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<sub>B</sub>) 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 molecules are monomethylated by ErmN (TlrD) or dimethylated by ErmE. ErmE dimethylation confers high resistance to all the MLS<sub>B</sub> 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<sub>B</sub> 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<sub>B</sub>) 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<sub>B</sub> 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<sub>B</sub> 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<sub>B</sub> 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 linco-samides (6). Dimethylation of A2058 confers high resistance to all the MLS<sub>B</sub> drugs, and this is generally considered the classical MLS<sub>B</sub> resistance phenotype (44). The distribution of the two forms of Erm is generally consistent with these phenotypes: Erm monomethyltransferases are sufficient to ensure the survival of lincomamide-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<sub>B</sub> 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<sub>B</sub>-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.
is susceptible to all types of MLSB 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 MLSB and ketolide drugs. After methylation of A2058, the bacteria exhibited resistance patterns toward established MLSB 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 MLSB-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, Tc r; Kanr This study. 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 Kanr cassette was inserted into the middle of *rrmA* within its unique SmaI site. The *rrmA*::kan fragment was then cleaved with EcoRI/BglII 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 gene *ermN*), or pSDdiv (which contains the dimethyltransferase *ermE*). For each strain, approximately 10^4 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-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 (Table 2) was used in a polymerase chain reaction (PCR) to amplify a fragment of the target site of the 23S rRNA from strain *AS19*, leaving a 20-bp sequence on both sides of the target site. The PCR reaction was performed in a 40-μl volume containing 1× reaction buffer (Promega), 1 μM of each primer, 200 μM of each dNTP, 0.5 U of Taq polymerase, and 1 μl of the *AS19* DNA template. The reaction was performed in a thermocycler for 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The PCR fragments were digested with the restriction enzyme *BsrGI* and separated on a 2% agarose gel. The amount of dimethylation was determined by densitometry of the autoradiogram produced by excising the bands from the gel, radiography, and scanning the autoradiogram 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 MLSB 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 MLSB 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-RrmA.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (µg/ml)</th>
<th>No methylation</th>
<th>Monomethylation</th>
<th>Dimethylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythromycin</td>
<td>0.5</td>
<td>128</td>
<td>1,024</td>
<td></td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>0.25</td>
<td>32</td>
<td>512</td>
<td></td>
</tr>
<tr>
<td>Telithromycin</td>
<td>0.5</td>
<td>2</td>
<td>512</td>
<td></td>
</tr>
<tr>
<td>HMR 3004</td>
<td>0.25</td>
<td>0.5</td>
<td>256</td>
<td></td>
</tr>
<tr>
<td>Lincomycin</td>
<td>16</td>
<td>&gt;8,000</td>
<td>8,000</td>
<td></td>
</tr>
<tr>
<td>Clindamycin</td>
<td>2</td>
<td>512</td>
<td>512</td>
<td></td>
</tr>
<tr>
<td>Pristinamycin IA</td>
<td>32</td>
<td>64</td>
<td>&gt;128</td>
<td></td>
</tr>
</tbody>
</table>

*: 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-RrmA 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.

a: Insufficient amounts of pristinamycin IA were available for us to determine the absolute MIC for the dimethylation strain.

expression of the Erm mono- and dimethyltransferases.

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), while conferring about 8-fold resistance to telithromycin and HMR 3004 (Liu, unpublished data).

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 rrmA, on the AS19 chromosome. MICs of drugs for the AS19-RrmA strain are shown in Table 2. For the macrolide, ketolide, and lincosamide drugs, these are essentially identical to the results with the RrmA strain (taking into account that the RrmA strain grows slightly more slowly).

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 indirectly 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 Streptococcus luidians, 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).

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 rrmA, 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.
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 macroline 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 MLSB 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 MLSB 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 MLSB- and 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


Downloaded from http://aac.asm.org/ on March 28, 2021 by guest