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Induction of Efflux-Mediated Macrolide Resistance in *Streptococcus pneumoniae* 

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The antimicrobial efflux system encoded by the operon mef(E)-mel on the mobile genetic element MEGA in *Streptococcus pneumoniae* and other Gram-positive bacteria is inducible by macrolide antibiotics and antimicrobial peptides. Induction may affect the clinical response to the use of macrolides. We developed mef(E) reporter constructs and a disk diffusion induction and resistance assay to determine the kinetics and basis of mef(E)-mel induction. Induction occurred rapidly, with a >15-fold increase in transcription within 1 h of exposure to subinhibitory concentrations of erythromycin. A spectrum of environmental conditions, including competence and nonmacrolide antibiotics with distinct cellular targets, did not induce mef(E). Using 16 different structurally defined macrolides, induction was correlated with the amino sugar attached to C-5 of the macrolide lactone ring, not with the size (e.g., 14-, 15- or 16-member) of the ring or with the presence of the neutral sugar cladinose at C-3. Macrolides with a monosaccharide attached to C-5, known to block exit of the nascent peptide from the ribosome after the incorporation of up to eight amino acids, induced mef(E) expression. Macrolides with a C-5 disaccharide, which extends the macrolide into the ribosomal exit tunnel, disrupting peptidyl transferase activity, did not induce it. The induction of mef(E) did not require macrolide efflux, but the affinity of macrolides for the ribosome determined the availability for efflux and pneumococcal susceptibility. The induction of mef(E)-mel expression by inducing macrolides appears to be based on specific interactions of the macrolide C-5 saccharide with the ribosome that alleviate transcriptional attenuation of mef(E)-mel.

Macrolides are broad-spectrum antibiotics with complex macrocyclic 14-, 15-, or 16-member lactone rings that bind in the peptide exit tunnel of bacterial ribosomes and inhibit protein synthesis. Macrolides are often recommended as the empirical first-line treatment for upper respiratory bacterial infections, including pneumococcal infections and community-acquired pneumonia. However, bacterial resistance to macrolides is expanding worldwide in Gram-positive bacteria and is now present in almost a third of all invasive *Streptococcus pneumoniae* isolates (14). The two most common mechanisms of macrolide resistance in bacterial pathogens are modification of the bacterial ribosome, either by methylation or mutation, and extrusion of the drugs from the bacterial cell by an efflux pump. Genes of the erm (erythromycin ribosomal methylases) family of rRNA methylases confer high-level resistance to lincosamides and streptogramins, as well as macrolides (the MLS*\(_\text{B}\)* phenotype), and can be constitutive or inducible. In the inducible form, resistance develops only after exposure of the bacterium to the macrolide.

The best-studied mechanism of inducible MLS*\(_\text{B}\)* resistance involves the erm(C) gene found in *S. aureus* and other Gram-positive pathogens (4, 32, 33). Translation of erm(C) is attenuated in the absence of inducers due to secondary structures that render the ribosomal binding site inaccessible. Inducer-bound ribosomes stall during the translation of a 19-amino acid leader peptide upstream of erm(C), resulting in the refolding of the transcript to make the ribosome binding site available, thus promoting translation (32). A later comparison of inducers for erm(C) and erm(SV) in *Streptomyces viridochromogenes* showed that the specific sequence of the leader peptide determines the range of inducers (24). Macrolides with 14-membered rings and C-3 cladinose induced erm(C), and 16-membered macrolides induced erm(SV) (24). The lincosamide celesticetin induced both erm(C) and erm(SV), demonstrating a common inducer between the otherwise disparately induced genes (24). Attenuation of erm genes can also occur at the level of transcription, as with *erm(K)* of *Bacillus licheniformis* (28, 29). Transcriptional attenuation occurs when secondary structures in the transcript function like rho-independent terminators to terminate transcription short of the erm gene coding region.

Efflux-mediated macrolide resistance was first reported in *Staphylococcus epidermidis* and later in *Streptococcus pyogenes* and *S. pneumoniae* (30, 40, 45). In *S. epidermidis*, macrolide efflux is conferred by msr(A) and is induced by the 14- and 15-membered macrolides clarithromycin and azithromycin and the ketolide telithromycin but not by streptogramin B, even though the latter is a substrate for Msr(A)-mediated efflux. In streptococci, macrolide efflux is conferred by proteins encoded by mef(E), mef(A), or less commonly, mef(I), which share >90% identity. The mef genes are transcribed as an operon with the msr(A) homolog mel [also called msr(D)], comprising
a dual efflux pump (3, 12). In *S. pneumoniae*, mef(E)-mel is carried on the small mobile element MEGA, the predominant macrolide efflux determinant in the pneumococcus (15, 43). *In vitro*, MeF(E)/Mel confers moderate resistance (1 to 32 μg/ml) to 14- and 15-membered macrolides but not, reportedly, to 16-membered macrolides, lincosamides, or streptogramins (M phenotype) (45, 47). MeF(E)/Mel-mediated resistance has been shown to be induced by the 14- and 15-membered macrolides erythromycin, clarithromycin, and azithromycin but not to 16-membered macrolides (3, 49). Ketolides, such as telithromycin, are considered poor inducers of mef(E)-mel and retain antimicrobial activity against pneumococcal strains carrying these genes (34, 49).

*In vivo* exposure to macrolides or other potential inducers may result in resistance higher than the levels predicted by MICs determined in *vitro*. We recently reported that certain antimicrobial peptides (AMP), including the human AMP LL-37, activated transcription of mef(E)-mel and resulted in induced resistance to the macrolide erythromycin (50). This suggests that the MeF(E)/Mel efflux pump can be induced by human host defenses and, therefore, can be primed prior to clinical administration of macrolides. The goals of this study were to define the range of compounds, macrolides as well as other antibiotics and other conditions, that induce MeF(E)/Mel-mediated resistance, to elucidate the kinetics of induction, to determine the structural features of macrolides required for induction, and to develop a model for the mechanism of induction. To accomplish these goals, mef(E)-lacZ reporter fusion constructs and a disk diffusion-based assay were developed that allowed the assessment of both the induction of mef(E)-mel and efflux-mediated macrolide resistance.

**MATERIALS AND METHODS**

**Bacterial strains.** Construction of the mef(E)-lacZ reporter strain XZ7042 and the MEGA element deletion derivative XZ8004 from the erythromycin-resistant, MEGA-containing *S. pneumoniae* clinical isolate GA17457 was as previously described (50). The negative-control strain XZ7049 was generated by insertion of the promoterless lacZ of pPP2 (18) into bgaA of GA17457. Likewise, XZ7067 was generated by insertion of the comC-lacZ transcriptional fusion on plasmid pPC2 (18) into GA17457.

**Quantitative β-galactosidase assays.** The rate of induction of mef(E) by erythromycin was determined by adding 0.1 μg/ml erythromycin to mid-log-phase cultures (optical density at 600 nm [OD₆₀₀] ~0.3 to 0.4). Test strains were incubated at 37°C in parallel with uninduced cultures. At 30-min intervals, cultures were sampled for assessment of β-galactosidase (β-Gal) activity as described previously (35). To determine a transcriptional dose response of mef(E)-lacZ, parallel cultures of XZ7042 and XZ8004 were grown to mid-log phase (OD₆₀₀ ~0.3 to 0.4) and exposed to concentrations of erythromycin varying by 4 orders of magnitude or more. Each subculture was harvested 1 h after induction for assessment of β-galactosidase activity. All experiments were performed in duplicate, and assay readings were taken in triplicate.

**Antibiotics.** Antibiotic susceptibility disks were either obtained commercially or prepared by application of stock solutions to sterile blank disks, followed by drying for 15 min in a laminar flow hood. Susceptibility disks containing the macrolides azithromycin, clarithromycin, erythromycin, telithromycin, and tilmicosin were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ). Disks were prepared for the macrolides dirithromycin, josamycin, midecamycin, roxithromycin, and spiramycin (Sigma-Aldrich, St. Louis, MO) and for kitsamycin (MP Biomedicals, Solon, OH), oleandomycin (Crescent Chemical Co., Inc., Islandia, NY), troleandomycin (Enzo Life Sciences, Plymouth Meeting, PA), and tylosin (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The macrolide tulathromycin was obtained as the animal health product Draxxin from Pfizer Animal Health (Kalamazoo, MI). Commercially prepared antibiotic disks containing bacitracin, clindamycin, colistin, levofloxacin, lincomycin, polymyxin B, and trimethoprim-sulfamethoxazole (Becton, Dickinson and Company, Franklin Lakes, NJ) and quinupristin-dalfopristin (Remel, Lenexa, KS) were obtained. Disks were prepared as described above for the nonmacrolide antibiotics amoxicillin, ampicillin, penicillin, cefotaxime, ceftriaxone, cefuroxime, ciprofloxacin, chloramphenicol, tetracycline, gentamicin, kanamycin, spectinomycin, rifampin, and vancomycin (Sigma-Aldrich, St. Louis, MO) and mupirocin (AppliChem GmbH, Darmstadt, Germany). Cyclopentadecanolide was obtained from Acros Organics (Geel, Belgium).

### Susceptibility assays.**

The MIC of erythromycin was determined by Etest according to the manufacturer’s recommendations (AB bioMérieux, Solna, Sweden). Briefly, strains incubated overnight at 37°C in 5% CO₂ on Trypticase soy agar with 5% sheep blood (TSA-SB) (Becton, Dickinson and Company, Franklin Lakes, NJ) were suspended in Mueller-Hinton broth to a 0.5 McFarland standard and spread onto Mueller-Hinton agar with 5% sheep blood (MH-SB) (Becton, Dickinson and Company, Franklin Lakes, NJ). Etest strips were placed on the plate surface, and the plates were incubated overnight at 37°C in 5% CO₂. Susceptibility to other macrolides was determined by disk diffusion in accordance with the standards described by the Clinical and Laboratory Standards Institute (11). To determine the effects of erythromycin induction on resistance, MICs were determined by Etest as described above except that strains were incubated on TSA-SB supplemented with 1/10 MIC erythromycin prior to suspension and plating on MH-SB. Susceptibility to other macrolides was determined by disk diffusion in accordance with the standards described by the Clinical and Laboratory Standards Institute (11). Susceptibility disks were purchased or prepared as described above. All susceptibility data represent the means ± standard deviations of at least five independent replications and were analyzed by two-tailed, paired *t*-tests. *P* values of >0.05 were considered significant.

**Disk diffusion assay for mef(E)-lacZ induction.** The mef(E)-lacZ reporter strain XZ7042 was grown to mid-log phase in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY). The liquid culture was swabbed onto TSA supplemented with 300 U/ml catalase (Sigma-Aldridge, St. Louis, MO) and 0.032% X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside). Susceptibility disks infused with test compounds were immediately placed on the plates, and the plates were incubated at 37°C, 5% CO₂ for 24 h. Induction by synthetic competence-stimulating peptide-1 (CSP-1) (20) was tested by spotting 15 μl of a 100 ng/ml solution directly to the center of an indicator plate swabbed with the indicated strain. CSP-1 was synthesized by the Emory University Microchemical Facility.

### RESULTS

Characterization of erythromycin-induced expression of mef(E)-mel. The kinetics and dose dependence of mef(E)-mel induction were determined using the prototype macrolide erythromycin and a series of isogenic reporter strains that contained a mef(E)-lacZ transcriptional fusion in the *S. pneumoniae* strain GA17457 (Table 1) (50). The wild-type reporter strain XZ7042 contained a functional MEGA element encoding the MeF(E)/Mel efflux pump that confers resistance to erythromycin (MIC 12 μg/ml), while the MEGA deletion derivative, XZ8004, was erythromycin susceptible (MIC of 0.1 μg/ml) (50). A promoterless lacZ reporter strain, XZ7049, which was otherwise identical to XZ7042, served as a negative control. The induction of mef(E) transcription in XZ7042, expressed as β-Gal activity, was measured over time following the addition of subinhibitory concentrations of erythromycin (1/10 of the MIC). β-Gal activity increased rapidly upon the addition of erythromycin, by more than 7-fold within the first 30 min postinduction (Fig. 1a). Maximal induction (>15-fold increase) was obtained at 1 h (Fig. 1a). The β-Gal activities of XZ7042 cultures without induction were equivalent to those of the induced and uninduced promoterless negative control, indicating a very low level of constitutive expression from the mef(E) promoter in the absence of an inducer (Fig. 1a).

The role of a functional MEGA element in mef(E) induction was assessed by measuring the erythromycin dose response in XZ7042 and the MEGA deletion derivative XZ8004. The
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TABLE 1. Strains and plasmids used in this work

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. pneumoniae strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GA17457</td>
<td>Wild type, parent; MEGA Ery&lt;sup&gt;a&lt;/sup&gt;</td>
<td>50</td>
</tr>
<tr>
<td>XZ7042</td>
<td>GA17457 derivative; bgaA::mef(E)-lacZ MEGA Ery&lt;sup&gt;a&lt;/sup&gt;</td>
<td>50</td>
</tr>
<tr>
<td>XZ8004</td>
<td>GA17457 derivative; bgaA::mef(E)-lacZ MEGA:aad&lt;sup&gt;9&lt;/sup&gt; Ery&lt;sup&gt;a&lt;/sup&gt;</td>
<td>50</td>
</tr>
<tr>
<td>XZ7067</td>
<td>GA17457 derivative; bgaA::comC-lacZ MEGA Ery&lt;sup&gt;a&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>XZ7049</td>
<td>GA17457 derivative; bgaA::lacZ (promoterless) MEGA Ery&lt;sup&gt;a&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pPP2</td>
<td>pPP1 derivative, lacZ reporter gene with the htrA ribosomal binding site</td>
<td>18</td>
</tr>
<tr>
<td>pPC2</td>
<td>pPP2 derivative, carries comC-lacZ fusion</td>
<td>18</td>
</tr>
</tbody>
</table>

Strains were incubated with erythromycin concentrations beginning with 0.0012 µg/ml and increasing by one-half- or one-quarter-log intervals (Fig. 1b). In XZ8004, mef(E) was induced at lower erythromycin concentrations than in XZ7042 (0.012 and 0.04 µg/ml, respectively) and with higher maximal expression (205.3 Miller units [MU] and 106.9 MU, respectively) (Fig. 1b). However, the peak expression of mef(E) in XZ8004 occurred at a concentration equivalent to the erythromycin MIC (0.12 µg/ml) of the strain, while in XZ7042, peak expression was observed at a concentration (0.4 µg/ml) 30-fold less than the strain’s erythromycin MIC (12 µg/ml) (Fig. 1b). Expression in XZ8004 decreased rapidly with increasing erythromycin concentrations above the MIC (Fig. 1b), consistent with an inhibitory effect of erythromycin. The mef(E)-lac<sup>Z</sup> induction in XZ8004 indicated that a functional MEGA element was not required for the induction of mef(E)-mel; however, the strain’s susceptibility to the inducer and its ability to modify intracellular concentrations of the inducer contributed to the magnitude and duration of mef(E)-mel induction.

To evaluate mef(E)-mel expression over a range of inducers and inducer concentrations, a disk diffusion assay using the XZ7042 reporter strain was developed (42). In contrast to the quantitative β-Gal assays, this approach provided a concentration gradient, allowing the detection of mef(E)-lac<sup>Z</sup> induction without a priori knowledge of the MIC of the test compound or the time-dependent induction kinetics and was suitable for study of a broad range of potential inducers. Figure 1c shows the results of the disk diffusion assays, using erythromycin as the test compound, with the wild-type reporter XZ7042, the MEGA deletion strain XZ8004, and the promoterless control strain XZ7049. Strain XZ7042 showed a characteristic large blue halo, indicative of the induction of mef(E)-lac<sup>Z</sup>, surrounding a narrow zone of inhibited growth (Fig. 1c). As anticipated, the MEGA deletion strain XZ8004 showed a narrow range of induction surrounding a large zone of inhibition (Fig. 1c). This result was consistent with the results of the quantitative assay (Fig. 1b), showing a wide range of inducing concentrations for XZ7042 and a strong but narrow range of futile induction of mef(E) in strain XZ8004 in the absence of the resistance determinant MEGA and the efflux pump (Fig. 1b). The promoterless-lac<sup>Z</sup> strain XZ7049 showed the same zone of inhibition as XZ7042, as expected due to a functional MEGA element; however, no β-Gal activity (i.e., blue halo) was detected (Fig. 1c), confirming that the mef(E)-lac<sup>Z</sup> expression in the reporter strain XZ7042 depended exclusively on mef(E).
Environmental or antibiotic (nonmacrolide) stress and competence did not induce \textit{mef(E)}-lacZ. To assess the spectrum of inducers of \textit{mef(E)}-mel expression, different environmental conditions and antibiotic compounds were analyzed in the disk diffusion assay using XZ7042. Nonmacrolide antibiotics were chosen to include representatives of major antimicrobial classes with diverse cellular targets, thus providing a wide range of antibiotic stresses. Also tested were environmental conditions involved in the regulation of other genetic systems, as well as known substrates for multidrug efflux systems. None of the tested compounds and conditions induced \textit{mef(E)}-lacZ expression, with the exception of the previously described antimicrobial peptides LL-37, CRAMP38, and CRAMP39 (50). These results demonstrated that \textit{mef(E)}-mel induction is restricted to a narrow range of compounds, suggesting one or more well-defined mechanism(s) of induction.

In addition, competence was tested as a potentially inducing condition of \textit{mef(E)} induction. Competence induction in \textit{S. pneumoniae} has been known to affect the expression of a broad range of genes and is mediated by the \textit{com} regulon (1, 2, 21, 31). Prudhomme et al. (39) showed that the \textit{com} regulon was induced in response to antibiotic stress. Since it was unknown whether the conditions of the disk diffusion assay supported the development of natural competence, synthetic competence-stimulating peptide (CSP-1) was used to induce competence and monitor the effect on \textit{mef(E)}-lacZ induction in the lacZ disk diffusion assay. The results in Fig. 2 show that erythromycin but not CSP-1 induced \textit{mef(E)}-lacZ in XZ7042. As a control for competence development, a reporter strain, XZ7067, which carried the lacZ gene under the control of the promoter for the early competence gene \textit{comC}, was constructed (Table 1). XZ7067 was induced by CSP-1, confirming competence induction under the assay conditions. Erythromycin did not induce \textit{comC} (Fig. 2), which is consistent with the previous observation (36) that erythromycin does not induce competence. Thus, general environmental and antibiotic stresses, as well as the development of competence, did not induce \textit{mef(E)}-mel expression.

**Induction of \textit{mef(E)} by macrolides.** To understand the relationship between macrolide structure and \textit{mef(E)}-mel induction, a panel of 16 macrolides composed of 14-, 15-, and 16-membered compounds were studied using the disk diffusion assay. The 14-membered cladinosolides, compounds with the lactone ring replaced with the sugar cladinose at C-3 and the monosaccharide amino sugar desosamine at C-5 (e.g., erythromycin, clarithromycin, dirithromycin, and roxithromycin) (Fig. 3a), each strongly induced β-Gal activity over a wide induction zone (i.e., concentration gradient) (Fig. 3a). Induction was correlated with decreased susceptibility to these compounds in the MEGA-containing reporter strain but not in the susceptible MEGA deletion strain \((P < 0.001)\) (Fig. 3a and Table 2). In addition, preincubation of the reporter strains with a subinhibitory concentration of erythromycin (1/10 MIC, 1.2 µg/ml) further increased the resistance to these compounds for the wild-type MEGA-containing reporter strain \((P < 0.05)\) (Table 2) but not for the MEGA deletion strain. Thus, resistance in the wild-type strain required the efflux-encoding MEGA element and suggested that erythromycin, clarithromycin, clarithromycin, clarithromycin, and roxithromycin are substrates for MeF(E)/Mel-mediated efflux. These findings were consistent with those of earlier reports for 14- and 15-membered macrolides (3, 34, 49).

Two 14-membered macrolides, oleandomycin and troleandomycin, with the substitution of oleandrose for the cladinose in the lactone ring, were tested (Fig. 3c). The induction of \textit{mef(E)}-mel by oleandomycin resembled the strong induction observed with the cladinosolides; however, troleandomycin was a poor inducer of \textit{mef(E)} (Fig. 3c). Troleandomycin has three acetate substitutions of the hydroxyl groups of oleandomycin (Fig. 3c); the results suggest that one or more of these acetate groups interfered with \textit{mef(E)} induction. The wild-type reporter strain XZ7042 was significantly more susceptible to troleandomycin than to oleandomycin \((P < 0.001)\) (Table 2). To determine whether the increased susceptibility of XZ7042 to troleandomycin compared to its susceptibility to oleandomycin was due to the weak \textit{mef(E)} induction by troleandomycin, the reporter strain XZ7042 was preincubated with a subinhibitory concentration of erythromycin (0.12 µg/ml) to achieve high induction of \textit{mef(E)}-mel prior to susceptibility testing with troleandomycin. Under these conditions, the resistance to troleandomycin increased to the levels of resistance to oleandomycin, indicating that troleandomycin was a substrate for efflux, despite being a poor \textit{mef(E)}-mel inducer.

The ketolide telithromycin is a 14-membered macrolide that belongs to the ketolide subclass due to a C-3 ketone substitution instead of cladinose (Fig. 3b). Telithromycin retains excellent antimicrobial activity against MeF(E)/Mel-containing pneumococci (12, 49). However, more detailed reports showed that the efflux pump conferred small increases in pneumococcal MICs of telithromycin after preincubation with subinhibitory concentrations of telithromycin (12, 49) or in comparison to the MICs of clinical isolates with and without \textit{mef(E)}-mel (46). To determine whether the lack of telithromycin resistance was due to an inefficient induction of \textit{mef(E)}-mel expression or poor efflux of telithromycin by MeF(E)/Mel, the inducing ability of telithromycin was tested in the disk diffusion assay.
Telithromycin strongly induced mef(E), and the observed induction pattern was quite similar to that observed for the MEGA deletion mutant XZ8004 with erythromycin (Fig. 1c), i.e., a narrow zone of strong mef(E) induction surrounding a large zone of inhibition of growth, which indicated that the wild-type reporter strain remained susceptible to telithromycin despite the strong induction of mef(E)-lacZ. This was confirmed by telithromycin resistance (Table 2). As expected, preincubation with erythromycin had no significant effect on telithromycin resistance, although the MEGA mutant was more susceptible to telithromycin than the MEGA-containing strain (P < 0.001) (Table 2). These data suggest that telithromycin,

FIG. 3. Structure and disk diffusion induction assays for the 14- and 15-membered macrolides used in this study. Discs containing 15 μg/ml of each macrolide were placed onto TSA indicator plates spread with XZ7042. (a) Cladinosolides. Erythromycin and derivatives each contain a C-3 cladinose. Previously described interactions between cladinosolides and the 23S rRNA (E. coli numbering) are indicated by dashed arrows. 1, erythromycin; 2, clarithromycin; 3, dirithromycin; 4, roxithromycin. (b) Telithromycin. The C-11/C-12 carbamate with a aryl-alkyl side chain is responsible for increased ribosomal affinity and compensates for the absence of cladinose at C-3 (19). 1, erythromycin; 2, telithromycin. (c) Oleandomycins. Oleandomycin and troleandomycin differ only by the acetylation of hydroxyl groups at three positions in troleandomycin (R1, R2, and R3). The dashed arrow indicates interaction of the acetyl group of the oleandrose of troleandomycin and the 23S rRNA nucleotide U790 (E. coli numbering). 1, erythromycin; 2, oleandomycin; 3, troleandomycin. (d) 15-Membered azalides. Previously described interaction between azithromycin cladinose hydroxyl group and the 23S rRNA nucleotide G2505 (E. coli numbering, dashed arrow) (19). C-5 desosamine is indicated in red. 1, erythromycin; 2, azithromycin; 3, tulathromycin.
while a strong inducer of mef(E)-mel, was not sufficiently
excluded by the Mef(E)/Mel efflux pump to allow resistance to
develop.

The two 15-membered macrolides, azithromycin and tulath-
romycin (Table 2 and Fig. 3d), were studied. Tulathromycin
differs from azithromycin by a demethylation of N-10 and by a
propylamino side chain substitution on the C-3 cladinose (Fig.
differs from azithromycin by a demethylation of N-10 and by a
3d). However, while mef(E) induction by azithromycin was
comparsable to that of erythromycin, induction by tulathrom-
ycin was weaker. XZ7042 was slightly more susceptible to tu-
lathromycin than to azithromycin, suggesting that the increase
in antimicrobial activity of tulathromycin could be due to the
weaker induction of mef(E)-mel. To test this, XZ7042 was
preincubated with erythromycin (1.2 μg/ml). Increased resis-
tance to tulathromycin, to the level of resistance to azithromy-
cin, was noted. However, the weaker induction by tulathromy-
cin was still sufficient to render the MEGA-containing strain
XZ7042 less susceptible than the MEGA deletion strain
XZ8004 (Table 2).

16-Membered macrolides can induce mef(E)-mel expression.
A current paradigm is that the Mef(E)/Mel efflux pump does
not confer resistance to 16-membered macrolides (34). Possi-
ble explanations include the inability of 16-membered macro-
loides to induce the efflux pump, structural feature(s) of the
compounds that preclude them as substrates for the efflux
pump, or sequestration, e.g., at the ribosome, making these
compounds unavailable for efflux. Seven 16-membered macro-
loides, josamycin, kitasamycin, midecamycin, rosamicin, spir-
amycin, tilmicosin, and tylosin, were studied for induction of
mef(E)-lacZ (Fig. 4). Josamycin, kitasamycin, midecamycin,
spiramycin, and tylosin did not induce mef(E)-lacZ expression
(Fig. 4). Surprisingly, two 16-membered macrolides, tilmicosin
and rosamicin, induced the mef(E)-lacZ reporter (Fig. 4b). All
the 16-membered macrolides lacked a cladinose at C-3. While
the cladinose has been suggested as an important feature of
inducing 14- and 15-membered macrolides (8), the above-
noted 14- or 15-membered macrolides with modifications of
the cladinose (oleandomycin and troleandomycin) or with a
ketone substitution (telithromycin) at C-3 were also inducers.
In contrast, the common feature of the 16-membered inducers
(tilmicosin and rosamicin) and the inducing 14- and 15-mem-
ered macrolides, including telithromycin, was the presence of
a monosaccharide at C-5 (Fig. 4b). All noninducing macrolides
possessed a disaccharide moiety at this position. The require-
ment of a monosaccharide at C-5 was supported by the data for
rosamicin, which did not

### Table 2. Induction of the Mef(E)/Mel efflux pump and pneumococcal susceptibility

<table>
<thead>
<tr>
<th>Test drug</th>
<th>No. of ring members&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Side group at C-3</th>
<th>Presence of ac-AH at C-6&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Inhibition zone diameter (mm) for&lt;sup&gt;c&lt;/sup&gt;</th>
<th>XZ7042</th>
<th>XZ8004</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+ERY</td>
<td>-ERY</td>
<td>+ERY</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>14</td>
<td>Cla Des</td>
<td>−</td>
<td>9.7 ± 0.5</td>
<td>7.8 ± 0.4</td>
<td>29.0 ± 1.4</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>14</td>
<td>Cla Des</td>
<td>−</td>
<td>9.4 ± 0.5</td>
<td>8.2 ± 0.4</td>
<td>28.0 ± 2.0</td>
</tr>
<tr>
<td>Dirithromycin</td>
<td>14</td>
<td>Cla Des</td>
<td>−</td>
<td>8.1 ± 0.4</td>
<td>7.0 ± 0.8</td>
<td>22.2 ± 0.8</td>
</tr>
<tr>
<td>Roxithromycin</td>
<td>14</td>
<td>Cla Des</td>
<td>−</td>
<td>8.4 ± 0.5</td>
<td>6.4 ± 0.5</td>
<td>24.4 ± 1.1</td>
</tr>
<tr>
<td>Oleandomycin</td>
<td>14</td>
<td>Ole Des</td>
<td>−</td>
<td>9.6 ± 0.5</td>
<td>6.9 ± 0.9</td>
<td>19.6 ± 0.5</td>
</tr>
<tr>
<td>Troleandomycin</td>
<td>14</td>
<td>Ac-ole Ac-des</td>
<td>−</td>
<td>12.5 ± 1.0</td>
<td>7.3 ± 0.8</td>
<td>16.5 ± 0.5</td>
</tr>
<tr>
<td>Telithromycin</td>
<td>14</td>
<td>OH Des</td>
<td>−</td>
<td>20.6 ± 1.1</td>
<td>19.0 ± 1.4</td>
<td>31.2 ± 0.4</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>15</td>
<td>Cla&lt;sup&gt;b&lt;/sup&gt; Des</td>
<td>−</td>
<td>7.6 ± 0.5</td>
<td>6.4 ± 0.5</td>
<td>22.4 ± 1.1</td>
</tr>
<tr>
<td>Tulathromycin</td>
<td>15</td>
<td>Cla&lt;sup&gt;b&lt;/sup&gt; Des</td>
<td>−</td>
<td>8.7 ± 0.5</td>
<td>6.6 ± 0.5</td>
<td>17.4 ± 2.7</td>
</tr>
<tr>
<td>Josamycin</td>
<td>16</td>
<td>OH Myn-myr</td>
<td>+</td>
<td>23.4 ± 3.8</td>
<td>20.4 ± 1.1</td>
<td>22.2 ± 1.3</td>
</tr>
<tr>
<td>Kitasamycin</td>
<td>16</td>
<td>OH Myn-myr</td>
<td>+</td>
<td>23.2 ± 1.3</td>
<td>22.2 ± 1.3</td>
<td>23.2 ± 1.3</td>
</tr>
<tr>
<td>Midecamycin</td>
<td>16</td>
<td>OH Myn-myr</td>
<td>+</td>
<td>20.2 ± 1.3</td>
<td>19.8 ± 0.8</td>
<td>21.2 ± 0.8</td>
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<tr>
<td>Tylosin</td>
<td>16</td>
<td>OH Myn-myr</td>
<td>+</td>
<td>19.0 ± 0.7</td>
<td>19.4 ± 0.5</td>
<td>18.4 ± 1.1</td>
</tr>
<tr>
<td>Rosamicin</td>
<td>16</td>
<td>OH Des</td>
<td>+</td>
<td>19.0 ± 1.4</td>
<td>18.0 ± 0.7</td>
<td>19.2 ± 1.9</td>
</tr>
<tr>
<td>Spiramycin</td>
<td>16</td>
<td>OH Myn-myr</td>
<td>+</td>
<td>10.5 ± 1.2</td>
<td>8.2 ± 0.7</td>
<td>13.2 ± 0.9</td>
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<tr>
<td>Tilmicosin</td>
<td>16</td>
<td>OH Myn</td>
<td>+</td>
<td>10.5 ± 1.2</td>
<td>8.2 ± 0.7</td>
<td>13.2 ± 0.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of atoms in the lactone ring.
<sup>b</sup> Side group attached at the C-3 or C-5 position of the lactone ring. Cla, cladinose; Cla<sup>b</sup>, modified cladinose; Ole, oleandrose; Ac-ole, acetylated oleandrose; Des, desosamine; Ac-des, acetylated desosamine; Myn, mycaminose; Myr, mycarose.
<sup>c</sup> Complete resistance, defined by growth at the edge of the disk, was scored as 6 mm, i.e., equal to the diameter of the disk. All disks contained 15 μg of the macrolide.

Values in bold vary significantly (<i>P < 0.05</i>) from that of the uninduced control (XZ7042 + ERY) for the same drug. Shaded pairs of values are significantly different (i.e., <i>P < 0.05</i>).
disk diffusion assays for induction, in which no D-zone of clearing was produced by the noninducing macrolides in the presence of erythromycin (data not shown).

Still remaining was the question of whether the induction of mef(E) observed with the inducing 16-membered macrolides rosamycin and tilmicosin translated into increased resistance to these compounds. The strong, narrow induction ring observed in the disk-diffusion induction assay with tilmicosin indicated a narrow range of inducing concentrations; however, the small zone of inhibition indicated pneumococcal resistance to tilmicosin (Fig. 4b). Preincubation of XZ7042 with erythromycin resulted in further resistance to tilmicosin compared to the resistance of the uninduced control (Table 2), indicating that tilmicosin was subjected to Mef(E)/Mel-mediated efflux. Conversely, the induction of mef(E) by rosamycin (Fig. 4b) resembled that observed with telithromycin (Fig. 3b) and the MEGA deletion strain XZ8004 (Fig. 1c), and XZ7042 remained a susceptible phenotype despite induction (Table 2). Indeed, there was no change in susceptibility to rosamycin between the MEGA deletion strain XZ8004 and the wild-type strain XZ7042 despite the induction of mef(E)-mel (Table 2).

These data further demonstrate the uncoupling of macrolide induction of mef(E)-mel expression and macrolide efflux. The results with 16-membered macrolides further demonstrated that for induction of mef(E)-mel, a monosaccharide in position C-5 of the macrolide was required, but this was not sufficient to confer Mef(E)/Mel-mediated resistance. Thus, MEGA-containing isolates of S. pneumoniae remained susceptible to most 16-membered macrolides, independent of the capacity to induce mef(E)-mel.

**DISCUSSION**

Mechanisms of S. pneumoniae resistance to macrolides include modification of macrolide target sites on the 23S ribosomal subunit, either by mutation or erm methylases, and macrolide efflux by the Mef(E)/Mel efflux pump encoded on the MEGA element (16, 23, 44, 45, 47, 51).
North America and many other parts of the world, Mef(E)/Mel is the predominant mechanism of pneumococcal macrolide resistance (16, 43). Approximately half of the macrolide-resistant pneumococci isolated in the United States in 2006 contained mef(E)-mel as the sole macrolide resistance determinant, and an additional 25% contained both mef(E)-mel and erm(B) (22). The resistance levels conferred by Mef(E)/Mel in vitro (erythromycin 1 to 32 µg/ml) are lower than the levels conferred by ribosome modification; nevertheless, these levels are correlated with macrolide treatment failures (25, 26). Exposure in vivo to inducers of the efflux pump may lead to levels of resistance at the site of infection higher than those predicted by in vitro assays. Mef(E)/Mel resistance can be induced by up to a 200-fold increase in MICs (3, 49) and, therefore, could be of significant clinical impact. This background led us to further study the induction of macrolide efflux, in order to identify structural features that differentiate inducing and noninducing macrolides and to determine the relationship between induction and resistance.

A range of compounds and conditions were studied for the ability to induce the expression of the mef(E)-lacZ reporter using the disk diffusion assay. This assay allowed the efficient screening of a broad range of inducer concentrations and conditions. Nonmacrolide antibiotics or general environmental stresses and conditions (e.g., competence induction), except for certain antimicrobial peptides (50) and macrolides, did not induce the expression of mef(E)-mel. The nonmacrolide antibiotics studied represented different classes with distinct cellular targets, including ribosomal binding sites on 23S rRNA overlapping the macrolide binding site (chloramphenicol, lincomycin, linezolid, and streptogramins) and 16S rRNA binding drugs (e.g., aminoglycosides and tetracycline), suggesting that disruption of protein synthesis per se does not influence mef(E)-mel expression. Likewise, drugs that disrupt DNA synthesis, cell wall synthesis, or membrane integrity and various environmental conditions did not induce expression, indicating that the range of inducers for mef(E)-mel expression is quite specific.

The current paradigm for the Mef(E)/Mel efflux pump is that it confers resistance to 14- and 15-membered but not 16-membered macrolides (5, 9, 10, 17, 34, 36) and that 14- and 15-membered macrolides induce Mef(E)/Mel-mediated resistance (3, 36, 49). Our data confirm strong induction of mef(E)-mel expression by erythromycin, clarithromycin, and azithromycin, as previously reported (3, 49), and show that the most commonly studied 16-membered macrolides indeed did not induce mef(E)-mel expression. However, the use of a broader spectrum of structurally diverse macrolides revealed 14-membered and 15-membered macrolides with reduced ability to induce mef(E)-mel expression and 16-membered macrolides that induced mef(E)-mel. In addition, the ketolide telithromycin, despite retaining excellent activity against mef(E)-containing pneumococci, strongly induced mef(E) expression. Telithromycin has not previously been demonstrated to induce mef(E)-mel.

The induction of mef(E)-mel by 14- and 15-membered macrolides but not by common 16-membered macrolides coincides with differences in macrolide binding to the ribosome. Most 14- and 15-membered macrolides contain the monosaccharide desosamine at position C-5 of the lactone ring, whereas 16-membered macrolides typically have a C-5 disaccharide. C-5 substituents form extensive and distinct bonds with the ribosome (Fig. 3 and 4). The disaccharides in the 16-membered macrolides extend deep into the exit tunnel of the ribosome (19, 38), allowing the mycarose (Fig. 4) to interact with G2505 and U2506 of the 23S rRNA (Escherichia coli numbering) (Fig. 4), thus interfering with peptidyl transferase activity (19, 37, 38) and resulting in truncated peptides (19). Macrolides with C-5 monosaccharides do not interfere with peptidyl transferase activity but instead block the egress of the nascent peptide from the exit tunnel, leading to peptides up to 8 amino acids long (13, 19, 27, 38, 42).

We found that the inducing 16-membered macrolides tilmicosin and rosamicin, like inducing 14- and 15-membered macrolides, have a monosaccharide at C-5 and, therefore, are expected to have a similar affect on peptide synthesis. The weakly inducing 14-membered macrolide troleandomycin has acetylations of the C-3 and C-5 sugars that force a substantially altered conformation when bound to the ribosome, resulting in a location of the macrolide farther down the exit tunnel than inducing macrolides (6). The pivotal role of the C-5 substituent for induction, over and above that of the lactone ring size or the C-3 substituent, is also supported by our first identification of inducing 16-membered macrolides, which additionally lack the C-3 cladinose that is typically found in the common 14- and 15-membered macrolides. Taken together, the induction mechanism involves specific binding of the macrolide to the ribosome and, in particular, the C-5 substituent of the macrolide.

Specific macrolide binding to the ribosome is also required for the induction of macrolide resistance by many erm methylyses (24, 28, 33, 41, 48) in mechanisms that are controlled by transcriptional or translational attenuation. Weisblum (48) found that translational attenuation of erm(C) in Staphylococcus aureus is alleviated by 14- but not by 16-membered macrolides (48) and proposed that when bound by 14-membered macrolides, ribosomes stall due to constriction of the exit tunnel after a short peptide is synthesized. Ribosome stalling at the end of the leader peptide synthesis promotes refolding of the transcript into an antiattenuator structure (48). Conversely, C-5 disaccharides of many 16-membered macrolides disrupt ribosyltransferase activity, preventing synthesis of the leader peptide such that the erm transcript remains folded in the attenuator structure (19).

We propose a similar mechanism for the induction of mef(E)-mel and efflux-mediated macrolide resistance in pneumococci. Macrolides with C-5 disaccharides may prevent the synthesis of an unidentified regulatory leader peptide and, thus, may not induce. Macrolides with a monosaccharide at C-5 promote stalling at the precise residue of the leader peptide due to the length of the nascent peptide that is synthesized before being blocked in the ribosomal exit tunnel. This is independent of the lactone ring size or the C-3 sugar substituent.

The Mef(E)/Mel efflux pump does not confer resistance to common 16-membered macrolides. Induction of the efflux pump with erythromycin prior to exposure to the 16-membered macrolides demonstrated that pneumococcal susceptibility to these macrolides was not due to a failure to induce
The lack of efflux of 16-membered macrolides could result either because of an unavailability of the compounds for export by the efflux pump due to high-affinity ribosomal binding or because the macrolides are not recognized as substrates. Most 16-membered macrolides have a C-6 acetylaldehyde that can form a covalent bond with the RNA, resulting in high-affinity binding to the ribosome (19). In contrast, the 14- and 15-membered macrolides, which are known substrates for the efflux pump, bind ribosomes through hydrophobic interactions and hydrogen bonding and are easily dissociated from the ribosome (7, 19, 37, 42). Of the two 16-membered macrolides, rosamycin and tilmicosin, that induced mef(E)-mel expression, only tilmicosin had reduced activity against MEGA-containing pneumococci, suggesting that it was subject to efflux. The primary structural difference between these two compounds is the C-6 acetylaldehyde of rosamycin (but not tilmicosin), which has the potential to form the covalent bond effecting efflux by Mef(E)/Mel. Furthermore, telithromycin, with 700-fold higher affinity for the ribosome than erythromycin, is predicted to have limited availability for export by Mef(E)/Mel. Despite strong induction of the efflux pump by telithromycin, pneumococci remain susceptible. Thus, because of high-affinity ribosomal binding, most 16-membered macrolides and the 14-membered ketolide are not effectively available for efflux even if mef(E)-mel is induced, and pneumococcal isolates remain susceptible to these compounds.

To summarize, induction of the mef(E)-mel operon was shown to be limited to a narrow range of macrolides and antimicrobial peptides. The model indicates that induction is mediated by ribosomal binding and that the inducing 14-, 15-, and 16-membered macrolides contain a monosaccharide in position C-5 of the lactone ring. The C-5 monosaccharide forms a characteristic macrolide-ribosome-mRNA complex that is consistent with an antiattenuation-based mechanism for mef(E)-mel regulation. Most 16-membered macrolides and the 14-membered ketolide have high-affinity binding to the ribosome, and this strong ribosomal binding limits the availability for efflux from the pneumococcus, even in the presence of Mef(E)/Mel induction. The antimicrobial peptides, such as LL-37 and CRAMP, that induce are not expected to bind ribosomes, and thus, induction by these agents is anticipated to occur by an alternate pathway. Investigations are under way to confirm and define the proposed macrolide transcriptional attenuator mechanism and to study the basis for induction by antimicrobial peptides.

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REFERENCES


