Activity of the Ketolide Telithromycin Is Refractory to Erm Monomethylation of Bacterial rRNA

Mingfu Liu and Stephen Douthwaite*

Department of Biochemistry and Molecular Biology, University of Southern Denmark, DK-5230 Odense M, Denmark

Received 18 July 2001/Returned for modification 18 November 2001/Accepted 7 February 2002

Methylation of specific nucleotides in rRNA is one of the means by which bacteria achieve resistance to macrolides-lincosamides-streptogramin B (MLS_B) and ketolide antibiotics. The degree of resistance is determined by how effectively the rRNA is methylated. We have implemented a bacterial system in which the rRNA methylations are defined, and in this study we investigate what effect Erm mono- and dimethylation of the rRNA has on the activity of representative MLS_B and ketolide antibiotics. In the test system, >80% of the rRNA molecules are monomethylated by ErmN (TlrD) or dimethylated by ErmE. ErmE dimethylation confers high resistance to all the MLS_B and ketolide drugs. ErmN monomethylation predictably confers high resistance to the lincosamides clindamycin and lincomycin, intermediate resistance to the macrolides clarithromycin and erythromycin, and low resistance to the streptogramin B pristinamycin IA. In contrast to the macrolides, monomethylation only mildly affects the antimicrobial activities of the ketolides HMR 3647 (telithromycin) and HMR 3004, and these drugs remain 16 to 250 times as potent as clarithromycin and erythromycin. These differences in the macrolide and ketolide activities could explain the recent reports of variation in the MICs of telithromycin for streptococcal strains that have constitutive *erm* MLS_B resistance and are highly resistant to erythromycin.

Many clinically useful antibiotics exert their antimicrobial activity by inhibiting the bacterial ribosome (14, 41). Such drugs include the macrolide-lincosamide-streptogramin B (MLS_B) antibiotics that bind to and block the function of the bacterial large (50S) ribosomal subunit (10, 25, 30, 31). Despite their potentially powerful antimicrobial activity, MLS_B drugs have unfortunately only offered a limited respite from the many pathogens that have rapidly acquired resistance to these drugs. Several different types of resistance to MLS_B drugs are encountered in clinical strains, and one of the most widespread is that conferred by the *erm* gene family (23, 44, 45). The *erm* genes encode methyltransferases that modify 23S rRNA at nucleotide A2058 (39). Nucleotide A2058 can be seen from recent X-ray diffraction data on crystals of 50S subunit-drug complexes to be central in the binding site of MLS_B drugs (35). The crystallography data reveal how methylation of A2058 by Erm would perturb the geometry of the drug-rRNA interaction and prevent efficient drug binding.

Erm methyltransferases are widespread in bacteria, and although they all modify nucleotide A2058, members of the Erm family can differ according to whether they add one or two methyl groups to this nucleotide. The physiological effects of mono- and dimethylation are clearly discernible. Monomethylation of A2058 confers low to intermediate resistance to macrolide and streptogramin B drugs but high resistance to lincosamides (6). Dimethylation of A2058 confers high resistance to all the MLS_B drugs, and this is generally considered the classical MLS_B resistance phenotype (44). The distribution of the two forms of Erm is generally consistent with these pheno-

types: Erm monomethyltransferases are sufficient to ensure the survival of lincosamide-producing actinomycetes (6), whereas Erm dimethyltransferases are found in bacteria that produce macrolide and streptogramin B antibiotics, as well as in drug-resistant pathogens (8).

MLS_B drugs, and in particular the macrolides, have been derivatized in a multitude of ways in efforts to improve their antimicrobial properties, particularly against resistant pathogens (4). One of the most recent derivatives to reach the advanced stages of clinical trials is the ketolide telithromycin, which is based on the 14-membered ring macrolide erythromycin (3). Telithromycin has shown great promise against many gram-positive pathogens that are erythromycin resistant (2, 19, 27, 28, 36). However, there have been reports of great variation in the efficacy of telithromycin against some *erm*-constitutive strains that are highly resistant to erythromycin (21). For instance, in MLS_B-resistant *Streptococcus pyogenes* the efficacy of telithromycin depends to a large extent on whether a strain carries *ermA*(TR) or *ermB*. More intriguing is the observation that *ermB*-constitutive strains of *S. pyogenes* are generally resistant to telithromycin, while strains of *Streptococcus pneumoniae* that constitutively express a virtually identical version of *ermB* remain sensitive to this drug (21).

The molecular mechanisms causing these differences in telithromycin resistance are currently unclear. This is due in part to the many differences between streptococcal species and serotypes, which confound a direct comparison of the resistance phenotypes. In the present study we circumvent these problems by investigating the mode of action of telithromycin in a single, well-characterized system. We selected *Escherichia coli* as the test organism, because this is currently the only bacterium in which the structures and the modifications of the ribosomal components have been comprehensively mapped (17, 33). A highly permeable strain of *E. coli* was chosen that

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Biology, University of Southern Denmark, DK-5230 Odense M, Denmark. Phone: (45) 65 50 23 95. Fax: (45) 65 50 24 67. E-mail: srd@bmb.sdu.dk.

TABLE 1. Bacterial strains and plasmids used in this study^a

Strains and vectors	Description	Reference or source
<i>E. coli</i> strains		
AS19	Permeable mutant of <i>E. coli</i> B strain	37
AS19-RrmA ⁻	AS19 <i>rrmA::kan</i> , Kan ^r	This study
DH10B	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80d <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>endA1</i> <i>araD139</i> Δ(<i>ara</i> , <i>leu</i>)7697 <i>galU</i> <i>galK</i> λ <i>mpsL</i> <i>nupG</i>	Life Technologies
JC7623	F ⁻ <i>thr-1</i> <i>ara-14</i> <i>leu-6</i> <i>proA2</i> <i>lacY1</i> <i>sbcC201</i> <i>tsx-33</i> <i>galK2</i> <i>sbcB15</i> <i>his-4</i> <i>recC22</i> <i>recB21</i> <i>rpsL31</i> <i>xyl-5</i> <i>mtl-1</i> <i>argE3</i> <i>thi-1</i>	<i>E. coli</i> Genetic Stock Center
Vectors		
Bacteriophage P1	Wild type	Laboratory collection
Plasmids		
pMAK705	Temperature-sensitive pSC101 replicon; Cm ^r	18
pSD184	Derivative of pACYC184, Tc ^r ; Kan ^r	This study
pSD57c	<i>ermN/tlrD</i> cloned into pSD184; Tc ^r	This study
pSDdiv	<i>ermE</i> cloned into pSD184; Tc ^r	This study
pSD707	<i>rrmA::kan</i> fragment cloned into pMAK705; Cm ^r Kan ^r	This study

^a Antibiotic abbreviations: Kan, kanamycin; Cm, chloramphenicol; Tc, tetracycline.

is susceptible to all types of MLS_B drugs. This strain was equipped with plasmids that facilitate the regulated expression of an *erm* monomethyltransferase gene (*tlrD*) (48), recently reclassified as *ermN* (32), or an *erm* dimethyltransferase gene (*ermE*) (42). The strain was then challenged with a series of MLS_B and ketolide drugs. After methylation of A2058, the bacteria exhibited resistance patterns toward established MLS_B drugs that were completely consistent with the phenotypes described above. However, the bacteria with A2058 monomethylation remained unexpectedly sensitive towards the ketolide antibiotics. This resistance phenotype is distinctly different from that of the established macrolide drugs erythromycin and clarithromycin and could be connected with the variable efficacy of telithromycin against MLS_B-resistant gram-positive pathogens.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacteria and plasmids used in this study are listed in Table 1. *E. coli* strain DH10B was used for all the cloning steps, unless otherwise stated. Plasmid pSD184 is a derivative of pACYC184 and was used to clone the A2058 monomethyltransferase gene *tlrD* from *Streptomyces fradiae* and the dimethyltransferase gene *ermE* from *Saccharopolyspora erythraea* (Table 1). The *tlrD* and *ermE* genes were isolated using PCR primers corresponding to the ends of the gene sequences available in the database. The cloned genes were placed under the control of a strong *E. coli* phage T5 promoter (5) with dual *lac* operator sites. Plasmid pMAK705 has a temperature-sensitive pSC101 replicon and was used for inactivation of *rrmA* on the *E. coli* chromosome.

All *E. coli* strains were grown at 37°C in LB medium (34) unless otherwise stated. The following antibiotics were used at the indicated concentrations to maintain the plasmids: ampicillin (Bristol-Myers Squibb), 100 μg/ml; chloramphenicol (Sigma), 25 μg/ml; kanamycin (Sigma), 25 μg/ml; and tetracycline (Sigma), 12.5 μg/ml.

Construction of *rrmA*-inactivated mutants. The *rrmA* gene encodes an rRNA methyltransferase. We inactivated *rrmA* in the hyperpermeable *E. coli* B strain AS19 before using this strain in the MIC studies. Briefly, *rrmA* was amplified by PCR and a Kan^r cassette was inserted into the middle of *rrmA* at its unique *Sna*BI site. The *rrmA::kan* fragment was then cleaved with *Eco*RI/*Bgl*II and cloned into the same sites of pMAK705, resulting in plasmid pSD707. *E. coli* strain AS19 could not be transformed by pMAK705-based plasmids, and therefore we inactivated *rrmA* first in JC7623, a transformable *E. coli* strain. JC7623 was transformed with pSD707 at 30°C and then cultured at 44°C in the presence of chloramphenicol and kanamycin. Cells were serially passaged at 30°C in

medium containing only kanamycin, before selecting for chloramphenicol sensitivity. The *rrmA::kan* marker on the chromosome of mutant JC7623 was then transferred into AS19 by phage P1-mediated transduction, generating the new strain AS19-RrmA⁻. The RrmA⁻ phenotype and lack of G745 methylation were confirmed by reverse transcriptase primer extension on the 23S rRNA (16, 24).

Determination of MIC. MICs were determined for the AS19 and AS19-RrmA⁻ strains by using the agar dilution method according to the NCCLS guidelines (26). Cells contained plasmid pSD184 (the empty plasmid without a methyltransferase gene), pSD57c (which contains the *erm* monomethyltransferase *ermN/tlrD*), or pSDdiv (which contains the dimethyltransferase *ermE*). For each strain, approximately 10⁴ cells were applied to the surface of LB agar plates containing serial twofold dilutions of clindamycin, clarithromycin (Abbott), erythromycin (Sigma), lincomycin (Sigma), pristinamycin IA, or telithromycin or HMR 3004 (Aventis). Tetracycline was present in the media (to maintain the plasmids) together with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) (to induce the plasmid *lac* promoter), and the plates were incubated for approximately 20 h. The MICs were measured a minimum of three times and were highly reproducible.

Quantification of dimethylation at A2058. The level of ErmE dimethylation at A2058 was measured by a procedure adapted from that of Sigmund et al. (38, 42). A primer which is complementary to nucleotides 2061 to 2078 of the 23S rRNA was extended with reverse transcriptase in the presence of dTTP and ddCTP. The extension reaction was blocked at one of two nucleotides: either at the dimethylated A2058 or in unmethylated rRNA molecules at G2057 by incorporation of ddCTP. Primer extension products were analyzed on denaturing polyacrylamide gels, and the degree of methylation was calculated by scanning the relative intensities of these two bands using a PhosphorImager (Storm 840; Molecular Dynamics).

RESULTS

The test strain. *E. coli* is currently the only organism in which the nucleotide modifications in the rRNA have been comprehensively mapped (33). As nucleotide modifications can influence drug binding, this bacterium was selected for the present study. The main disadvantage of working with *E. coli* is its gram-negative wall and outer membrane that act as a barrier to most MLS_B drugs. To overcome this problem, several permeable *E. coli* mutants were screened, and strain AS19 was chosen for its ability to absorb a wide range of MLS_B drugs (Table 2). Although these MICs remain appreciably higher than those for gram-positive bacteria, they are low enough to make this strain a useful experimental model. The MICs for this permeable strain are less than 1/50 (macrolides erythro-

TABLE 2. Effects of rRNA methylation on the MICs of MLS_B antibiotics on the permeable *E. coli* strain AS19-RmA^{-a}

Antibiotic	MIC (μg/ml)		
	No methylation	Monomethylation	Dimethylation
Erythromycin	0.5	128	1,024
Clarithromycin	0.25	32	512
Telithromycin (HMR 3647)	0.5	2	512
HMR 3004	0.25	0.5	256
Lincomycin	16	>8,000	8,000
Clindamycin	2	512	512
Pristinamycin IA ^b	32	64	>128

^a MICs were compared for the 14-membered ring macrolides erythromycin and clarithromycin, the ketolides HMR 3674 (telithromycin) and HMR 3004, the lincosamides lincomycin and clindamycin, and the streptogramin B pristinamycin IA. *E. coli* strain AS19-RmA⁻ contained either the empty plasmid pSD184 (no methylation), plasmid pSD57c with *ermN/tlrD* (monomethylation), or plasmid pSDdiv with *ermE* (dimethylation). The MICs were consistently reproduced for all the drugs in a minimum of three independent experiments.

^b Insufficient amounts of pristinamycin IA were available for us to determine the absolute MIC for the dimethylated strain.

mycin and clarithromycin), approximately 1/10 (ketolides) (11), and about 1/20 (clindamycin and lincomycin) those for the wild-type *E. coli*.

E. coli 23S rRNA contains an m¹G745 modification (16), which is situated close to the MLS_B drug binding site on the ribosome (35). This modification is absent in other pathogens such as *S. pneumoniae*, *S. pyogenes*, *Staphylococcus aureus*, and *Haemophilus influenzae* (our unpublished observations), and we were uncertain what effect this modification could have on MLS_B drug binding. We therefore inactivated the gene encoding the G745-specific methyltransferase, *rrm4*, on the AS19 chromosome. MICs of drugs for the AS19-RmA⁻ strain are shown in Table 2. For the macrolide, ketolide, and lincosamide drugs, these are essentially identical to the results with the RmA⁺ strain (taking into account that the RmA⁻ strain grows slightly more slowly).

Expression of the Erm mono- and dimethyltransferases. Well-characterized *erm* genes encoding a monomethyltransferase (*ermN/tlrD*) and a dimethyltransferase (*ermE*) were chosen for the study. The genes were placed under control of the *lac* promoter in the low-copy-number plasmid pSD184. The resistance phenotypes for mono- and dimethylation of 23S rRNA were confirmed by use of a set of MLS_B drugs (Table 2). Monomethylation conferred intermediate resistance to the two 14-membered ring macrolides erythromycin and clarithromycin, while conferring high resistance to the lincosamides lincomycin and clindamycin. Dimethylation conferred high resistance to all of these drugs. Resistance to the streptogramin B drug pristinamycin IA was also consistent with that expected for mono- and dimethylation of the rRNA, although an absolute value for the MIC for the *ermE* strain was not determined.

The degree of ErmE modification was ascertained biochemically using primer extension with reverse transcriptase, and it was shown that 82% of the ribosomes in this strain were dimethylated at nucleotide A2058. Monomethylation of A2058 cannot be measured by this method, and consistent with this, rRNA from the strain expressing TlrD showed no reverse transcriptase stop at A2058. The proportion of ribosomes monomethylated by TlrD was consequently estimated indi-

rectly by comparison of MICs. All other factors being equal, mono- and dimethylation of A2058 confer the same level of lincosamide resistance. Thus, from the MICs for the *tlrD* and *ermE* strains on clindamycin and lincomycin (Table 2) it appears that TlrD has methylated at least as great a proportion of the ribosomes as ErmE. Conceivably, a fraction of the remaining 18% of ribosomes in the *ermE* strain could have been monomethylated, and thus the estimations given here should be regarded as minimal levels of methylation.

Monomethylated ribosomes remain sensitive to the ketolides. Monomethylation of 23S rRNA nucleotide A2058 offered only a slight growth advantage in the presence of ketolide antibiotics. The ketolide HMR 3004, which has strong antimicrobial activity against gram-positive organisms (12), was the most-effective drug tested here. HMR 3004 showed a potency 256 times that of erythromycin and 64 times that of clarithromycin at inhibiting the monomethylated strain. The antimicrobial activity of HMR 3647 (telithromycin) against the monomethylated strain was slightly lower than that of HMR 3004, although it was appreciably more potent than the macrolides (Table 2). These results are consistent with those from the gram-positive bacterium *Streptomyces lividans*, where monomethylation of A2058 confers 50- to 100-fold resistance to erythromycin and clarithromycin (29; M. Liu, unpublished data), while conferring about 8-fold resistance to telithromycin and HMR 3004 (Liu, unpublished data).

The structural features of the macrolide and ketolide drugs that are responsible for the differences in their antimicrobial activities are confined to the 3 and the 11/12 positions of the macrolactone ring. The ketolides used here have a keto group at the 3 position (in contrast with a cladinose sugar residue in the macrolides), and the ketolides are derivatized with an alkyl-aryl extension from a carbamate group at the 11/12 position (3, 20).

DISCUSSION

We have investigated how the susceptibility of bacteria to MLS_B and ketolide antibiotics is affected by defined rRNA modifications. Here we have customized the rRNA methylation pattern in *E. coli*: one methylation has been removed by knocking out the 23S rRNA G745 methyltransferase gene *rrm4*, and methylation has been introduced at A2058 via plasmid-encoded *erm* mono- and dimethyltransferases. Problems of cell permeability to drugs commonly encountered in gram-negative bacteria have been avoided by choice of a hyperpermeable *E. coli* strain. The drug MICs for this strain are appreciably lower than those required to inhibit most wild-type gram-negative bacteria, although they remain higher than MICs for susceptible gram-positive bacteria.

One of the antibiotics tested, telithromycin (HMR 3647), has shown considerable promise as an effective antimicrobial against gram-negative pathogens such as *H. influenzae* and *Moraxella catarrhalis*, as well as against a range of gram-positive organisms, including most erythromycin-resistant isolates of *S. pneumoniae* (3). However, some puzzling telithromycin phenotypes have been observed in a few clinical isolates. In particular, these include the higher efficacy of telithromycin against *S. pyogenes* isolates with *ermA*(TR) compared to isolates with *ermB*. Even more striking is the incongruity between

the telithromycin resistance seen in *ermB*-constitutive *S. pyogenes* and the telithromycin susceptibility in *ermB*-constitutive *S. pneumoniae* (21). ErmB and ErmA(TR) are both A2058 dimethyltransferases. The phenotypic consequences of effective (82% or greater) dimethylation at A2058 are clear from the test system used here, with high resistance being conferred to all the macrolide and ketolide antibiotics (Table 2). Why then is comparable ketolide resistance not observed in the strains of *S. pyogenes* and *S. pneumoniae*?

As there is no evidence of auxiliary resistance mechanisms such as *mef*-encoded efflux in the highly resistant streptococci (21), the reason for the variation in resistance levels is undoubtedly linked with how effectively these strains alter the ketolide target site on their ribosomes. The simplest explanation is that the streptococcal species dimethylate their rRNA with different efficiencies. This could be caused by variation in the expression of *erm*, leading to a corresponding variation in the proportions of dimethylated and unmethylated ribosomes in the cell. Such differences in dimethylation by ErmB in streptococcal strains have recently been shown to correlate with their levels of resistance to ketolide drugs (49). While a higher proportion of unmethylated ribosomes would explain the lower ketolide resistance in some of the constitutive *erm* strains, it does not explain their concurrent high levels of erythromycin resistance.

An important piece of the puzzle could be connected with the way in which Erm dimethylates nucleotide A2058. This process occurs in two steps, each requiring addition of a methyl group from an *S*-adenosylmethionine donor molecule (47). In vitro kinetic studies on the dimethyltransferases ErmC and ErmSF (now ErmS) showed that these enzymes function as monomethyltransferases under conditions where the concentration of *S*-adenosylmethionine is limiting (9, 22, 47). The streptococcal ErmB and ErmA(TR) could conceivably also act as monomethyltransferases if the metabolic conditions within the bacterium, such as availability of *S*-adenosylmethionine, were unfavorable. As shown here, monomethylation of A2058 confers intermediate resistance to the macrolides erythromycin and clarithromycin (Table 2). It appears, therefore, that streptococcal strains containing a mixture of monomethylated and dimethylated ribosomes would have a macrolide-resistant phenotype. This contrasts with the situation for the ketolides, where cells remain susceptible despite monomethylation at A2058. The ketolides presumably bind avidly to monomethylated ribosomes, causing them to abort protein synthesis at an early stage of mRNA translation.

While A2058 in domain V of 23S rRNA is a key nucleotide for the interaction of MLS_B and ketolide antibiotics, the binding site for these drugs additionally involves contributions from nucleotides in domain II of the rRNA (20, 25, 46) as well as from the ribosomal proteins L4 and L22 (13, 15). The ribosomal proteins do not directly intrude into the MLS_B site, but rather they influence the conformation of the site via their interactions with the rRNA (1, 35). The nature of the drug site is thus complex, and as a consequence of this, several other structural changes in addition to Erm methylation confer drug resistance. Thus, base substitution at A2058 and neighboring nucleotides (43), nucleotide methylation in domain II of the rRNA (24), or mutations in the L4 and L22 ribosomal proteins (7, 40) can confer resistance to all or to subsets of the MLS_B-

ketolide drugs. In principle, structural changes in any of these ribosomal components could be a factor contributing to streptococcal drug resistance phenotypes. However, the most pertinent question about these strains that needs to be addressed is whether the disparities in drug resistance levels correlate with variation in the degree of *erm*-encoded dimethylation at nucleotide A2058.

ACKNOWLEDGMENTS

Sidney R. Kushner, University of Georgia, is thanked for providing plasmid pMAK705.

This work was supported by grants from the European Commission 5th Framework Program (QLK2-2000-00935), Aventis Pharma, The Danish Biotechnology Instrument Center (DABIC), and the Danish Grundforskningsfond's Nucleic Acid Center.

REFERENCES

- Ban, N., P. Nissen, J. Hansen, P. B. Moore, and T. A. Steitz. 2000. The complete atomic structure of the large ribosomal subunit at 2.4 Å resolution. *Science* **289**:905–920.
- Boswell, F. J., J. M. Andrews, J. P. Ashby, C. Fogarty, N. P. Brenwald, and R. Wise. 1998. The in-vitro activity of HMR 3647, a new ketolide antimicrobial agent. *J. Antimicrob. Chemother.* **42**:703–709.
- Bryskier, A. 2000. Ketolides: telithromycin, an example of a new class of antibacterial agents. *Clin. Microbiol. Infect.* **6**:661–669.
- Bryskier, A. J., J. P. Butzler, H. C. Neu, and P. M. Tulkens. 1993. Macrolides: chemistry, pharmacology and clinical uses. Arnette Blackwell, Paris, France.
- Bujard, H., R. Gentz, M. Lanzer, D. Stuber, M. Muller, I. Ibrahimi, M. T. Hauptle, and B. Dobberstein. 1987. A T5 promoter based transcription-translation system for the analysis of proteins *in vivo* and *in vitro*. *Methods Enzymol.* **155**:416–433.
- Calcutt, M. J., and E. Cundliffe. 1990. Cloning of a lincosamide-resistant determinant from *Streptomyces caelestis*, the producer of celesticetin, and characterization of the resistance mechanism. *J. Bacteriol.* **172**:4710–4714.
- Chittum, H. S., and W. S. Champney. 1994. Ribosomal protein gene sequence changes in erythromycin-resistant mutants of *Escherichia coli*. *J. Bacteriol.* **176**:6192–6198.
- Cundliffe, E. 1990. Recognition sites for antibiotics in rRNA, p. 479–490. In W. Hill, A. Dahlberg, R. A. Garrett, P. B. Moore, D. Sclessinger, and J. Warner (ed.), *The structure, function, and evolution of ribosomes*. American Society for Microbiology, Washington, D.C.
- Denoya, C., and D. Dubnau. 1989. Monomethylating and dimethylating activities and kinetic studies of the ErmC 23S ribosomal RNA methyltransferase. *J. Biol. Chem.* **264**:2615–2624.
- Douthwaite, S. 1992. Interaction of the antibiotics clindamycin and lincomycin with *Escherichia coli* 23S ribosomal RNA. *Nucleic Acids Res.* **20**:4717–4720.
- Douthwaite, S., L. H. Hansen, and P. Mauvais. 2000. The macrolide-ketolide inhibition of MLS-resistant ribosomes is improved by alternative drug interaction with domains II of 23S rRNA. *Mol. Microbiol.* **36**:183–192.
- Ednie, L. M., S. K. Spangler, M. R. Jacobs, and P. C. Appelbaum. 1997. Susceptibilities of 228 penicillin- and erythromycin-susceptible and -resistant pneumococci to RU 64004, a new ketolide, compared with susceptibilities to 16 other agents. *Antimicrob. Agents Chemother.* **41**:1033–1036.
- Gabashvili, I. S., S. T. Gregory, M. Valle, R. Grassucci, M. Worbs, M. C. Wahl, A. E. Dahlberg, and J. Frank. 2001. The polypeptide tunnel system in the ribosome and its gating in erythromycin resistance mutants of L4 and L22. *Mol. Cell* **8**:181–188.
- Gale, E. F., E. Cundliffe, P. E. Reynolds, M. H. Richmond, and M. J. Waring. 1981. *The molecular basis for antibiotic action*. John Wiley and Sons, London, United Kingdom.
- Gregory, S. T., and A. E. Dahlberg. 1999. Erythromycin resistance mutations in ribosomal proteins L22 and L4 perturb the higher order structure of 23S ribosomal RNA. *J. Mol. Biol.* **289**:827–834.
- Gustafsson, C., and B. C. Persson. 1988. Identification of the *rmA* gene encoding the 23S rRNA m¹G745 methyltransferase in *Escherichia coli* and characterization of an m¹G745-deficient mutant. *J. Bacteriol.* **180**:359–365.
- Gutell, R. R., N. Larsen, and C. R. Woese. 1994. Lessons from an evolving rRNA: 16S and 23S rRNA structures from a comparative perspective. *Microbiol. Rev.* **58**:10–26.
- Hamilton, C. M., M. Aldea, B. K. Washburn, P. Babitzke, and S. R. Kushner. 1989. New method for generating deletions and gene replacements in *Escherichia coli*. *J. Bacteriol.* **171**:4617–4622.
- Hamilton-Miller, J. M., and S. Shah. 1998. Comparative in-vitro activity of ketolide HMR 3647 and four macrolides against gram-positive cocci of known erythromycin susceptibility status. *J. Antimicrob. Chemother.* **41**:649–653.

20. Hansen, L. H., P. Mauvais, and S. Douthwaite. 1999. The macrolide-ketolide antibiotic binding site is formed by structures in domains II and V of 23S ribosomal RNA. *Mol. Microbiol.* **31**:623–631.
21. Jalava, J., J. Kataja, H. Seppälä, and P. Huovinen. 2001. In vitro activities of the novel ketolide telithromycin (HMR 3647) against erythromycin-resistant *Streptococcus* species. *Antimicrob. Agents Chemother.* **45**:789–793.
22. Kovalic, D., R. B. Giannattasio, J. Hyung-Jong, and B. Weisblum. 1994. 23S rRNA domain V, a fragment that can be specifically methylated in vitro by the ErmSF (TlrA) methyltransferase. *J. Bacteriol.* **176**:6992–6998.
23. Leclercq, R., and P. Courvalin. 1991. Bacterial resistance to macrolide, lincosamide, and streptogramin antibiotics by target modification. *Antimicrob. Agents Chemother.* **35**:1267–1272.
24. Liu, M., F. Kirpekar, G. P. van Wezel, and S. Douthwaite. 2000. The tylosin resistance gene *tlrB* of *Streptomyces fradiae* encodes a methyltransferase that targets G748 in 23S rRNA. *Mol. Microbiol.* **37**:811–820.
25. Moazed, D., and H. F. Noller. 1987. Chloramphenicol, erythromycin, carbomycin and vernamycin B protect overlapping sites in the peptidyl transferase region of 23S ribosomal RNA. *Biochimie* **69**:879–884.
26. NCCLS. 1993. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. M7–A3. 3rd ed. p. 13–17. National Committee for Clinical Laboratory Standards, Villanova, Pa.
27. Okamoto, H., S. Miyazaki, K. Tateda, Y. Ishii, and K. Yamaguchi. 2000. Comparative in vitro activity of telithromycin (HMR 3647), three macrolides, amoxicillin, cefdinir and levofloxacin against Gram-positive clinical isolates in Japan. *J. Antimicrob. Chemother.* **46**:797–802.
28. Pankuck, G. A., M. A. Visalli, M. R. Jacobs, and P. C. Appelbaum. 1998. Susceptibilities of penicillin- and erythromycin-susceptible and -resistant pneumococci to HMR 3647 (RU 66647), a new ketolide, compared with susceptibilities to 17 other agents. *Antimicrob. Agents Chemother.* **42**:624–630.
29. Pernodet, J.-L., S. Fish, M. H. Blondelet-Rouault, and E. Cundliffe. 1996. The macrolide-lincosamide-streptogramin B resistance phenotypes characterized by using a specifically deleted, antibiotic-sensitive strain of *Streptomyces lividans*. *Antimicrob. Agents Chemother.* **40**:581–585.
30. Porse, B. T., and R. A. Garrett. 1999. Sites of interaction of streptogramin A and B antibiotics in the peptidyl transferase loop of 23 S rRNA and the synergism of their inhibitory mechanisms. *J. Mol. Biol.* **286**:375–387.
31. Poulsen, S. M., C. Kofoed, and B. Vester. 2000. Inhibition of the ribosomal peptidyl transferase reaction by the mycarose moiety of the antibiotics carbomycin, spiramycin and tylosin. *J. Mol. Biol.* **304**:471–481.
32. Roberts, M. C., J. Sutcliffe, P. Courvalin, L. B. Jensen, J. Rood, and H. Seppälä. 1999. Nomenclature for macrolide and macrolide-lincosamide-streptogramin B resistance determinants. *Antimicrob. Agents Chemother.* **43**:2823–2830.
33. Rozenski, J., P. F. Crain, and J. A. McCloskey. 1999. The RNA modification database: 1999 update. *Nucleic Acids Res.* **27**:196–197.
34. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
35. Schlünzen, F., R. Zarivach, J. Harms, A. Bashan, A. Tocilj, R. Albrecht, A. Yonath, and F. Franceschi. 2001. Structural basis for the interaction of antibiotics with the peptidyl transferase centre in eubacteria. *Nature* **413**:814–821.
36. Schülin, T., C. B. Wennersten, R. C. Moellering, Jr., and G. M. Eliopoulos. 1998. In-vitro activity of the new ketolide antibiotic HMR 3647 against gram-positive bacteria. *J. Antimicrob. Chemother.* **42**:297–301.
37. Sekiguchi, M., and S. Iida. 1967. Mutants of *Escherichia coli* permeable to actinomycin. *Proc. Natl. Acad. Sci. USA* **58**:2315–2320.
38. Sigmund, C. D., M. Ettayebi, A. Borden, and E. A. Morgen. 1988. Antibiotic resistance mutations in ribosomal RNA genes of *Escherichia coli*. *Methods Enzymol.* **164**:673–690.
39. Skinner, R., E. Cundliffe, and F. J. Schmidt. 1983. Site of action of a ribosomal RNA methylase responsible for resistance to erythromycin and other antibiotics. *J. Biol. Chem.* **258**:12702–12706.
40. Tait-Kamradt, A., T. Davies, P. C. Appelbaum, F. Depardieu, P. Courvalin, J. Petitpas, L. Wonderack, A. Walker, M. R. Jacobs, and J. Sutcliffe. 2000. Two new mechanisms of macrolide resistance in clinical strains of *Streptococcus pneumoniae* from Eastern Europe and North America. *Antimicrob. Agents Chemother.* **44**:3395–3401.
41. Vázquez, D. 1979. Inhibitors of protein synthesis. Springer-Verlag, Berlin, Germany.
42. Vester, B., and S. Douthwaite. 1994. Domain V of 23S rRNA contains all the structural elements necessary for recognition by the ErmE methyltransferase. *J. Bacteriol.* **176**:6999–7004.
43. Vester, B., and S. Douthwaite. 2001. Macrolide resistance conferred by base substitutions in 23S rRNA. *Antimicrob. Agents Chemother.* **45**:1–12.
44. Weisblum, B. 1995. Erythromycin resistance by ribosome modification. *Antimicrob. Agents Chemother.* **39**:577–585.
45. Weisblum, B. 1998. Macrolide resistance. *Drug Resist. Updates* **1**:29–41.
46. Xiong, L., S. Shah, P. Mauvais, and A. S. Mankin. 1999. A ketolide resistance mutation in domain II of 23S rRNA reveals the proximity of hairpin 35 to the peptidyl transferase centre. *Mol. Microbiol.* **31**:633–639.
47. Zalacain, M., and E. Cundliffe. 1989. Methylation of 23S rRNA caused by *tlrA* (*ermSF*), a tylosin resistance determinant from *Streptomyces fradiae*. *J. Bacteriol.* **171**:4254–4260.
48. Zalacain, M., and E. Cundliffe. 1991. Cloning of *tlrD*, a fourth resistance gene, from the tylosin producer, *Streptomyces fradiae*. *Gene* **97**:137–142.
49. Zhong, P., Z. Cao, R. Hammond, Y. Chen, J. Beyer, V. D. Shortridge, L. Y. Phan, S. Pratt, J. Capobianco, K. A. Reich, R. K. Flamm, Y.-S. Or, and L. Katz. 1999. Induction of ribosomal methylation in MLS-resistant *Streptococcus pneumoniae* by macrolide and ketolides. *Microb. Drug Resist.* **5**:183–188.