RNA (1995), *1*:501-509. Cambridge University Press. Printed in the USA.

The conformation of 23S rRNA nucleotide A2058 determines its recognition by the ErmE methyltransferase

BIRTE VESTER,[1,2] LYKKE HAASTRUP HANSEN,[1] and STEPHEN DOUTHWAITE[1]

[1] Department of Molecular Biology, Odense University, DK-5230 Odense M, Denmark
[2] Institute of Molecular Biology, University of Copenhagen, DK-1307 Copenhagen K, Denmark

ABSTRACT

The ErmE methyltransferase confers resistance to MLS antibiotics by specifically dimethylating adenine 2058 (A2058, *Escherichia coli* numbering) in bacterial 23S rRNA. To define nucleotides in the rRNA that are part of the motif recognized by ErmE, we investigated both in vivo and in vitro the effects of mutations around position A2058 on methylation. Mutagenizing A2058 (to G or U) completely abolishes methylation of 23S rRNA by ErmE. No methylation occurred at other sites in the rRNA, demonstrating the fidelity of ErmE for A2058. Breaking the neighboring G2057–C2611 Watson–Crick base pair by introducing either an A2057 or a U2611 mutation, greatly reduces the rate of methylation at A2058. Methylation remains impaired after these mutations have been combined to create a new A2057–U2611 Watson–Crick base interaction. The conformation of this region in 23S rRNA was probed with chemical reagents and it was shown that the A2057 and U2611 mutations alone and in combination alter the reactivity of A2058 and adjacent bases. However, mutagenizing position G → A2032 in an adjacent loop, which has been implicated to interact with A2058, alters neither the ErmE methylation at A2058 nor the accessibility of this region to the chemical reagents. The data indicate that a less-exposed conformation at A2058 leads to reduction in methylation by ErmE. Nucleotide G2057 and its interaction with C2611 maintain the conformation at A2058, and are thus important in forming the structural motif that is recognized by the ErmE methyltransferase.

Keywords: ErmE methyltransferase; MLS antibiotic resistance; modified bases; rRNA structure; rRNA–protein interaction

INTRODUCTION

The ErmE methyltransferase from the Actinomycete *Saccharopolyspora erythraea* modifies 23S rRNA and thereby confers resistance to MLS (macrolide, lincosamide, and streptogramin B) antibiotics (Graham & Weisblum, 1979). *S. erythraea* is the producer of the macrolide antibiotic erythromycin (McGuire et al., 1952; Labeda, 1987), and this organism therefore modifies its rRNA to prevents its own growth from being inhibited by the drug. The modification by ErmE is a highly specific N6,N6-dimethylation of nucleotide A2058 (*Escherichia coli* 23S rRNA numbering) (Skinner & Cundliffe, 1982; Skinner et al., 1983), which is located in the peptidyl transferase loop within domain V of 23S rRNA (Noller, 1984). Erythromycin and other MLS antibiotics have been shown to interact with A2058 and neighboring bases in the loop (Moazed & Noller, 1987; Egebjerg & Garrett, 1991; Douthwaite, 1992a). Mutations at these bases confer antibiotic resistance (Vester & Garrett, 1987; Sigmund et al., 1988) by reducing the strength of the interaction between the drug and the rRNA (Douthwaite & Aagaard, 1993). Modification of the rRNA by ErmE probably confers resistance by the same mechanism.

ErmE has been cloned (Thompson et al., 1982; Bibb et al., 1985) and sequenced (Uchiyama & Weisblum, 1985; Dhillon & Leadlay, 1990), and has been expressed and isolated in a purified, active form from *S. erythraea* (Skinner & Cundliffe, 1982) and from *E. coli* (Vester & Douthwaite, 1994). In vitro studies showed that the enzyme acts on naked rRNA but not on rRNA that has been assembled into ribosomes (Skinner et al.,

Reprint requests to: Stephen Douthwaite, Department of Molecular Biology, Odense University, DK-5230 Odense M, Denmark; e-mail: srd@mol-bio.ou.dk.

1983). All the determinants for recognition are therefore within the rRNA and these are probably occluded in ribosomes by the r-proteins. Recently, it was shown that ErmE methylates a 625-nt domain V RNA fragment as effectively as intact 23S rRNA (Vester & Douthwaite, 1994). It was furthermore shown that methylation at A2058 is independent of magnesium ions, suggesting that the tertiary structure of the rRNA is not required for ErmE interaction, and that the enzyme recognizes a motif in the primary and secondary structures of the RNA. The helical structure immediately adjacent to A2058 (Fig. 1) is possibly the major recognition determinant for the ErmE interaction.

To test this idea, we investigated point mutations in *E. coli* 23S rRNA at A2058 and adjacent nucleotides. We chose to study mutations in the rRNA that have been shown previously to affect the tolerance of cells to erythromycin, probably by altering the conformation around A2058 in ribosomes (Douthwaite & Aagaard, 1993). Mutant 23S rRNAs were expressed from plasmid systems in *E. coli*. Methylation of mutant 23S rRNAs by ErmE was followed both in vivo and in vitro by reverse transcription from allele-specific priming sites in the rRNA. The effects of the mutations on the accessibility of this region were investigated by probing naked 23S rRNA with chemical reagents. We find a correlation between the conformation of A2058 and its methylation by ErmE.

RESULTS

ErmE expression in vivo

The ErmE methyltransferase was expressed from plasmids pREK47 and pREK48 in growing *E. coli* cells. The degree of methylation at A2058 in 23S rRNA was measured by primer extension from the 2060 primer (Fig. 2).

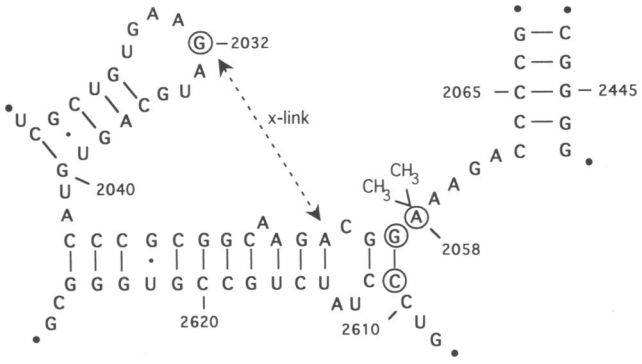

FIGURE 1. Secondary structure adjacent to the ErmE methylation site at A2058 in *E. coli* 23S RNA. The nucleotide positions at which point mutations were introduced (2032, 2057, 2058, and 2611) are circled. In the secondary structure (Noller, 1984), nucleotides 2057, 2058, and 2611 are at the peptidyl transferase loop. The proximity of G2032 to these nucleotides is suggested by a crosslink (Döring, et al., 1991).

Expression of ErmE from the single copy pREK47 plasmid under noninducing conditions methylated only 3% of 23S rRNA molecules. However, the enzyme was substantially induced either by amplifying the plasmid copy number at 42 °C, or by addition of IPTG to derepress the *lac* promoter. ErmE expression from plasmid pREK48 was markedly higher under noninducing conditions, methylating 64% of the rRNA, and expression was only slightly increased by addition of IPTG (Fig. 2). This suggests that cells possess insufficient *lac* repressor to shut down effectively the *lac* promoter on pREK48.

Expression of ErmE in vivo confers high levels of MLS drug resistance in *E. coli*, and this resistance is considerably greater than that conferred by the rRNA mutations. Because ErmE methylates naked 23S rRNA and not subunits or ribosomes (Skinner et al., 1983), methylation in cells must occur before ribosomes are fully assembled.

In vivo ErmE methylation of mutant 23S rRNA

23S rRNAs with mutations at or close to the ErmE target base, A2058, were expressed from pFK plasmids in *E. coli* cells (Table 1). Cells transformed with pFK plasmids contain a mixture of the plasmid-coded 23S rRNA

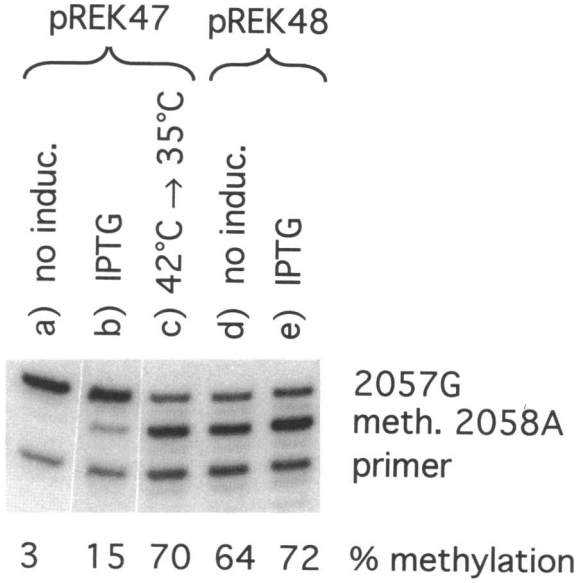

FIGURE 2. In vivo ErmE methylation of wild-type 23S rRNA. 23S rRNA was isolated from cells containing plasmid pREK47 or pREK48. The autoradiogram shows reverse transcriptase extension from the 2060 primer, which terminates before position A2058 in methylated rRNA (meth. 2058A) or by incorporation of ddCTP at G2057 in unmethylated rRNA. The rRNA was extracted from cells in which *ermE* was induced with 0.5 mM IPTG (lanes b and e) or by heating to 42 °C for 45 min and then returning to 35 °C for 120 min (lane c). The constitutive expression of *ermE* from the two plasmids is indicated in lanes a and d (no induction). The degree of methylation for each lane was determined by densitometer scanning of the relative intensities of the A2058 methylation band and the G2057 band.

TABLE 1. Plasmids encoding mutant 23S rRNA.[a]

Plasmid	Mutations[b] in 23S rRNA	Phenotype	Methylation[c]	References[d]
pFK1	None	Ery^S	++	–
pFK2	G2057 → A	Ery^R	+	Ettayebi et al. (1985)
pFK3	A2058 → U	Ery^R	–	Sigmund et al. (1984)
pFK5	A2058 → G	Ery^R	–	Vester and Garrett (1987)
pFK6	G2032 → A	Ery^{HS}	++	Douthwaite (1992b)
pFK10	C2611 → U	Ery^R	+	Vannuffel et al. (1992)
pFK11	G2057 → A +C2611 → U	Ery^r	+	This study

[a] All plasmids are constructed from, and are similar in structure to, pKK3535 (Brosius et al., 1981), which is a pBR322-derivative (Bolivar et al., 1977) containing the *E. coli rrn*B operon.
[b] In addition to the mutations listed here, each of the pFK plasmids has the specific priming site at 23S rRNA position 2140.
[c] Visual estimations of the in vivo methylation at 2058 using the 2140 primer: ++, strongly methylated by ErmE methyltransferase; +, reduced methylation; –, no methylation. The relative amounts of methylation (+ and ++) were accurately quantified in the in vitro experiments.
[d] Original references for the erythromycin phenotypes. Ery^S, erythromycin sensitive; Ery^R, resistant; Ery^{HS}, hypersensitive; Ery^r, slightly resistant.

and wild-type 23S rRNA encoded by the chromosome. The two types of molecule were distinguished by allele-specific priming. The sequence around position 2140 was altered in plasmid-coded 23S rRNA, allowing specific analysis of chromosome- or plasmid-coded rRNAs by reverse transcriptase extensions from oligonucleotide primers complementary to the different sequences at 2140 (Aagaard et al., 1991).

Total rRNA was isolated from cells co-transformed with a pREK plasmid and a pFK plasmid. The rRNAs were analyzed with chromosome- or plasmid-specific 2140 primers. Extension with reverse transcriptase stops at N6,N6-dimethylated adenine (Zalacain & Cundliffe, 1989), resulting in a clear autoradiogram band corresponding to ErmE methylated A2058. Changing this target base to a uracil (in pFK3) or a guanine (in pFK5) completely abolished ErmE methylation (Table 1). Methylation by ErmE was greatly reduced, but remained detectable, after breaking the Watson–Crick base pair adjacent to A2058 by either a G → A2057 mutation (pFK2) or a C → U2611 mutation (pFK10). Methylation remained reduced after the mutations were combined in pFK11 to give an alternative A2057–U2611 pair. Mutagenesis of G → A2032 in the nearby hairpin loop (Fig. 1) had no effect on ErmE methylation at A2058.

In vitro methylation of mutant 23S rRNAs

The ErmE methyltransferase was isolated in concentrated active form from *E. coli* cells containing heat-amplified plasmid pREK47 (Vester & Douthwaite, 1994). The conditions for ErmE methylation can be readily controlled in vitro, allowing a more precise comparison of the effects of the different 23S rRNA mutations than can be obtained in vivo. Unmethylated rRNA was extracted from cells containing the pFK plasmids (Table 1). The rRNAs were incubated in vitro for up to 90 min with a relatively small amount of ErmE in order to follow the methylation kinetics. Methylation was analyzed by reverse transcription from the 2140 and 2060 primers. The in vitro data confirmed the findings of the in vivo studies: mutagenizing A2058 abolishes ErmE methylation; A2057 and/or U2611 substantially reduce the degree of methylation; and A2032 has no effect. The presence of the mutant 23S rRNAs did not detectably influence the methylation of the wild-type 23S rRNA. Analyses of the A2057 and U2611 mutant rRNAs from the 2140 primers are shown in Figure 3 (data for the 2032 and 2058 mutations are not shown). rRNAs encoded by the chromosome or by plasmid pFK1 (which has no mutations in the 23S rRNA ErmE target region) were methylated to identical levels, showing that the allele-specific priming sites at position 2140 had no effect on ErmE activity. The rRNAs were screened throughout domain V with additional primers. No sites of artefactual methylation were found under the conditions used here (data not shown).

In the case of the 23S rRNA A2057 mutants, methylation in vitro was accurately quantified by extension from the 2060 primer. The 2060 primer is extended by reverse transcriptase on both chromosome- and plasmid-coded rRNAs, and terminates either at A2059 (because of the A2058 methylation) or, if the rRNA is unmethylated, on incorporation of ddCTP at the next guanine. Thus, transcription stops at G2057 on wild-type rRNA, or at G2056 on rRNA with the A2057 mutation (Fig. 4). The relative rates of methylation of wild-type and A2057 mutant rRNAs were measured by densitometer scanning of these autoradiogram bands intensities, as shown in Figure 5. The methylation of 23S rRNA with the A2057 mutation (and with A2057 plus U2611) was approximately one fifth that of wild-type rRNA.

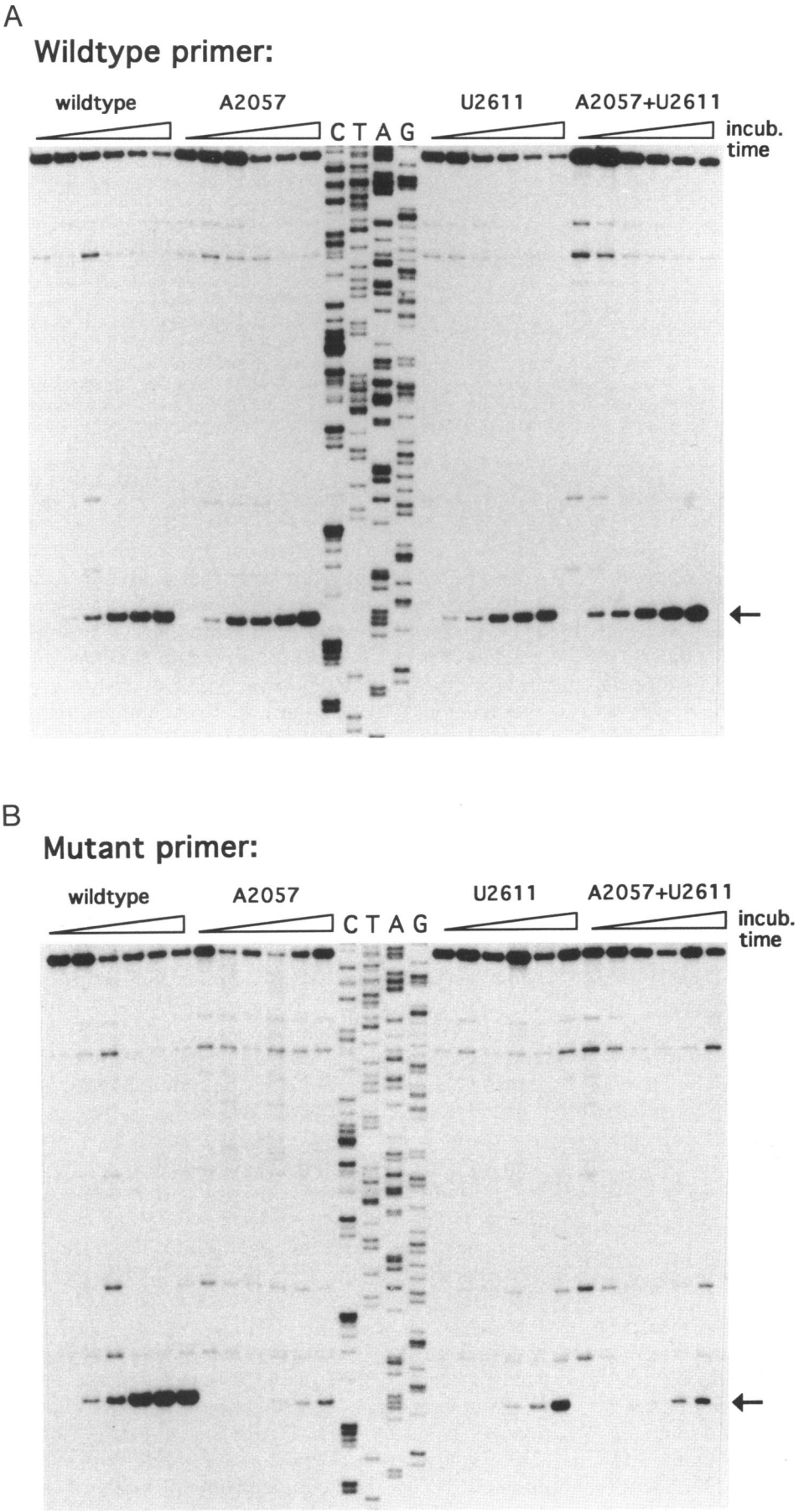

FIGURE 3. Allele-specific primer extensions to compare the relative methylation of wild-type and mutant rRNAs. rRNAs were isolated from cells containing plasmid pFK1 ("wild type"), pFK2 (A2057), pFK10 (U2611), or pFK11 (A2057+U2611). The 23S rRNAs from these cells are heterogeneous mixtures of wild-type (chromosome-coded) and mutant (plasmid-coded) molecules. The rRNAs were methylated in vitro for 0, 3, 6, 12, 40, and 90 min, as indicated by the increasing incubation time above the gel lanes. 23S rRNAs were then analyzed by primer extension from nucleotide position 2140 using a primer specific for chromosome-coded RNA (**A:** wild-type primer), or for plasmid-coded RNA (**B:** mutant primer). Arrow indicates the stop at A2059 caused by methylation at A2058. The band at the top of the gel represents a break at the weak phosphodiester bond between U1915 and A1916. Sequence lanes C, T, A, and G are dideoxy sequences performed on a single-stranded M13 bacteriophage template encoding the 23S rRNA gene.

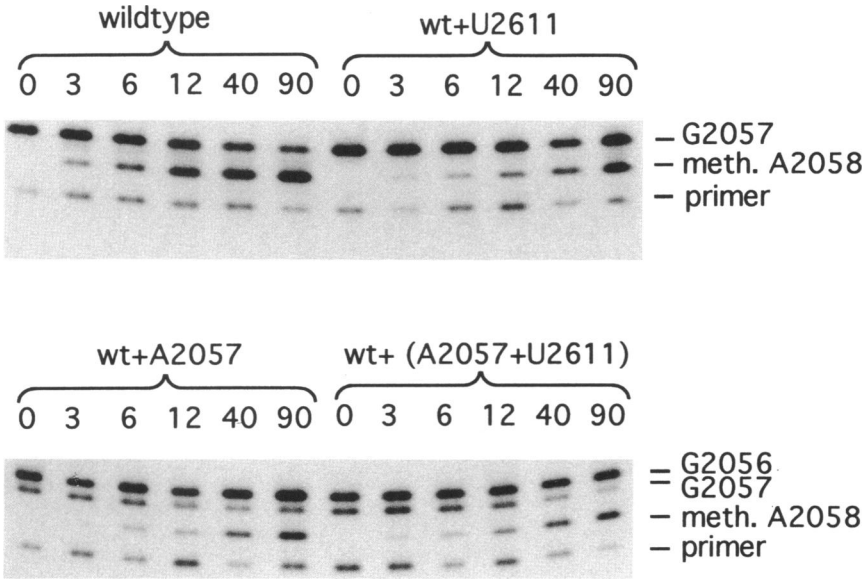

FIGURE 4. In vitro methylation of wild-type and mutant rRNAs analyzed from the 2060 primer. 23S rRNAs are mixtures of chromosome-coded, wild-type (wt) molecules and plasmid-coded molecules. These were isolated from cells containing plasmid pFK1 (wild type), pFK10 (wt+U2611), pFK2 (wt+A2057), or pFK11 (wt+A2057+U2611). Methylation reactions with ErmE were carried out for the times (in min) indicated above each lane. Reverse transcriptase extension from the 2060 primer with dTTP and ddCTP results in termination either at A2059, directly before methylated nucleotide A2058 (meth. A2058), or at the next guanosine. Transcription therefore terminates at G2057 in wild-type rRNA encoded by the chromosome, and in rRNA encoded by pFK1 and pFK10. Transcription on rRNA with the A2057 mutation (from pFK2 and pFK11) terminates at G2056. The different rates of disappearance of the G2056 and G2057 bands thus reflect differences in rates of methylation of the A2057 mutant and wild-type rRNAs.

Structural analysis of 23S rRNAs by chemical probing

The target base for ErmE at A2058 lies within the peptidyl transferase region of 23S rRNA (Fig. 1). This region has a well-defined and phylogenetically conserved secondary structure (Gutell et al., 1994). To determine the extent to which the mutations perturb this structure, we probed mutant 23S rRNAs with the chemical reagents DMS (which reacts with unpaired adenines and cytosines) and kethoxal (which reacts with unpaired guanines). Accessible bases in the rRNAs were analyzed by allele-specific primer extension from position 2140 with the wild-type primer (for chromosome-coded rRNA) and the mutant primer (for plasmid-coded rRNA) as shown in Figure 6. The wild-type rRNA encoded by the chromosome and by pFK1 produced the same DMS and kethoxal modification patterns. In the peptidyl transferase loop of wild-type rRNA, the relative degrees of accessibility to DMS are A2062 > A2058 > A2059 > A2060. G2061 is highly accessible to kethoxal. Nucleotides in the helix from 2043 to 2057 (most notably, C2055) are moderately accessible to DMS and kethoxal modification.

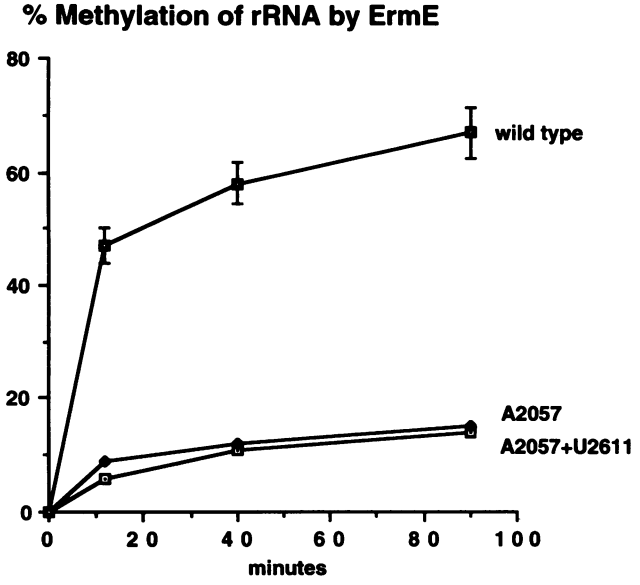

FIGURE 5. Kinetics of in vitro methylation of wild-type and mutant 23S rRNAs by ErmE. Methylation of the rRNAs were measured by densitometric analysis of autoradiograms (see Fig. 4). Methylation rates of the rRNAs with the A2057 mutation were measured directly by disappearance of the G2056 band; wild-type rRNA methylation was measured by disappearance of the G2057 band. Methylation of the A2057 and the A2057+U2611 mutant rRNAs were 22% and 21% that of wild-type rRNA, respectively (after 90 min incubation under the conditions described in the Materials and methods). The means and standard errors of three experiments are shown.

On mutagenesis of the rRNA at 2057 or 2611, G2061 remains highly accessible, whereas the reactivities at both A2058 and A2062 are reduced (Fig. 6). The G to A change at 2057 additionally renders this position reactive to DMS. The accessibility of A2057 is not reduced by the compensatory change U2611. Despite this, there are good indications that positions 2057 and 2611 interact. First, the C→U2611 change increases the kethoxal modification at G2057; and second, the reduction in the kethoxal modification at G2056 suggests that the G2056–C2612 interaction is stabilized by A2057–U2611. At the small internal loop adjacent to these base pairs, the accessibility of C2055 is markedly reduced by the 2057 and 2611 changes. Further structural rearrangement within this helix is suggested by the shift in accessibility from G2053 to A2052 on introduction of the U2611 mutation.

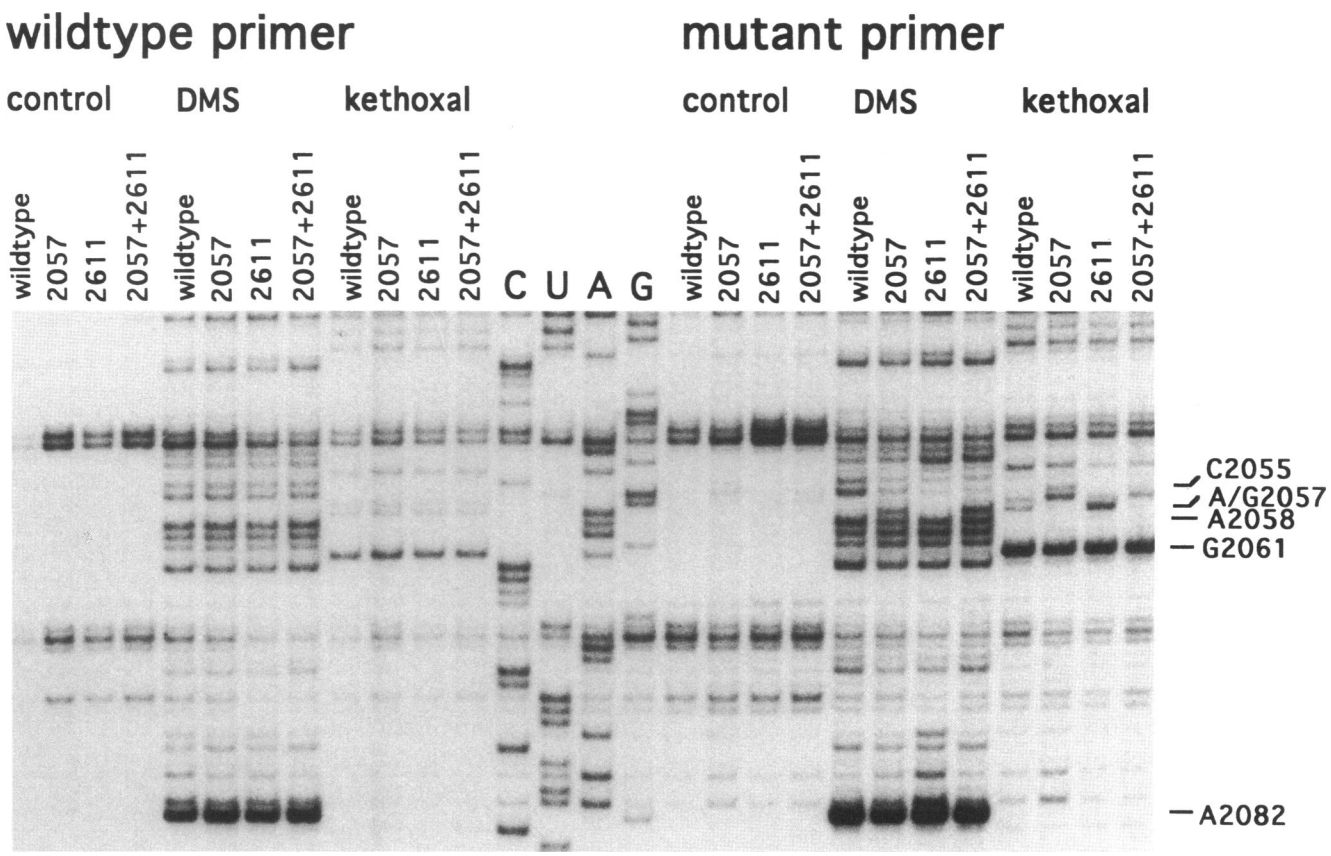

FIGURE 6. Structural analysis of the peptidyl transferase loop in naked 23S rRNA. rRNAs were isolated from plasmid-containing cells as indicated by the individual lane headings: wild type, plasmid pFK1; 2057, pFK2; 2611, pFK10; 2057+2611, pFK11. Renatured rRNAs were modified with DMS or kethoxal (control samples were unmodified) prior to allele-specific primer extension from the 2140 region with reverse transcriptase. Extension reactions on the left of the sequence lanes (C, U, A, G) were with a primer specific for the chromosome-coded wild-type rRNA; extensions on the right were with a primer specific for plasmid-coded rRNA (mutant primer).

DISCUSSION

Methylation by ErmE is specific for base A2058 in domain V of 23S rRNA. ErmE methylates this same position in phylogenetically distant bacterial 23S rRNAs (Skinner et al., 1983), indicating that it recognizes a distinct and conserved motif in the rRNA. We investigated A2058 and neighboring bases that have been implicated as having structural and functional connections with A2058 (Table 1), and that are thus possibly part of the ErmE recognition motif. Mutations at these bases affect the tolerance of cells toward erythromycin (Table 1). Disrupting the end of the RNA helix adjacent to A2058, by changing either A2057 or U2611, confers erythromycin resistance, as previously reported (Ettayebi et al., 1985; Vannuffel et al., 1992). Combining these bases to form a new Watson–Crick base pair reduces the level of erythromycin resistance, although cells remain more tolerant to the drug than wild-type cells.

We found it advantageous to study the effects of rRNA mutations on ErmE methylation both in vivo and in vitro. Studies in vivo avoid the problems of artefactual conformations that can be imposed on RNA during its isolation for in vitro studies (Uhlenbeck, 1995). On the other hand, in systems such as this, with readily purifiable RNA and protein components, the reaction kinetics can be more easily controlled and observed during in vitro studies. We found that ErmE efficiently methylates *E. coli* 23S rRNA both in vivo and in vitro (Figs. 2, 4). In addition, the rRNA mutations had similar effects on in vivo and in vitro methylation. It thus seems unlikely that the isolation procedures significantly affected the rRNA (or the methyltransferase), and we therefore chose in vitro systems to follow the reaction kinetics.

Mutagenesis of the target base produced the predictable result that ErmE methylation is abolished. Both of the mutant bases inserted at position 2058 (guanine and uracil) lack a correctly positioned exocyclic amine group that can be methylated by the enzyme. It has previously been shown, using radioactive S-adenosyl methionine, that ErmE exclusively methylates A2058 in wild-type rRNA (Skinner et al., 1983). On mutagen-

esis of 2058, none of the adjacent adenines nor any other base within domain V become modified by the enzyme. The lack of methylation at other sites emphasizes the high fidelity of ErmE for nucleotide A2058.

Altering the G2057–C2611 base pair next to A2058 in the rRNA secondary structure markedly reduced the methylation by ErmE. Breaking the base pair by introduction of either the A2057 or the U2611 mutation lowered the methylation rate by about the same degree (Fig. 3). The methylation kinetics were followed quantitatively for the A2057 mutant rRNA, and for A2057 in combination with U2611 (Fig. 5). The lower methylation by ErmE correlates with a change in the relative accessibilities of adenines in the peptidyl transferase loop, with A2058 becoming less exposed (Fig. 6). Structural rearrangements within the adjacent helix, particularly at C2055, are also evident. The G2057–C2611 base pair is thus important in maintaining the wild-type rRNA conformation at and adjacent to nucleotide A2058.

Nucleotide G2032 has been placed close to the peptidyl transferase loop in ribosomes by crosslinking (Fig. 1; Döring et al., 1991) and genetic studies (Douthwaite, 1992b). Transition of G to A at position 2032 confers resistance to lincomycin antibiotics (Cseplö et al., 1988), but hypersensitivity to erythromycin (Douthwaite, 1992b). Chemical probing of ribosomes with A2032 revealed no structural changes around the A2058 region (Douthwaite & Aagaard, 1993). However, the combination of A2032 and G2058 mutations in the same rRNA caused loss of drug resistance and disruption of ribosome function, probably due to the pronounced distortion of the peptidyl transferase loop region. These results indicate a close functional interaction between position 2032 and the ErmE target at 2058. Mutation at G2032 did not, however, affect the ability of ErmE to methylate A2058. Furthermore, ErmE methylation of A2032 mutant rRNA conferred erythromycin resistance as high as wild-type rRNA methylated by ErmE (Table 1). The structural perturbation that destroys rRNA function in the A2032/G2058 double mutant clearly does not occur in rRNA with A2032/N6,N6-dimethylated A2058.

A previous study showed that ErmE methylates a domain V fragment of 23S rRNA as well as intact 23S rRNA (Vester & Douthwaite, 1994). This has also been shown to be the case for the related methyltransferase ErmSF (Kovalic et al., 1994). Furthermore, magnesium ion depletion, causing loss of tertiary structure in the rRNA, has little effect on ErmE methylation at A2058 (Vester & Douthwaite, 1994). These findings suggest that the ErmE recognition motif lies within the primary and secondary structure of 23S rRNA domain V. Consistent with this, nucleotide G2032, and its possible tertiary interaction close to A2058, is unnecessary for ErmE methylation. The helix adjacent to A2058 is unstable in naked rRNA, probably oscillating between paired and open states. Despite this, the G2057–C2611 interaction at the end of the helix is essential for maintaining the accessibility of A2058 and its efficient methylation by ErmE. It is envisaged that this pair is a part of the ErmE recognition motif that is directly contacted, and possibly stabilized, during interaction with ErmE.

MATERIALS AND METHODS

Plasmids

The *ermE* methyltransferase gene was brought under control of the *lac* promoter in the R1-derivative plasmid pREK47 (Vester & Douthwaite, 1994). This type of plasmid is present in the cell as a single copy per chromosome when the incubation temperature is kept below 37 °C, but it exhibits runaway replication at 42 °C, generating up to 1,000 copies/cell (Larsen et al., 1984). Plasmid pREK48 was formed by deleting a 250-bp *Bgl* II fragment from pREK47. This inactivates the *copB* gene, leading to a constitutive copy number of 8–10 plasmids per chromosome at temperatures below 37 °C, but the plasmid still exhibits runaway replication at higher temperatures (Larsen et al., 1984). The pREK plasmids additionally encode *aph*, conferring kanamycin resistance.

Mutant 23S rRNAs were expressed from pFK plasmids (Table 1). These are pBR322 derivatives (Bolivar et al., 1977) that confer ampicillin resistance and additionally encode the entire rRNA operon, *rrnB* (Brosius et al., 1981). The 23S rRNA gene sequence in pFK plasmids has been altered around position 2140 to allow allele-specific analysis of plasmid-coded rRNA (Aagaard et al., 1991). The pFK-plasmids with mutations at 23S rRNA positions 2032, 2057, and 2058 have been described previously (Douthwaite & Aagaard, 1993). The 2611 mutation was created by site-directed mutagenesis (Kunkel et al., 1987), and pFK10 and pFK11 plasmids containing this mutations (Table 1) were constructed in the same manner as the other pFK plasmids. Manipulations with plasmid DNA were carried out by standard procedures (Sambrook et al., 1989) and cells were transformed as described by Hanahan (1985).

Bacterial strain and growth conditions

E. coli strain DH1 (Sambrook et al., 1989) was used as the plasmid host. Cells with pREK plasmids were grown in LB medium with kanamycin at 50 mg/L; the incubation temperature was no higher than 35 °C to avoid runaway replication of the plasmid (Larsen et al., 1984). Cells with pFK plasmids were grown in LB medium with ampicillin at 100 mg/L. The drug resistance phenotypes conferred by the 23S rRNA mutations were investigated on agar plates containing erythromycin (Douthwaite, 1992b). The pREK and pFK plasmids are compatible and were present in the same cell for the in vivo methylation studies.

Isolation of rRNA

Cultures of cells harboring pREK and/or pFK plasmids were grown to 0.4 A450 units. rRNA was isolated as described previously (Vester & Douthwaite, 1994). Briefly, cells were harvested by centrifugation and were washed and resuspended

in TMN buffer (50 mM Tris·HCl, pH 7.8, 10 mM $MgCl_2$, 100 mM NH_4Cl). Cells were lysed by sonication and cell debris was removed by centrifugation. Ribosomes were pelleted by centrifugation and were extracted with phenol and chloroform. rRNA was recovered by ethanol precipitation. The rRNA from cells containing ErmE methyltransferase (encoded on pREK plasmids) was analyzed directly by primer extension to assay for in vivo methylation. RNA isolated from cells without pREK plasmids was used as a substrate for in vitro methylation assays and for the structural probing studies.

In vitro methylation

rRNA (3.5 μg per sample) was renatured in 25 μL buffer A (20 mM Tris·HCl, pH 7.8, 10 mM $Mg(OAc)_2$, 100 mM NH_4Cl, 6 mM β-mercaptoethanol, 10% glycerol) by heating at 50 °C for 5 min followed by 10 min at 30 °C. Fifty nanograms purified methyltransferase (Vester & Douthwaite, 1994) and 1.5 mM S-adenosyl-methionine were preincubated in 25 μL buffer A at 30 °C for 10 min prior to addition to the rRNA. Methylation reactions were carried out at 30 °C for up to 90 min. Reactions were stopped by extracting with phenol followed by chloroform extraction, and the rRNA was recovered by precipitation with ethanol.

Analysis of methylation by allele-specific priming

Methylation at A2058 was assayed by extension with reverse transcriptase (Life Sciences) from oligodeoxynucleotide primers hybridized to the 23S rRNA 2140 region. This region had been changed in plasmid-coded rRNA to give a unique sequence complementary to the primer 5′-CCATGCATGCTG GCGCA (mutant primer). The same region in chromosome-coded 23S rRNA is complementary to the primer 5′-CCATGC AGACTGGCGTC (Aagaard et al., 1991). 5′-^{32}P-end-labeled primers were extended with the four dNTPs and transcription products were analyzed on sequencing gels as described by Moazed et al. (1986).

Quantitative analysis of methylation

A quantitative measurement of methylation was obtained by adapting the technique described by Sigmund et al. (1988). The oligodeoxynucleotide primer 5′-GTAAAGGTTCACGG GGTC (the 2060 primer) is complementary to 23S rRNA bases 2061–2078, which are adjacent to the ErmE methylation site at A2058. The 5′-^{32}P-end-labeled 2060 primer was hybridized to rRNA and extended using reverse transcriptase together with dTTP and ddCTP. Transcripts were run on 14 × 16.5 × 0.02 cm 13% polyacrylamide/7 M urea gels. Extension terminates directly before A2058 in methylated rRNA, or at the first guanine encountered by reverse transcriptase in unmethylated rRNA. The intensities of bands on gel autoradiograms were estimated by scanning with an LKB Ultroscan XL laser densitometer.

Fidelity of methylation

The remainder of domain V in the 23S rRNAs was screened with primers complementary to sequences 2275–2291, 2603–2619, and 2677–2694. Primers were extended with reverse transcriptase and the four dNTPs to detect any artefactual methylation sites in the rRNA.

Structural probing of mutant 23S rRNA

Ten micrograms of rRNAs isolated from cells that harbored plasmid pFK1, pFK2, pFK10, or pFK11 (Table 1) were renatured by warming for 5 min at 50 °C in 200 μL of 20 mM HEPES, pH 7.8, 100 mM NH_4Cl, 10 mM $Mg(OAc)_2$, 1 mM dithiothreitol, 10% glycerol. Samples were preincubated for 10 min at 30 °C, before modification (for 30 min at 30 °C) with either dimethyl sulfate (DMS: 2 μL of a 1:6 (v/v) dilution in ethanol) or kethoxal (3 μL of a 40 mg solution in 30% (v/v) ethanol). Reactions were stopped and rRNA was recovered as described by Moazed et al. (1986). The sites of modification in mutant and wild-type 23S rRNAs were analyzed by allele-specific priming, as described above.

ACKNOWLEDGMENT

We thank Leo Zeef for discussions. The research was supported by grants from the Carlsberg Foundation.

Received May 9, 1995; returned for revision June 5, 1995; revised manuscript received June 22, 1995.

REFERENCES

Aagaard C, Rosendahl G, Dam M, Powers T, Douthwaite S. 1991. Specific structural probing of plasmid-coded ribosomal RNAs from *Escherichia coli*. *Biochimie 73*:1439–1444.

Bibb MJ, Janssen GR, Ward JM. 1985. Cloning and analysis of the promoter region of the erythromycin-resistance gene (*ermE*) of *Streptomyces erythraeus*. *Gene 41*:E357–E368.

Bolivar F, Rodriguez RL, Greene PJ, Betlach MC, Heyneker HL, Boyer HW, Crosa JH, Falkow S. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene 2*:95–113.

Brosius J, Ullrich A, Raker MA, Gray A, Dull TJ, Gutell RR, Noller HF. 1981. Construction and fine mapping of recombinant plasmids containing the rrnB ribosomal RNA operon of *E. coli*. *Plasmid 6*:112–118.

Cseplö A, Etzold T, Schell J, Schreier PH. 1988. Point mutation in the 23S rRNA genes of four lincomycin resistant *Nicotiana plumbaginiflora* mutants could provide new selectable markers for chloroplast transformation. *Mol Gen Genet 214*:295–299.

Dhillon N, Leadlay PF. 1990. A repeated decapeptide motif in the C-terminal domain of the ribosomal RNA methyltransferase from the erythromycin producer *Saccharopolyspora erythraea*. *FEBS Lett 262*:189–193.

Douthwaite S. 1992a. Interaction of the antibiotics clindamycin and lincomycin with *Escherichia coli* 23S ribosomal RNA. *Nucleic Acids Res 20*:4717–4720.

Douthwaite S. 1992b. Functional interactions within 23S rRNA involving the peptidyltransferase center. *J Bacteriol 174*:1333–1338.

Douthwaite S, Aagaard C. 1993. Erythromycin binding is reduced in ribosomes with conformational alterations in the 23S rRNA peptidyl transferase loop. *J Mol Biol 232*:725–731.

Döring T, Greuer B, Brimacombe R. 1991. The three dimensional folding of ribosomal RNA: Localization of a series of intra-RNA crosslinks in 23S RNA induced by treatment of *Escherichia coli* 50S subunits with *bis*-(2-chloroethyl)-methylamine. *Nucleic Acids Res 19*:3517–3524.

Egebjerg J, Garrett RA. 1991. Binding sites of the antibiotics pactamycin and celesticetin on ribosomal RNAs. *Biochimie 73*:1145–1149.

Ettayebi M, Prasad SM, Morgan EA. 1985. Chloramphenicol-erythromycin resistance mutations in a 23S rRNA gene of *Escherichia coli*. *J Bacteriol 162*:551–557.

Graham MY, Weisblum B. 1979. 23S ribosomal ribonucleic acid of macrolide producing streptomycetes contains methylated adenine. *J Bacteriol 137*:1446–1467.

Gutell RR, Larsen N, Woese CR. 1994. Lessons from an evolving rRNA: 16S and 23S rRNA structures from a comparative perspective. *Microbiol Reviews 58*:10–26.

Hanahan D. 1985. Techniques for transformation of *Escherichia coli*. In: Glover DM, ed. *DNA cloning: A practical approach, vol 1*. Oxford: IRL Press. pp 109–135.

Kovalic D, Giannattasio RB, Hyung-Jong J, Weisblum B. 1994. 23S rRNA domain V, a fragment that can be specifically methylated in vitro by the ErmSF (TlrA) methyltransferase. *J Bacteriol 176*: 6992–6998.

Kunkel TA, Roberts JD, Zakour R. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol 154*:367–382.

Labeda DP. 1987. Transfer of the type strain of *Streptomyces erythraeus* (Waksman 1923) Waksman and Henrici 1948 to the genus *Saccharopolyspora* Lacy and Goodfellow 1979 as *Saccharopolyspora erythraea* sp. nov., and designation of a neotype strain for *Streptomyces erythraeus*. *Int J Systematic Bacteriol 37*:19–22.

Larsen JEL, Gerdes K, Light J, Molin S. 1984. Low-copy-number plasmid-cloning vectors amplifiable by derepression of an inserted foreign promoter. *Gene 28*:45–54.

McGuire JM, Bunch RL, Anderson RC, Boaz HE, Flynn EH, Powell HM, Smith JW. 1952. "Ilotycin", a new antibiotic. *Antibiot Chemother 2*:281–283.

Moazed D, Noller HF. 1987. Chloramphenicol, erythromycin, carbomycin and vernamycin B protect overlapping sites in the peptidyl transferase region of 23S ribosomal RNA. *Biochimie 69*:879–884.

Moazed D, Stern S, Noller HF. 1986. Rapid chemical probing of conformation in 16S ribosomal RNA and 30S ribosomal subunits using primer extension. *J Mol Biol 187*:399–416.

Noller HF. 1984. Structure of ribosomal RNA. *Annu Rev Biochem 53*:119–162.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: A laboratory manual, 2nd ed*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Sigmund CD, Ettayebi M, Borden A, Morgan EA. 1988. Antibiotic resistance mutations in ribosomal RNA genes of *Escherichia coli*. *Methods Enzymol 164*:673–690.

Sigmund CD, Ettayebi M, Morgan EA. 1984. Antibiotic resistance mutations in 16S and 23S ribosomal RNA genes of *Escherichia coli*. *Nucleic Acids Res 12*:4653–4663.

Skinner R, Cundliffe E. 1982. Dimethylation of adenine and resistance of *Streptomyces erythraeus* to erythromycin. *J Gen Microbiol 128*:2411–2416.

Skinner RH, Cundliffe E, Schmidt FJ. 1983. Site of action of a ribosomal RNA methylase responsible for resistance to erythromycin and other antibiotics. *J Biol Chem 258*:12702–12706.

Thompson CJ, Kieser T, Ward JM, Hopwood DA. 1982. Physical analysis of antibiotic resistance genes from *Streptomyces* and their use in vector construction. *Gene 20*:51–62.

Uchiyama H, Weisblum B. 1985. *N*-methyl transferase of *Streptomyces erythraeus* that confers resistance to the macrolide-lincosamide-streptogramin B antibiotics: Amino acid sequence and its homology to cognate *R*-factor enzymes from pathogenic bacilli and cocci. *Gene 38*:103–110.

Uhlenbeck OC. 1995. Keeping RNA happy. *RNA 1*:4–6.

Vannuffel P, Giambattista MD, Morgan EA, Cocito C. 1992. Identification of a single base change in ribosomal RNA leading to erythromycin resistance. *J Biol Chem 267*:8377–8382.

Vester B, Douthwaite S. 1994. Domain V of 23S rRNA contains all the structural elements necessary for recognition by the ErmE methyltransferase. *J Bacteriol 176*:6999–7004.

Zalacain M, Cundliffe E. 1989. Methylation of 23S rRNA caused by *tlrA* (*ermSF*), a tylosin resistance determinant from *Streptomyces fradiae*. *J Bacteriol 171*:4254–4260.