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EmtA, a rRNA methyltransferase conferring high-level evernimicin resistance

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Summary

Enterococcus faecium **strain 9631355 was isolated from animal sources on the basis of its resistance to the growth promotant avilamycin. The strain also exhibited high-level resistance to evernimicin, a drug undergoing evaluation as a therapeutic agent in humans. Ribosomes from strain 9631355 exhibited a dramatic reduction in evernimicin binding, shown by both cell-free translation assays and direct-binding assays. The resistance determinant was cloned from strain 9631355; sequence alignments suggested it was a methyltransferase and therefore it was designated *emtA* for *e*vernimicin *m*ethyl*t*ransferase. Evernimicin resistance was transmissible and *emtA* was localized to a plasmid-borne insertion element. Purified EmtA methylated 50S subunits from an evernimicin-sensitive strain 30-fold more efficiently than those from a resistant strain. Reverse transcription identified a pause site that was unique to the 23S rRNA extracted from resistant ribosomes. The pause corresponded to methylation of residue G2470 (*Escherichia coli* numbering). RNA footprinting revealed that G2470 is located within the evernimicin-binding site on the ribosome, thus providing an explanation for the reduced binding of the drug to methylated ribosomes.**

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Introduction

There is growing alarm regarding the prolific use of antibiotics and the concomitant spread of drug resistance among human clinical strains, particularly among the enterococci (Huycke *et al*., 1998). The localization of resistance determinants to plasmids and conjugative transposons has facilitated their dissemination, resulting in both intraspecies and intergeneric gene transfer (Nobel *et al*., 1992). Extensive use of antibiotics, both therapeutically and as growth promotants in animal feed, has resulted in a similar problem in animals (Aarestrup *et al*., 1998a; Van den Bogaard and Stobberingh, 1999). Particular concern arises when animal isolates exhibit cross-resistance to antibiotics used to treat humans (Aarestrup, 1998; Aarestrup *et al*., 1998b). Such strains could transfer antibiotic resistance to human clinical strains. This route has been demonstrated both in the laboratory (Khan *et al*., 2000) and in the human gut (Shoemaker *et al*., 2001). Direct colonization of humans by resistant animal isolates would provide a more direct route; current data suggests that this occurs rarely (Van den Bogaard and Stobberingh, 1999).

Evernimicin, a novel oligosaccharide antibiotic (Ganguly *et al*., 1989), is active against a broad range of Gram-positive pathogenic bacteria (Jones and Barret, 1995). The drug binds a single high-affinity site on the 50S ribosomal subunit (McNicholas *et al*., 2000) and may inhibit protein synthesis by interfering with binding of initiation factor 2 (Belova *et al*., 2001). Consistent with its binding site, evernimicin resistance was caused by mutations in both *rplP* (encodes the 50S associated protein L16) in *Streptococcus pneumoniae* and *Staphylococcus aureus* (Adrian *et al*., 2000a; McNicholas *et al*., 2001) and in domain V of the 23S rRNA in *S. pneumoniae* and *Halobacterium halobium* (Adrian *et al*. 2000b; Belova *et al*., 2001). One source of *rplP* mutants is Enterococcal isolates from animals given the growth promotant avilamycin, a structural analogue of evernimicin (Aarestrup and Jensen, 2000; this study). These mutants also exhibited limited cross-resistance to evernimicin (Aarestrup, 1998). In this study, we characterize an enterococcal isolate from animals, which encodes a

methyltransferase that confers high-level evernimicin resistance through methylation of 23S rRNA.

Results

Identification of an E. faecium *strain encoding transmissible high-level evernimicin resistance*

Twenty avilamycin-resistant (minimum inhibitory concentration (MIC) $> 64\ \mu g\ ml^{-1}$) enterococcal strains (eleven *Enterococcus faecalis* and nine *E. faecium*) were obtained from the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (Aarestrup *et al.*, 1998a). Nineteen strains exhibited low-level cross-resistance to evernimicin (MIC $< 12\ \mu g\ ml^{-1}$) and all had single point mutations in *rplP*. A similar result was reported recently (Aarestrup and Jensen, 2000). *E. faecium* strain 9631355 exhibited an evernimicin MIC $> 256\ \mu g\ ml^{-1}$ and had no mutations in either *rplP* or the 23S rDNA. The evernimicin-resistant phenotype was transferable; using the recipient strain 27270RF (evernimicin MIC $0.5\ mg\ ml^{-1}$), we obtained evernimicin-resistant transconjugants (MIC $> 256\ \mu g\ ml^{-1}$) at an average frequency of 1×16^{-6} per recipient colony-forming unit in liquid media. No other drug-resistance markers appeared to be co-transferred. The transconjugants generated above transferred evernimicin resistance to strain 27270S at the same frequency as strain 9631355 (data not shown). There were no differences in growth rates between isogenic recipient (27270RF) and transconjugant (27270TC1) strains (27270TC1 was grown in both the presence and absence of evernimicin).

High-level evernimicin resistance is associated with an alteration in the ribosomes

We measured the susceptibility of ribosomes, isolated from strains exhibiting varying levels of evernimicin resistance, to inhibition by evernimicin in a cell-free translation assay. The S100 extract, which provides the ancillary factors required for protein synthesis, was isolated from strain 27270 (evernimicin MIC $0.5\ \mu g\ ml^{-1}$). Ribosomes were isolated from strain 27270, strain 9508106 (which carries a *rplP* mutation (MIC $4\ \mu g\ ml^{-1}$)) and strain 9631355 (MIC $> 256\ \mu g\ ml^{-1}$). In the absence of drug, the ribosomes all synthesized similar amounts of labelled protein (data not shown). Addition of evernimicin inhibited ribosomes from strain 27270 in a dose-dependent manner; 95% inhibition occurred at approximately $0.3\ \mu g\ ml^{-1}$ (195 nM, Fig. 1). To achieve 95% inhibition of ribosomes from strain 9508106 (the *rplP* mutant) required eightfold more evernimicin. Ribosomes from strain 9631355 showed no inhibition at $160\ \mu g\ ml^{-1}$ ($100\ \mu M$). The above susceptibilities were mirrored by the capacity of the ribosomes to bind [^{14}C]-evernimicin (Fig. 2). Similar to previous data (McNicholas *et al.*, 2000), wild-type ribosomes exhibited a Kd of 200 nM and a 1:1 stoichiometry of bound drug to ribosomes. Ribosomes from strain 9508106 exhibited a Kd of 400 nM. Under the conditions used here, we did not detect binding of [^{14}C]-evernimicin to ribosomes from strain 9631355. We repeated the binding assay using ribosomes from isogenic recipient (27270RF) and transconjugant (27270TC1) strains. Ribosomes from strain 27270RF exhibited a Kd of 100 nM. We were unable to detect binding to ribosomes from strain 27270TC1 (data not shown). In contrast, both sets of ribosomes bound [^{14}C]-erythromycin with a Kd of 50 nM (data not shown) that is similar to a previous determination (Pestka, 1976).

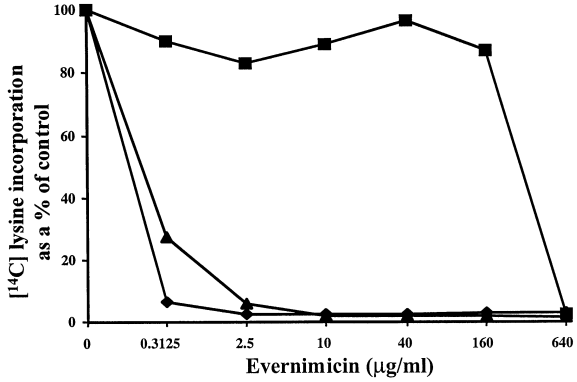

Fig. 1. Effect of evernimicin on cell-free translation reactions. Reactions were performed using an S100 extract isolated from the evernimicin-sensitive *E. faecium* strain 27270 and ribosomes isolated from the following *E. faecium* strains; 27270 (evernimicin MIC $0.5\ \mu g\ ml^{-1}$, ◆); 9508106 (a *rplP* mutant, MIC $4\ \mu g\ ml^{-1}$, ▲) and 9631355 (MIC $> 256\ \mu g\ ml^{-1}$, ■). For each set of ribosomes, incorporation of [^{3}H]-lysine into hot TCA-precipitable material is expressed as a percentage of the control (no antibiotic) reaction.

Cloning and sequence analysis of the evernimicin-resistance determinant, emtA

A plasmid library, constructed from total cellular DNA

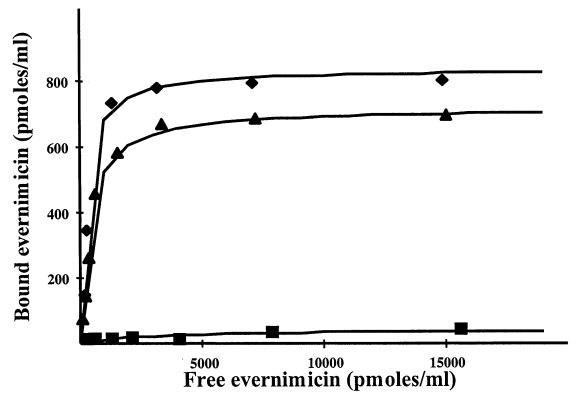

Fig. 2. Non-linear regression analysis of [^{14}C]-evernimicin binding to 70S ribosomes. Binding was performed with ribosomes isolated from the following *E. faecium* strains: 27270 (evernimicin MIC $0.5\ \mu g\ ml^{-1}$, ◆); 9508106 (MIC $4\ \mu g\ ml^{-1}$; ▲) and 9631355 (MIC $> 256\ \mu g\ ml^{-1}$, ■). The amount of bound [^{14}C]-evernimicin was calculated from a standard curve.

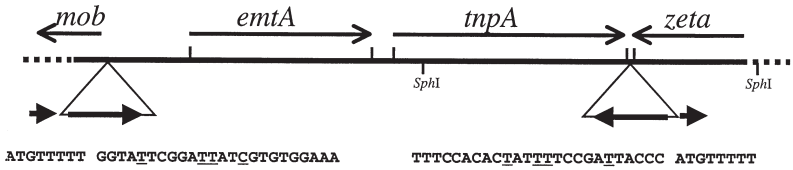

Fig. 3. Diagrammatic representation of the open reading frames (ORFs) present in the cloned fragment conferring high-level evernimicin resistance. The size and orientation of the various ORFs are shown as the *Sph*I restriction sites used to generate pPAM21. Hatched lines represent vector sequences. Shown below is the DNA sequence of the inverted repeats and the flanking direct repeats; mismatches in the inverted repeats are underlined.

from strain 27270TC1, was screened for clones conferring high-level evernimicin resistance. We identified two identical clones; the 3837 bp insert in pPAM19 had the coding potential for two full-length open reading frames (ORFs) and two truncated ORFs (Fig. 3). Deletion of the *Sph*I fragment from pPAM19 did not impair evernimicin resistance, implying that the ORF labelled *emtA* encoded the resistance determinant (Fig. 3). Sequence alignments using PSI-BLAST (Altschul *et al*., 1997) produced a number of weak matches to EmtA; the best was a hypothetical protein (accession number BAA79525) from *Aeropyrum pernix* whose GenBank entry indicated that it contained an 'adenine-specific DNA methylase signature'. Similarly, the top hits, using IMPALA (Schaffer *et al*., 1999) and the threading algorithm PROFIT (Flockner *et al*., 1997), were the UbiE methyltransferase family of proteins and a cobalt-precorrin-4 methyltransferase respectively. Therefore we labelled the resistance ORF *emtA* for *e*vernimicin *m*ethyl*t*ransferase.

EmtA *is located on a plasmid-borne transposable element*

emtA and a second full-length ORF, labelled *tnpA*, are flanked by imperfect inverted repeats (24 bp at the 5′-end and 25 bp at the 3′-end with four mismatched bases) that are themselves flanked by 8 bp AT-rich direct repeats (Fig. 3). TnpA exhibited 37% identity and 58% similarity with TnpA from IS*Ps1* (Bolognese *et al*., 1999). Based on these features, the transposon appears to belong to the ISL3 family (Mahillon and Chandler, 1998). The truncated ORF at the 3′-end of the insert exhibited 96% homology to the terminal half of *zeta*, a gene present on numerous Gram-positive plasmids including pDB101 from *S. pyogenes* (Ceglowski and Alonso, 1994). Immediately downstream of *zeta,* on pDB101, is a cluster of ORFs including *epsilon*, *delta* and *gamma*. To determine whether *emtA* is linked to the same ORFs in 27270TC1, we performed a polymerase chain reaction (PCR) analysis using chromosomal DNA and an *emtA*-specific primer in combination with primers specific to the 5′-ends of ORFs *zeta*, *epsilon*, *delta* and *gamma*. In all cases, we obtained PCR products of a size consistent with the gene arrangement seen in pDB101 (data not shown). By sequencing the region upstream of *emtA* in 27270TC1, we identified an ORF with 80% homology to *mob*, a gene located on a *Lactococcus lactis* plasmid (Wang and Macrina, 1995). Plasmid analysis revealed that strain 9631355 contained multiple plasmids; 27270RF was plasmid-free and 27270TC1 harboured a single plasmid (estimated using restriction analysis to be 40 kb) similar in size to a plasmid in strain 9631355 (data not shown). Southern blotting localized *emtA* to the large plasmid in 27270TC1 (data not shown) and treatment of strains 9631355 and 27270TC1 with novobiocin, an agent known to interfere with plasmid replication, cured evernimicin resistance (loss of *emtA* was confirmed by PCR analysis) at frequencies of 2% and 15% respectively.

EmtA preferentially methylates the 50S subunit of sensitive ribosomes

Sequences encoding a hexa-histidine tag were appended to the 3′-end of *emtA* and the modified gene was cloned into pET29a generating pPAM25. In HS294, an evernimicin-sensitive *Escherichia coli* strain (MIC 4 μg ml^{-1}), pPAM25 conferred IPTG-dependent, high-level evernimicin resistance. Growth of BL21(pPAM25) in

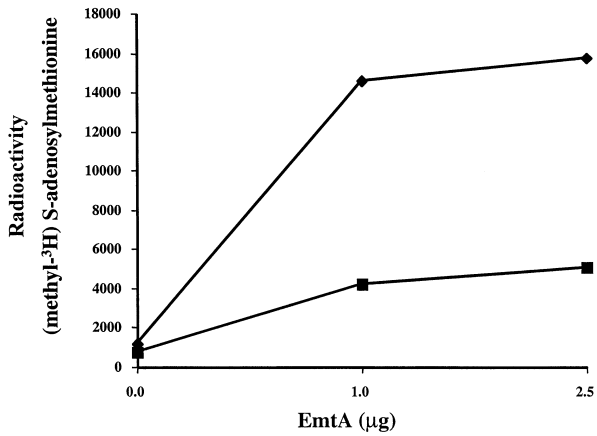

Fig. 4. Methylation of 70S ribosomes. Ribosomes, from isogenic recipient (evernimicin MIC 0.5 μg ml^{-1}; 27270RF, ◆) and transconjugant (evernimicin MIC > 256 μg ml^{-1}; 27270TC1, ■) strains were incubated with varying amounts of purified EmtA and [methyl-^{3}H]-S-adenosylmethionine. After 45 min at 37°C, the reactions were stopped by addition of a large excess of unlabelled SAM and unincorporated label removed by passage through a size exclusion column.

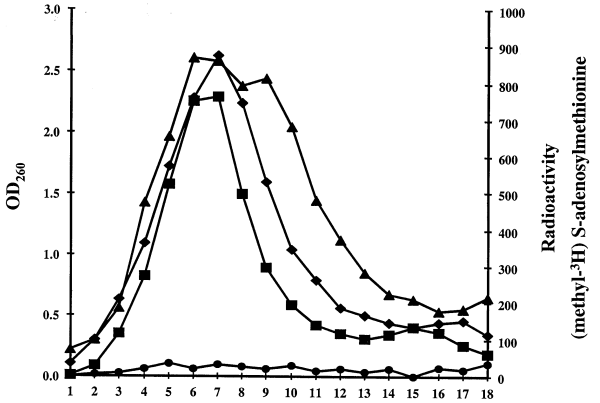

Fig. 5. Fractionation of *in vitro* methylated 50S ribosomal subunits. 50S subunits from recipient strain 27270RF (evernimicin MIC 0.5 μg ml^{-1}) and transconjugant strain 27270TC1 (evernimicin MIC > 256 μg ml^{-1}) were incubated with purified EmtA and [methyl-^{3}H]-S-adenosylmethionine as described in *Experimental procedures*. The reactions were resolved on sucrose gradients and for each fraction the optical density at 260 nm (27270RF, ■ and 27270TC1, ◆) and radioactivity (27270RF, ▲ and 27270TC1, ●) were determined.

the presence of IPTG did not result in detectable overexpression of EmtA. Nevertheless, EmtA was purified from cell lysates; the protein was 90% pure and migrated at the predicted molecular weight (data not shown). Methylation of intact ribosomes from isogenic donor and transconjugant strains, using purified EmtA and [methyl-^{3}H]-S-adenosylmethionine ([methyl-^{3}H]-SAM), resulted in less than twofold discrimination between evernimicin-resistant and -sensitive strain ribosomes (data not shown). Lowering the magnesium content of the reaction buffer from 10 to 1 mM increased overall labelling fourfold and also resulted in a modest (fourfold) discrimination between sensitive and resistant ribosomes (Fig. 4). Label incorporation was linear up to 30 min, after which no further increases were observed, and was abolished by either heat denaturation of EmtA (2 min at 100°C) or by increasing the proportion of unlabelled SAM in the reaction 100-fold (data not shown). Addition of evernimicin also inhibited methylation in a dose-dependent manner; maximal inhibition, 50% of the control value, occurred at an evernimicin concentration of 250 nM. Attempts to methylate rRNA extracted from evernimicin-sensitive ribosomes were unsuccessful. To identify which subunit was being methylated, we methylated 70S ribosomes and then resolved the individual ribosomal subunits on sucrose gradients. In reactions utilizing evernimicin-sensitive ribosomes, the majority of the radiolabel was associated with the 50S subunit and with a species that migrated slightly slower than the 50S subunit (data not shown). Again, evernimicin-resistant ribosomes were labelled fourfold less efficiently and the label was associated primarily with the 30S subunit (data not shown). We repeated the assay using purified ribosomal subunits (Fig. 5). Comparison of the amount of label associated with fraction #7, which corresponds to the centre of the 50S peak, revealed that subunits from a sensitive strain were labelled 30-fold more efficiently than those from a resistant strain. The 30S subunits from both resistant and sensitive strains were not methylated (data not shown). Approximately 1% of the evernimicin-sensitive 50S subunits were methylated and addition of increased amounts of EmtA to the reaction did not increase label incorporation significantly (data not shown). To determine whether *in vitro* methylation blocked evernimicin binding, we methylated 50S subunits using unlabelled SAM and then added either [^{14}C]-evernimicin or [^{14}C]-erythromycin at four times the measured Kd. Methylation had no effect on the binding of either drug (data not shown). Finally, to test the effect of magnesium on methylation, we methylated 50S subunits in buffer containing either 1 or 10 mM of magnesium; methylation was reduced approximately fourfold at the higher concentration.

The target site for methylation by EmtA overlaps the evernimicin binding site

To identify the target site for EmtA methylation, we performed primer extension on 23S rRNA extracted

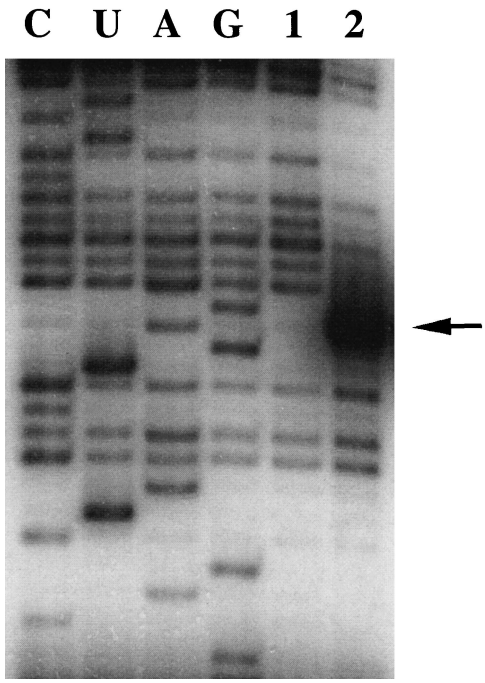

Fig. 6. Identification of the methylation site. 23S rRNA was extracted from ribosomes isolated from isogenic recipient (evernimicin MIC 0.5 μg ml^{-1}), 27270RF (1) and transconjugant (evernimicin MIC > 256 μg ml^{-1}), 27270TC1 (2) strains. Both batches of rRNA were reverse transcribed from primer #3 (see *Experimental procedures*) and the products resolved on denaturing polyacrylamide gels. A sequencing ladder, utilizing the same primer as above and 23S rRNA from strain 27270RF, was run on the same gel. An arrow indicates the pause site.

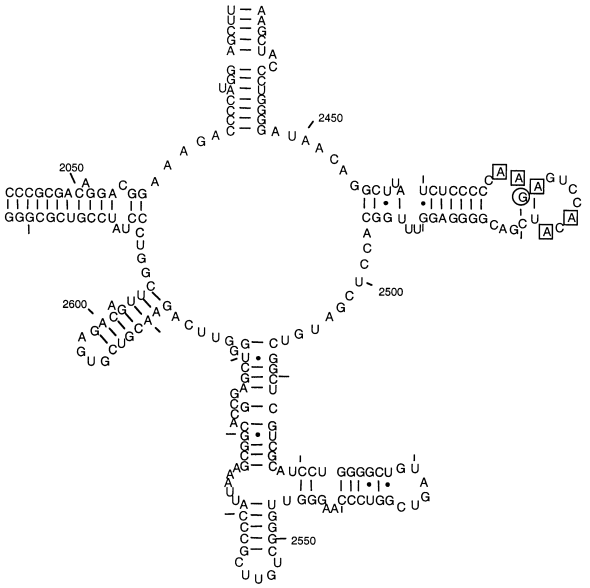

Fig. 7. Location of the sites of EmtA methylation and evernimicin binding. The peptidyl transferase loop (domain V) from *E. faecium* 27270 is diagrammatically represented. Residues protected by evernimicin from modification by dimethyl sulphate are boxed and the residue methylated by EmtA is circled.

from ribosomes from isogenic evernimicin-sensitive and -resistant strains. We identified a single pause site, unique to rRNA isolated from resistant ribosomes (Fig. 6), that corresponded to methylation of guanine 2470 in helix 89 (*E. coli* numbering, Fig. 7). We used two additional primers that annealed to different sites to confirm this data (data not shown). Evernimicin was footprinted previously to helices 89 and 91 in ribosomes from *E. coli* and *H. halobium* (Belova *et al*., 2001). We repeated this analysis using *E. faecium* ribosomes; here we scanned nucleotides 2400–2650 only. When bound to sensitive ribosomes, the drug protected a number of adenine residues flanking the proposed methylation site (Fig. 6). In contrast, we saw no protection when using evernimicin-resistant ribosomes (data not shown).

Discussion

Prior to this study, evernimicin resistance had been associated solely with mutations in genes encoding ribosomal components, specifically *rplP* and the 23S rDNA. In this study, we characterized an *E. faecium* strain capable of mediating transfer of high-level evernimicin resistance. Probable resistance mechanisms included drug efflux, drug modification and ribosome modification. To differentiate between these possibilities, we examined the interaction of evernimicin with ribosomes isolated from the resistant strain. The ribosomes exhibited a dramatic reduction in evernimicin binding and were refractory to inhibition by evernimicin in cell-free translation assays, suggesting that they had been modified in some manner. We cloned the gene responsible and demonstrated that it was functional in *E. coli*, suggesting the target site for modification is conserved between both Gram-positive and -negative organisms. Sequence analysis indicated that the resistance gene encoded a protein with a SAM-binding site. Therefore it was designated *emtA* for *e*vernimicin *m*ethyl*t*ransferase.

Initial attempts to demonstrate methyltransferase activity, using purified EmtA, yielded low levels of methylation and no discrimination between ribosomes from sensitive and resistant strains. Reducing the magnesium content of the reaction buffer increased overall levels of methylation and resulted in sensitive ribosomes being methylated between three- and fourfold more efficiently than resistant ribosomes. This discrimination was increased to 30-fold when the substrate for methylation was purified 50S subunits. Thus, it would appear that the *in vitro* methylation reaction accurately mimics *in vivo* methylation in terms of site specificity. The requirement for low magnesium may either be a physical characteristic of the enzyme or may stem from the fact that 70S ribosomes dissociate under low magnesium conditions that, in turn, may unmask a previously hidden site of methylation. Methylation reactions using purified 50S subunits exhibited a similar magnesium dependency suggesting that the effect is a characteristic of the enzyme (see below).

Only 1% of the purified 50S subunits were methylated by EmtA *in vitro* and therefore it was not surprising that methylation did not block evernimicin binding. One explanation for the low levels of methylation is that EmtA is only partially functional. This may be a result of his-tagging the enzyme or due to damage sustained during purification. However, this seems unlikely as adding more EmtA to a reaction did not result in a significant increase in methylation. Alternatively, the intact 50S subunit may not be the preferred substrate for EmtA. In the sucrose fractionation experiments using both intact ribosomes and purified subunits, the labelled SAM was associated with both the 50S subunit and with a species that migrated slightly more slowly. It is conceivable that this species, which may represent a 50S precursor particle, is the preferred substrate for EmtA and, as such, may be present in low amounts in our ribosome preparations. Preliminary data suggested that the ErmC methylase recognizes and interacts with a similar precursor particle (Champney, 2001). Furthermore, it has been postulated that evernimicin interacts with a 50S assembly intermediate and in some manner blocks ribosome assembly (Champney and Tober, 2000).

A single pause site in the 23S rRNA, that was unique to rRNA isolated from resistant ribosomes, was identified by primer extension. The pause site corresponded to methylation of G2470. A number of lines of evidence strongly suggest that the evernimicin binding site overlaps

G2470. Footprinting of evernimicin, bound to ribosomes isolated from *E. coli*, *H. halobium* (Belova *et al*., 2001) and *E. faecium* (this study) identified rRNA contact points in the same hairpin. Similarly, mutations that confer evernimicin resistance in *S. pneumoniae* and *H. halobium* were also mapped to this hairpin; the nearest nucleotide substitution was located at residue 2471 (Adrian *et al*., 2000b; Belova *et al*., 2001). Finally, addition of evernimicin to the methylation reactions inhibited methylation of ribosomes in a dose-dependent manner.

The origin of *emtA* is intriguing; EmtA had no significant homology with the Erm family of methylases and the best match from public databases was an archeal protein with a putative methyltransferase function. Recently, a large gene cluster encoding enzymes required for evernimicin biosynthesis in *Micromonospora carbonacea* was sequenced (unpublished data). EmtA exhibited 25% identity and 42% similarity with EvrH, an ORF of unknown function from this cluster. Finally, two rRNA methyltransferases, AviRa and AviRb, that conferred resistance to avilamycin, were recently cloned from the avilamycin producer *Streptomyces viridochromogenes* (Weitnauer *et al*., 2001). AviRa had no homology to proteins in the data bases and AviRb resembled the SpoU family of methyltransferases. However, despite the strong structural similarity between avilamycin and evernimicin, neither protein exhibited significant homology to either EmtA or EvrH.

Experimental procedures

Bacterial strains and growth conditions

Strains and plasmids are listed in Table 1. Evernimicin was isolated at Schering-Plough Research Institute and solubilized as the clinical formulation at 16 μg ml^{-1}. Avilamycin was a gift from Elanco Animal Health; all other antibiotics were purchased from Sigma Chemical Co. MIC determinations were performed using tryptic soy broth, antibiotics were added in doubling dilutions and the MIC recorded as the lowest antibiotic concentration that inhibited growth after 16 h at 37°C.

Genetic techniques

Spontaneous mutants of *E. faecium* strain ATCC 27270, resistant to either streptomycin (27270S) or to both fusidic acid and rifampicin (27270RF), were selected on the appropriate plates. For liquid matings, equal volumes of fresh overnight cultures (grown in brain–heart infusion medium) of donor and recipient strains were mixed, incubated at 37°C for 4 h and plated on selective media. Arbitrarily primed polymerase chain reaction (PCR), using p38 (5′-CGGTGGCGAA-3′) and BOX (5′-CTACGGCAAGGC GACGCTGACG-3′), was used to confirm the identity of transconjugants. Novobiocin curing of plasmids was performed as described (McHugh and Schwartz, 1977).

Manipulation and sequencing of DNA

DNA manipulations were carried out according to standard procedures (Sambrook *et al*., 1989). DNA sequencing was performed using an ABI Prism Big Dye Terminator Cycle Sequencing Kit and the products resolved using a 310 Genetic Analyser. Transformation (Shepard and Gilmore, 1995) and extraction of plasmid DNA from *E. faecium* were performed as described (Woodford *et al*., 1993).

Cloning of emtA

Total DNA was extracted from *E. faecium* 27270TC1 by incubating cells in 0.5% of SDS/0.1 mg ml^{-1} proteinase K for

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Origin	Relevant genotype or phenotype[a]	Source or reference
E. faecium			
ATCC 27270		Wild-type strain	Laboratory stock
9508106		Animal isolate Av^r Ev^r	Aarestrup *et al*. (1998a)
9631355		Animal isolate Av^r Ev^r	Aarestrup *et al*. (1998a)
27270RF	ATCC 27270	Rif^r Fus^r	This study
27270S	ATCC 27270	Str^r	This study
27270TC1	Conjugation (9631355 × 27270RF)	Rif^r Fus^r Ev^r	This study
E. coli strains			
XL Blue 1		*recA1*	Laboratory stock
BL21 (λDE3)		λ lysogen carrying T7 polymerase	Laboratory stock
HS294		*lacZ*::T7 polymerase, highly drug-susceptible strain	Laboratory stock
Plasmids			
pLI51		Shuttle vector Ap^r Cm^r	Lee *et al*. (1987)
pPAM19	pLI51	Ev^r Ap^r Cm^r	This study
pPAM21	pPAM19	Deletion of the *Sph*I fragment Ev^r Ap^r Cm^r	This study
pET29a		T7 based over expression plasmid Kan^r	Novagen
pPAM25	pET29a	*emtA* with 6 histidine codons at 3′-end Kan^r Ev^r	This study

a. Ap^r, ampicillin-resistant; Av^r, avilamycin-resistant; Ev^r, evernimicin-resistant; Cm^r, chloramphenicol-resistant; Fus^r, fusidic acid-resistant; Kan^r, kanamycin-resistant; Str^r, streptomycin-resistant.

1 h at 37°C. After phenol–chloroform extraction, the DNA was precipitated with ethanol. *Sau*3A fragments (size range of 3–5 kb) were ligated into the *Bam*H1 site of pLI51. The library was amplified via passage through *E. coli* XL1-Blue. Twofold coverage of the insert in pPAM19 was performed by the Midland Certified Reagent Co. (GenBank accession number AF403298).

Ribosome isolation and cell free translation reactions

Cells were lysed by passage through a microfluidizer and ribosomes purified as described previously (Staehlin and Marglott, 1971). Ribosomes and S100 extracts were resuspended and dialysed, respectively, against B3 (10 mM of $MgCl_2$, 20 mM of Tris (pH 7.8), 30 mM of NH_4Cl, 0.1 mM of EDTA and 6 mM of β-mercaptoethanol). Ribosomal subunits were obtained by fractionating ribosomes on 10–30% sucrose gradients formed in B4 (B3 with 1 mM of $MgCl_2$ and 100 mM of NH_4Cl), subunit-containing fractions were washed and concentrated using Microcon 30 filter devices (Amicon). Cell free translation reactions using a polyA template and [^{3}H]-lysine (specific activity 89 Ci mmol^{-1}, NEN) were performed as described (Mahmood *et al*., 1991) and labelled proteins quantified by liquid scintillation counting (McNicholas *et al*., 2000).

Binding assays

Binding reactions using [^{14}C]-evernimicin (specific activity 8 mCi mmol^{-1}, Hesk *et al*., 1999) and [^{14}C]-erythromycin (specific activity 55 mCi mmol^{-1}, NEN) were performed as described previously (McNicholas *et al*., 2000).

Purification of EmtA

emtA was PCR amplified with the following primers to append six histidine codons to the 3′-end of the gene (5′-CA TATGACTCTTTATTTTGCGAGCG-3′ and 5′-GGGATCCTT AGTGGTGGTGGTGGTGGTGCTTCCTTTTAAGTCCATC T-3′) and cloned into pET29a-generating pPAM25. EmtA was purified from BL21(pPAM25) as follows: after resuspension in lysis buffer (50 mM of Na_2HPO_4 (pH 8.0), 300 mM of NaCl, 10 mM of imidazole, 0.1% of β-mercaptoethanol, 10 μg ml^{-1} of lysozyme, 0.1 M of PMSF, 0.1% of Triton X-100 and 10% of glycerol), cells were disrupted in a microfluidizer. Cleared lysates, obtained by centrifugation at 14 000 *g* for 15 min, were incubated with Qiagen's Ni-NTA slurry. After washing and elution (according to the manufacturer's protocol, Qiagen), EmtA was dialysed against 50 mM of Tris-HCl (pH 7.4), 40 mM of KCl, 4 mM of $MgCl_2$, 10 mM of DTT and 10% of glycerol.

In vitro *methylation assays*

Methylase reactions (50 μl) were performed in buffer C (50 mM of Tris-HCl [pH 7.4], 40 mM of KCl, 10 mM DTT and either 10 or 1 mM of $MgCl_2$) containing 20 mM of SAM, 0.5 μM of methyl [^{3}H]-SAM (specific activity 85 Ci mmol^{-1}, NEN), 5 U of RNasin (Promega) and 12 pmoles of either 70S ribosomes or rRNA (phenol extracted from 70S ribosomes). Reactions were incubated for 45 min at 37°C and then chased for 15 min with 200 mM of SAM. Labelled ribosomes were separated from unincorporated label by centrifugation through size exclusion columns (McNicholas *et al*., 2000) or by fractionation on 10–30% sucrose gradients.

Primer extension and footprinting assays

Primer extension, using primers designed to amplify the entire 23S rRNA in 150 bp increments, was performed as described by Stern *et al*. (1988). Footprinting of evernimicin, bound to ribosomes using dimethyl sulphate, was performed as described by Merryman and Noller (1988) using primers #1–3 (5′-CGGCAGATAGGGACCGA-3', 5′-CAGCCCCAG GATG-3' and 5′-CAGCCCCAGGATG-3').

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