalysis and/or binding to the RNA substrate.

Interactions between the C-domain and the core domain of EfmM were examined to evaluate whether these regions might participate in rRNA binding. The C1 and C2 domains are tightly packed on the core domain (binding surface area of 1876 Å²): the C1 domain interacts mainly with the MTase domain over a surface area of 896 Å², while the main interactions of the C2 domain are with the N1 domain and extend over a surface area of 528 Å². In the C1 domain, strand β 9 interacts with β C of the MTase domain, thereby extending the β -sheet of the MTase domain. Additional hydrogen bonds are created between residues belonging to the four domains of EfmM and stabilize the domain interfaces (Fig. 5C). Notably, D431 contacts Y56 and is constrained in such a way that its Ramachandran angles lie in a disallowed region. Interestingly, corresponding residues that could make

FIGURE 5. (A) A site on the *E. coli* 30S subunit (PDB ID code 3I1M; Zhang et al. 2009). (Gray ribbon) rRNA; (green cartoon) r-protein S12. (Red sticks) Nucleotides C1404 and C1407; (gray sticks) nucleotides U1406, G1405, G1494, G1497, and A1519. (Red dashed lines) Hydrogen bonds responsible for tertiary interactions between C1404 and A1519 and between G1497 and A1519. The view illustrates that C1404 is less accessible than C1407 since the Watson-Crick pair C1404–G1497 makes a tertiary interaction with A1519. (B) Electrostatic surface representation of EfmM. Blue and red represent regions of positive and negative electrostatic potential calculated using Grasp (Nicholls et al. 1991). Domains of EfmM are labeled; the SAM cofactor and nucleotide C1404 are visible within the negatively charged cleft. (C) Interface regions between domains N1, MTase, C1, and C2 in EfmM. The color code is the same as in Figure 4. (Sticks) Residues involved in hydrogen bonds at the interface; (dashed lines) hydrogen bonds. (D) Same view as in C but for *E. coli* RsmF. The color code is the same as that used for EfmM.

hydrogen bonds between the C-domain and the core domain are absent in the *E. coli* RsmF (Fig. 5D), and these regions interface in a different manner. Superimposition of the core domains of RsmF and EfmM shows that the position of the C1 domain deviates significantly in the two enzymes (Fig. 4B), and it can thus be imagined that different adjustments of the interfaces between the core domain and the C domain of RsmF and EfmM contribute to the specificity for their target nucleotide. Notably, if the C1 domain is excluded, the RMSD value between the two proteins drops to 1.46 Å (314 C α atoms compared) compared to 2.5 Å when the entire proteins are superimposed (453 C α atoms compared). The most important deviations concern the positions of $\alpha 6$ and the following peptide (Fig. 4B). It is therefore notable that the connective belts connecting the MTase domains to $\alpha 6$ have markedly different sequences in the two enzymes (Fig. 1).

The structure of the RsmF protein from *T. thermophilus* recently became available (Demirci et al. 2010) during revision of the present manuscript. *T. thermophilus* RsmF displays a more extensive activity than the *E. coli* RsmF and EfmM enzymes and catalyzes m⁵C formation at three posi-

tions—C1400, C1404, and C1407—near the ribosomal A-site. The *T. thermophilus* RsmF protein structure is similar to that of EfmM, and the two structures are superimposable with an RMSD of 1.38 Å for 369 C α atoms compared (Fig. 4D). The largest deviation is within the C2 domain. Accordingly, the residues at the interface between the N1 and C2 domains in *T. thermophilus* RsmF are markedly different than in EfmM. Such differences are particularly obvious when the contacts of domain N1 with the $\beta 12$ – $\alpha 8$ turn and the $\beta 14$ – $\beta 15$ turn are compared (Figs. 1, 4).

The extended specificity shown by the *T. thermophilus* RsmF enzyme has not been observed for EfmM. The MS data clearly show that EfmM does not methylate C1400 (Fig. 3D), and, likewise, C1407 remains unmethylated when EfmM is expressed in the *E. coli* Δ rsmF strain (Fig. 3C). We note, however, that C1407 is methylated in the *E. faecium* 16S rRNA (Fig. 3D) and that there is no other obvious rsmF ortholog in the *E. faecium* genome that might encode the methyltransferase for this modification. This caveat aside, the substrate specificities of the *E. coli* RsmF, the *T. thermophilus* RsmF, and the *E. faecium* EfmM enzymes are distinct, and the structural data suggest that this is brought about difference in the

properties and positioning of their C-terminal domains. Possibly, the enzymes melt the rRNA structure around the A-site in a similar manner but differ in the specific interactions that accommodate their respective target base(s) into the active site. Understanding the molecular mechanisms by which these enzymes recognize and methylate their specific nucleotide targets awaits the resolution of co-crystal structures of complexes with the 30S ribosomal subunit.

Novel mechanism of aminoglycoside resistance

We have identified and characterized an enzyme from *E. faecium* CIP 54-32 that methylates the C5-position of nucleotide C1404 in the ribosomal A-site. This modification leads to moderate resistance to a subset of aminoglycosides including kanamycin and tobramycin of the 4,6-disubstituted-2-deoxystreptamine family. Examination of the crystal structure of kanamycin bound to the A-site (François et al. 2005) shows that C1404 is located in the immediate vicinity of the antibiotic-binding cleft, where the nucleotide C5 atom is 3.9 Å from the N3'' of kanamycin (Fig. 6A,B). Importantly, the N3'' atom of kanamycin is hydrogen-bonded to a water molecule, the presence of which is incompatible with a C5-methyl group on nucleotide C1404.

Geneticin and the near-identical drug gentamicin are themselves methylated at the N3'' and are thus even bulkier at this position than kanamycin and tobramycin; however, the N3'' aminomethyl group of geneticin and gentamicin does not require (or allow) hydration (François et al. 2005). The m⁵C1404 modification confers no resistance to gentamicin (Table 1), and thus the aminomethyl group can presumably be accommodated into the A-site binding pocket, despite initial appearances to the contrary (Fig. 6C). This suggests that the mechanism of resistance is not merely due to a steric clash between the drugs and m⁵C1404, but involves preventing the solvation of kanamycin and tobramycin at their N3'' position. Furthermore, Efm does not confer resistance to amikacin, which has no methyl group on its N3'' atom (Table 1). It can be imagined that amikacin with its bulky substitution

in the second ring sits differently within the binding site and takes on a slightly different position relative to the C5 of C1404.

BLAST searches reveal close EfmM homologs in Gram-positive bacteria; protein sequences with >60% identity were found in the genomes of enterococcal species. Other close homologs (45% to 50% identity) could be identified in many Gram-positive bacteria, for instance, *Lactobacillus* spp., *Pediococcus* spp., *Streptococcus* spp., and *Leuconostoc* spp. Homologs to RsmF are found in Gram-negative bacteria, principally in Enterobacteriaceae.

The *efmM* gene is located in the proximity of IS6770 in the chromosome of *E. faecium* strains, as well as in *Enterococcus faecalis* and some *Lactobacillus* strains, raising the possibility that this gene might be mobile. Although chromosome-coded homologs of *efmM* are widely distributed among Gram-positive bacteria, conferring low-level antibiotic resistance might not be the main function of EfmM. The position of m⁵C1404 at the junction between the ribosomal A-site and P-site in 16S rRNA suggests that this modification might also play a more basic role in protein synthesis by influencing codon–anticodon interactions.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The plasmids and strains used in cloning and insertional inactivation experiments are listed in Supplemental Table S2. Strains were grown in brain heart infusion (BHI) or Luria Bertani (LB) broth and agar at 37°C, unless otherwise indicated.

Antibiotic susceptibility was tested by disk diffusion on Mueller-Hinton (MH) medium according to the standards of the Comité de l'Antibiogramme de la Société Française de Microbiologie (<http://www.sfm.asso.fr>). MICs of antimicrobial agents for *E. coli* and *E. faecium* strains were determined on MH or LB agar by Etest (AB Biodisk).

Inactivation of the *aac(6')-Ii* and *efmM* genes

In Gram-positive bacteria, pGhost (Maguin et al. 1996) confers erythromycin resistance and replicates at 28°C but is lost above 37°C, allowing insertional inactivation in *E. faecium*. To generate an *aac(6')-Ii* knockout strain, the *E. faecium* CIP 54-32 chromosomal sequence surrounding the *aac(6')-Ii* gene was amplified using primers *aac6'* F and *aac6'* R (Supplemental Table S1) containing KpnI and XbaI sites, respectively, and cloned in the same sites of pGhost5. A chloramphenicol acetyltransferase (*cat*) cassette, amplified using plasmid pJIR 750 as a template (Bannam and Rood 1993) with primers *cat* F2 and *cat* R2 containing AccI and EcoRI sites, respectively (Supplemental Table S1), was ligated into the *aac(6')-Ii* gene cleaved at the same sites forming pAT856 [pGhostΔ*aac(6')-Ii*/*cat*] that was transformed into *E. faecium* CIP54-32 as described (Cruz-Rodriguez and Gilmore 1990).

A similar approach was used to create an *efmM* knockout derivative. The *E. faecium* genomic sequence surrounding the *efmM* gene was amplified using primers *efm* F3 and *efm* R3 (Supplemental Table S1) containing KpnI and EcoRI sites, respectively, cloned in

FIGURE 6. Aminoglycoside interactions at the ribosomal A site on 16S rRNA. (A) 16S rRNA is shown in gray indicating the C5-positions of cytosines (yellow spheres) and uracils (blue spheres). (Blue) Kanamycin (PDB ID code 2ESI; François et al. 2005). (Pink sphere) The water molecule involved in antibiotic binding. (B) The same structure from a different angle. (C) Superimposition of (blue) kanamycin (PDB ID code 2ESI; François et al. 2005), (magenta) geneticin (PDB ID code 1MWL; Vicens and Westhof 2003), and (green) tobramycin (PDB ID code 1LC4).

the same sites of pGhost5. A *cat* cassette amplified with primers cat F1 and cat R1 containing BglII and ClaI sites, respectively (Supplemental Table S1), was ligated into the same sites of *efmM* leading to pAT857 (pGhost5 Ω *efmM/cat*). Gene replacement was obtained by transfer of culture in liquid LB medium from permissive (28°C) to nonpermissive (42°C) temperature, selection with chloramphenicol (10 μ g/mL), and screening for loss of erythromycin resistance (10 μ g/mL).

An 800-bp *efmM* intragenic fragment was amplified with primers *efmintra* F and *efmintra* R (Supplemental Table S1) containing, respectively, PstI and XbaI sites, and cloned in the same sites of pGhost5 leading to pAT858 (pGhost5 Ω 800 bp *efmM*). To generate a doubly inactivated *E. faecium* CIP54-32, pAT858 was electrotransformed into *aac(6')-II* inactivated BM4681. Gene disruption was achieved by switching cultures from 28°C to 42°C with selection for erythromycin resistance (10 μ g/mL). Potential knock-out strains were screened by PCR mapping and sequencing with internal primers (Supplemental Table S1).

Site-directed mutagenesis of the *efmM* gene

The structural gene for EfmM was amplified with primers EfmF1 and EfmR1 (Supplemental Table S1) and cloned in pBAD/His between the KpnI and EcoRI sites under the control of an arabinose promoter generating pAT854 (pBAD/His Ω *efmM*). Mutagenesis was carried out according to the QuickChange site-directed mutagenesis method (Stratagene) with the oligonucleotides and their complements listed in Supplemental Table S1 using pAT854 as the template. Overexpression of the mutated proteins was checked by SDS-PAGE, and antibiotic susceptibility of the hosts was determined by disk diffusion.

Expression, purification of EfmM and of substrates for the methylation assay

E. coli TOP10 carrying pAT854 (pBAD/His Ω *efmM*) was grown at 37°C in 0.5 L of LB broth containing ampicillin (50 μ g/mL) and induced with 0.2% (w/v) L-arabinose at an A_{650} of 0.6. The culture was then incubated for 4 h at room temperature. Cells were harvested by centrifugation at 4°C and used immediately or stored at -80°C. The cells were washed in buffer A (20 mM sodium phosphate at pH 8.0 containing 500 mM NaCl, and 20 mM imidazole), resuspended in the same buffer, and disrupted by sonication. Cellular debris was removed by centrifugation, and the supernatant was collected, filtered (0.45 μ m), and applied to a HisTrap Fast Flow column (GE Healthcare) previously equilibrated with buffer A. The enzyme was eluted with a linear gradient of 20 to 500 mM imidazole over 30 mL. Fractions containing the protein were analyzed for purity by SDS-PAGE, and the pure fractions were pooled, dialyzed against 10 mM HEPES (pH 7.5), 10 mM MgCl₂, 50 mM NH₄Cl, and 3 mM β -mercaptoethanol, and concentrated with a Centrprep-30 concentrator (Amicon). Protein concentration was determined on the basis of A_{280} assuming an extinction coefficient of 71,640 M⁻¹ cm⁻¹ as calculated by ProtParam of the ExPASy proteomic server (<http://www.expasy.org>).

Tight-coupled 70S ribosomes and 30S and 50S ribosomal subunits from *E. coli* MRE600 were purified on sucrose gradients; 16S rRNA was extracted from 30S subunits with phenol/chloroform and was recovered by ethanol precipitation (Moazed et al. 1986).

In vitro methylation assays were performed using S-adenosyl-L-[methyl-³H] methionine (81.9 Ci/mmol, 0.55 mCi/mL; PerkinElmer) as described previously (Liou et al. 2006; Schmitt et al. 2009). Briefly, substrates were incubated with EfmM (15 mM) and SAM (10 mM) at 30°C in 50 mM HEPES (pH 7.5), containing 10 mM MgCl₂, 100 mM NH₄Cl, and 5 mM β -mercaptoethanol in a final volume of 20 μ L. The reaction was stopped at 3, 6, 9, 20, 40, and 60 min by adding ice-cold 5% (w/v) trichloroacetic acid and 20 μ L of carrier RNA from yeast (4 mg/mL). Precipitates were collected on GF/C filters (Whatman) that were immersed in picofluor scintillation mixture (Packard) and counted in a Beckman LS6500 scintillation counter after 24 h.

RNase protection assay

16S rRNA was extracted from 30S ribosomal subunits methylated in vitro by EfmM. One picomole of [methyl-³H]-labeled 16S rRNA was hybridized with 100 pmol of a deoxyoligonucleotide 16S-1421 or 16S-1507 (Supplemental Fig. S1; Supplemental Table S1) in 50 μ L of hybridization buffer (40 mM morpholineethanesulfonic [MES] acid at pH 6.4, 400 mM NaCl, 9 mM EDTA, and 80% [v/v] formamide). The sample was incubated for 10 min at 85°C, cooled at room temperature for 15 min, and diluted with 450 μ L of RNase buffer containing 20 units of RNase T1 (Roche). The digestion was performed for at least 1 h at 37°C and stopped by adding ice-cold 5% trichloroacetic acid. Precipitates were collected and counted in a scintillation counter.

Analysis of 16S rRNA by mass spectrometry

16S rRNA was extracted from 30S ribosomal subunits isolated from *E. faecium* CIP54-32 and *E. coli* MRE600 and C79yebU-1 (Δ *rsmF*) strains harboring pBAD/His or pAT854 (pBAD/His Ω *efmM*). The 16S rRNA C1378–G1432 sequence was isolated by hybridization to complementary 55-mer deoxyoligonucleotides (Supplemental Fig. S1; Supplemental Table S1; Andersen et al. 2004; Andersen and Douthwaite 2006). The masses of the RNase T1 fragments were recorded on a Micromass MALDI Q-TOF Ultima instrument (Waters) run in the positive ion mode. Spectra were analyzed and smoothed using the *m/z* program Masslynx (Micromass). Fragments of particular interest were selected for further analysis by tandem MS.

Purification and crystallization of EfmM

To improve protein expression, the EfmM gene was re-cloned into pET28/16 (Chastanet et al. 2003) to give pAT859 (pET28/16 Ω *efmM*). *E. coli* B834 cells (Novagen) containing pET28 Ω *efmM* were used for the production of selenomethionylated EfmM with an N-terminal His₆ purification tag followed by a thrombin cutting site. Bacteria were grown overnight at 37°C in 1 L of autoinducible selenomethionine medium (Studier 2005) containing ampicillin (50 μ g/mL) and chloramphenicol (34 μ g/mL). Cells were disrupted by sonication in 40 mL of buffer A (10 mM HEPES at pH 7.5, 3 mM 2-mercaptoethanol, 500 mM NaCl, 0.1 mM PMSF). After centrifugation, the supernatant was loaded onto a column (3 mL) containing Talon affinity resin (Clontech). The resin was washed with 50 mL of buffer A followed by 50 mL of buffer A supplemented with 10 mM imidazole. The protein was eluted with buffer A containing 125 mM imidazole. The N-terminal-His₆ tag was removed using 0.25 U of thrombin (Sigma) per milligram of

the eluted protein during overnight dialysis at 4°C against buffer A containing 5 mM CaCl₂. The dialyzed protein was loaded onto a column (3 mL) containing Talon affinity resin equilibrated in buffer A, recovered in the flow-through, and dialyzed against buffer B (10 mM HEPES at pH 7.5, 3 mM 2-mercaptoethanol, 150 mM NaCl). The protein was loaded onto a column (3 mL) containing S-Sepharose resin (Amersham) equilibrated in buffer B and eluted with a 120-mL gradient from 0.15 to 1 M NaCl in 10 mM HEPES (pH 7.5) and 3 mM 2-mercaptoethanol. Fractions containing the protein were pooled and concentrated using a centricon-30 concentrator (Amicon).

Crystals were obtained using the vapor diffusion method at 24°C in 25% PEG3350 buffered with 0.1 M HEPES-HCl (pH 7.5). They belonged to space group P₄₁2₁2 and diffracted to 2.28 Å resolution (Table 2). The structure was solved by single-wavelength anomalous diffraction methods at the peak of Se absorption ($\lambda = 0.979$ Å). Data, collected at the Proxima 1 beamline of synchrotron SOLEIL (Saclay, France), were processed using XDS (Kabsch 1988) and programs of the CCP4 suite (Collaborative Computational Project Number 4 1994). The positions of six Se sites were determined using SHELX (Sheldrick 2008). The Se sites and the associated B factors were then refined using PHASER (McCoy et al. 2007), and the experimental electron density map was generated after solvent flattening using PARROT (Cowtan 2010). The quality of the map was sufficient to trace an initial model manually, using that of RsmF (PDB; ID 2FRX) as a guide. The model was refined by multiple cycles of energy minimization using PHENIX and manual rebuilding using O (Jones et al. 1991). The final model showed an R_{free} of 25.2% ($R = 19.8\%$) with good geometry (Table 2).

Nucleotide sequence and PDB accession numbers

The nucleotide sequence of *efmM* and of flanking regions has been deposited in the GenBank database under accession number GU584085. Coordinates were deposited at the PDB with ID code 3M4X.

SUPPLEMENTAL MATERIAL

Supplemental material can be found at <http://www.rnajournal.org>.

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