Macrolide Efflux in *Streptococcus pneumoniae* Is Mediated by a Dual Efflux Pump (*mel* and *mef*) and Is Erythromycin Inducible

Karita D. Ambrose,† Rebecca Nisbet, and David S. Stephens

Departments of Medicine and Microbiology and Immunology,

Emory University School of Medicine,

Atlanta, Georgia 30322, and VA Medical Center, Decatur, Georgia 30033

Received 11 March 2005/Returned for modification 14 April 2005/Accepted 8 July 2005

Macrolide resistance in *Streptococcus pneumoniae* due to efflux has emerged as an important worldwide clinical problem over the past decade. Efflux is mediated by the genes of the genetic element mega (macrolide efflux genetic assembly) and related elements, such as Tn1207.1. These elements contain two adjacent genes, *mef* (*mefE* or *mefA*) and the closely related *mel* gene (*msrA* homolog), encoding a proton motive force pump and a putative ATP-binding cassette transporter homolog, and are transcribed as an operon (M. Del Grosso et al., J. Clun. Microbiol. 40:774–778, 2004; K. Gay and D. S. Stephens, J. Infect. Dis. 184:56–65, 2001; and M. Santagati et al., Antimicrob. Agents Chemother. 44:2585–2587, 2000). Previous studies have shown that Mef is required for macrolide resistance in *S. pneumoniae*; however, the contribution of Mel has not been fully determined. Independent deletions were constructed in *mefE* and *mel* in the serotype 14 macrolide-resistant strains GAL16638 (erythromycin [Em] MIC, 8 to 16 μg/ml) and GAL17719 (Em MIC, 2 to 4 μg/ml), which contain allelic variations in the mega element. The MICs to erythromycin were significantly reduced for the independent deletion mutants of both *mefE* and *mel* compared to those of the parent strains and further reduced threefold to fourfold to Em MICs of <0.15 μg/ml with *mefE* and *mel* double mutants. Using quantitative reverse transcription-PCR, the expression of *mefE* in the *mel* deletion mutants was increased more than 10-fold. However, in the *mefE* deletion mutants, the expression of *mel* did not differ significantly from the parent strains. The expression of both *mefE* and *mel* was inducible by erythromycin. These data indicate a requirement for both Mef and Mel in the novel efflux-mediated macrolide resistance system in *S. pneumoniae* and other gram-positive bacteria and that the system is inducible by macrolides.

**Streptococcus pneumoniae** is a leading cause of respiratory infections, which include otitis media, sinusitis, and pneumonia. Antibiotic treatment of these infections has become increasingly problematic due to an emergence of resistance to both penicillin and non-β-lactam antibiotics. During the last decade, a rapid increase in the resistance of *S. pneumoniae* to macrolides has been observed in the United States (3, 12, 13, 19, 43).

The major mechanisms of macrolide resistance in *S. pneumoniae* are target modification and drug efflux. Genetic determinants conferring macrolide resistance by target modification include *erm* and mutations in the 23S rRNA and ribosomal proteins. The *erm* gene product methylates the peptidyl transferase center of newly synthesized 23S rRNA, thereby conferring cross-resistance to lincomamides and streptogramin B (MLS phenotype) (30, 42). Mutations in the 23S rRNA and ribosomal proteins L4 and L22 have also been reported and can confer macrolide-lincomamide (ML) and macrolide-streptogramin B (MS) resistance phenotypes when different mutations are combined (5, 21, 26, 28).

Throughout the world, rapidly increasing rates of macrolide resistance have been due primarily to the second major mechanism of macrolide resistance in *S. pneumoniae*, efflux linked to the gene product of *mef* (14, 20, 37, 39). Mef belongs to the major facilitator superfamily of efflux pumps and carries a proton motive force pump that is specific for 14- and 15-membered macrolides (M phenotype) (7, 40). Two variants, *mefE* and *mefA*, with >90% protein sequence homology, are found in isolates of *S. pneumoniae* (9, 10, 14, 15). Macrolide resistance due to the presence of MefE accounts for the majority of macrolide-resistant pneumococcal strains isolated in the United States (12, 13).

The genetic elements harboring both *mefA* and *mefE* in *S. pneumoniae* are localized on conjugative transposon-related elements (15, 36). The *mefE* gene is present on the 5.4- or 5.5-kb mega (macrolide efflux genetic assembly) element that confers macrolide resistance to susceptible *S. pneumoniae* (15), and *mefA* is found on the closely related elements Tn1207.1 (36) and Tn1207.3 (35). Other genetic elements have subsequently been identified to contain mega-like regions, including Tn09, the chimeric element in *S. pyogenes* composed of a transposon inserted into a prophage (1, 11), and elements found in viridans streptococci (6). Both *mefE* and *mefA* are part of an operon in mega that includes a downstream gene, *mel*, a homolog of *msrA* (15, 36). In staphylococci, *msrA* encodes a 488-amino-acid ATP-binding cassette (ABC) transporter protein which results in an energy-dependent efflux of erythromycin (34). ABC transporter proteins typically contain two ATP-binding domains located cytoplasmically that interact with two hydrophobic domains (22). Both MsrA and Mel contain ATP-binding domains characteristic of ABC transporters; however, they lack hydrophobic segments carrying the trans-
membrane domains. Although MsrA is predicted to interact with chromosomally encoded transmembrane complexes, MsrA was sufficient in conferring resistance to macrolides and streptogramin B (MS phenotype) (32). In *S. pneumoniae*, mefE and mel are cotranscribed as an operon and are predicted to be a dual efflux pump in *S. pneumoniae* (15). The two allelic forms of mefE, 5.4 or 5.5 kb, differ in the presence or absence of a 99-bp insertion between mefE and mel. Here we describe the requirement of both MefE, the proton motive force pump homolog, and Mel, the homolog of an ATP-binding cassette transporter, in macrolec efflux in *S. pneumoniae*.

### MATERIALS AND METHODS

**Bacterial strains and plasmids.** Bacterial strains and plasmids used in this study are listed in Table 1. Serotype 14 pneumococcal isolates GA16638 and GA17719 and other erythromycin-resistant *S. pneumoniae* isolates were obtained as part of an active, population-based surveillance program of invasive pneumococcal disease in metropolitan Atlanta. Surveillance and isolate collection methods have been described previously (14, 15, 18). The initial antimicrobial susceptibility of isolates was assessed according to guidelines established by the Clinical and Laboratory Standards Institute (formerly NCCLS) (8). Isolates not susceptible to erythromycin (MIC, &gt;0.5 μg/ml) were further classified by antibiogram and molecular studies. GA16638 and GA17719 are M phenotype macrolide-resistant isolates (erythromycin [Em] MICs, 8 to 16 μg/ml), and GA17719 is M phenotype macrolide-resistant isolate (erythromycin sensitive; Ap', ampicillin resistant; Kn', kanamycin resistant. × indicates transformation (or crossing) of the plasmid into the strain. Primers are listed in Table 2.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Derivationa</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GA16638</strong></td>
<td>Parent type 14, type 2 mega, Em'</td>
<td>This study</td>
</tr>
<tr>
<td><strong>GA17719</strong></td>
<td>Parent type 14, type 1 mega, Em'</td>
<td>This study</td>
</tr>
<tr>
<td><strong>KA3000/01</strong></td>
<td>pKA309 × GA16638 ΔmefE Em'</td>
<td>This study</td>
</tr>
<tr>
<td><strong>KA3005/04</strong></td>
<td>pKA312 × GA16638 ΔmefE Em'</td>
<td>This study</td>
</tr>
<tr>
<td><strong>KA3005/06</strong></td>
<td>pKA321 × GA16638 ΔmefE Δmel Em'</td>
<td>This study</td>
</tr>
<tr>
<td><strong>ATCC 49619</strong></td>
<td></td>
<td>26</td>
</tr>
</tbody>
</table>

**E. coli** DH5α plasmids

- pSF151
- pWA101: TOP10 pCR2.1::0.34- and 0.56-→kb KG7/KG11 PCR product from GA16638
- pKA309: DH5α pSF151::double-fragment ligation of KpnI/XbaI insert of pWA101 and KpnI/PstI insert of pWA103 ligated to XbaI/PstI of vector
- pKA310: TOP10 pCR2.1::0.66-→kb KG20/KG41R PCR fragment from GA16638
- pKA312: DH5α pSF151::double-fragment ligation of SacI/EcoRV fragment from pWA103 ligated to EcoRV/SpeI fragment of pKA310
- pKA321: DH5α pSF151::0.34-→kb EcoRI/SpeI fragment of pKA310 and 0.6-→kb XbaI/BamHI fragment of pKA310 ligated to EcoRI/BamHI of vector, Kn'

**a** Em', erythromycin resistant; Em', erythromycin sensitive; Ap', ampicillin resistant; Kn', kanamycin resistant.

**TABLE 1.** Oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>mefE-1</td>
<td>5'-GCT AGT GGA TCG TCA TGA TAG G-3'</td>
</tr>
<tr>
<td>mefE-2</td>
<td>5'-TTC CCG AAA CGG CTA AAC TGG T-3'</td>
</tr>
<tr>
<td>mefE-3F</td>
<td>5'-ATA TGG GCA GGG CAA GCA G-3'</td>
</tr>
<tr>
<td>mefE-4R</td>
<td>5'-CAT TGG CAG GAT GGC ACT AGT G-3'</td>
</tr>
<tr>
<td>mel-2F</td>
<td>5'-GAA GTT AAG ACG CAA GCA G-3'</td>
</tr>
<tr>
<td>mel-3R</td>
<td>5'-GGC ACG TTC CGC AAT AAA TT-3'</td>
</tr>
<tr>
<td>rpsE-2R</td>
<td>5'-GCA GAA TCT ATA CCC GAT GAT AGG-3'</td>
</tr>
<tr>
<td>KG7</td>
<td>See reference 15</td>
</tr>
<tr>
<td>KG8</td>
<td>See reference 15</td>
</tr>
<tr>
<td>KG10</td>
<td>5'-ACA CCT AGC TTG CTT ACA AGT G-3'</td>
</tr>
<tr>
<td>KG11</td>
<td>5'-GCA GAA TCA TCT ATC GAT GAT AGG-3'</td>
</tr>
<tr>
<td>KG20</td>
<td>5'-CTG TTC TGG TTA TGG GCC GAC C-3'</td>
</tr>
<tr>
<td>KG41R</td>
<td>5'-CAT GTC TGA CTT AAT ACT AGA G-3'</td>
</tr>
<tr>
<td>rpsE-1F</td>
<td>5'-ACG TCG TCT TCT TTG CTC A-3'</td>
</tr>
<tr>
<td>rpsE-2R</td>
<td>5'-CAT GGA CTA TTG TCA CCA AC-3'</td>
</tr>
<tr>
<td>fabK-1F</td>
<td>5'-TGA TGT GGA TGG TGG TCT CTC T-3'</td>
</tr>
<tr>
<td>fabK-2R</td>
<td>5'-GAA ACA AGC CCT GCG ATT TG-3'</td>
</tr>
</tbody>
</table>
FIG. 1. Schematic of mega element for strain GA16638 illustrating the locations of single mutations for \textit{mefE}, \textit{mel}, and a \textit{mefE mel} double mutant. ORF, open reading frame.

**FIG. 1.** Schematic of mega element for strain GA16638 illustrating the locations of single mutations for \textit{mefE}, \textit{mel}, and a \textit{mefE mel} double mutant. ORF, open reading frame.
TABLE 3. MICs of erythromycin for mega mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Em MIC (µg/ml) (±SEM)a</th>
<th>Etest</th>
<th>Microdilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA16638 (type 2 mega, parent)</td>
<td>15 (±1.00)b</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>GA16638 ΔmefE</td>
<td>1.13 (±0.16)b</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>GA16638 ΔmefE ΔmefE</td>
<td>0.68 (±0.04)b</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>GA16638 ΔmefE ΔmefE Δmel</td>
<td>0.15 (±0.01)b</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>GA17719 (type 1 mega, parent)</td>
<td>4.13 (±0.13)b</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>GA17719 ΔmefE</td>
<td>2.13 (±0.13)b</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>GA17719 Δmel</td>
<td>0.46 (±0.03)b</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>GA17719 ΔmefE Δmel</td>
<td>0.13 (±0.00)b</td>
<td>≤0.03</td>
<td></td>
</tr>
<tr>
<td>ATCC 49619</td>
<td>0.09 (±0.01)b</td>
<td>≤0.03</td>
<td></td>
</tr>
</tbody>
</table>

a Standard errors of the means are based on quadruplicate cultures.
b P < 0.0001 compared to results for the parent strains.

and Southern hybridizations (data not shown). The Etest erythromycin MICs were reduced for both independent mefE (13-fold) and mel (22-fold) deletion mutants compared to that of the parent strain (Table 3). Reductions in MICs were also obtained when the mefE (twofold) and the mel (ninefold) mutations were constructed in the clinical isolate GA17719 (serotype 14) (Table 3) (MIC, 4.13 ±0.3 µg/ml), which has a type 1 mega insert (15). In the mefE mel double mutants of both strains, erythromycin MICs were further reduced threefold to fourfold (Table 3) to MICs of <0.15 µg/ml. Similar changes were seen when MICs were determined by microdilution methods (Table 3). Also, the telithromycin MICs of both strains with deletions of mefE or mel or both were reduced from 0.5 µg/ml (GA16638) and 0.06 µg/ml (GA17719) to ≤0.03 µg/ml in mel, mef, and dual mutants. The antibiograms of the mutants otherwise remained unchanged compared to those of the parent strains.

Expression of mefE and mel. The mutations in mefE or mel may influence expression of the gene not mutated. The two allelic forms of mega, 5.4 and 5.5 kb, differ in their intergenic regions separating mefE and mel, which may also influence the expression of these genes. Using real-time quantitative RT-PCR, expression of mefE and mel was determined in the parent and mutant strains. In the wild-type strains, the genes were expressed as an operon and levels of expression of mefE and mel in the two allelic forms were similar. Mutations did not decrease the expression of the adjacent gene, and thus the construction of the mutations did not have a polar effect on expression. Expression of mefE in the mel mutants was increased more than 10-fold (Fig. 2A). The expression of mel in the mefE deletion mutants did not differ significantly from that in the parent strain (Fig. 2B). Expression profiles in GA16638 and GA17719 were similar with mefE and mel deletions.

Expression of mefE and mel is erythromycin inducible. Parent strain GA16638 grown in the presence of subinhibitory concentrations of Em induced the expression of both mefE and mel (Fig. 3). Levels of induction of gene expression by Em varied when different concentrations of Em were used. A concentration of 0.024 µg/ml of Em (500-fold less than the MIC) resulted in a 20-fold increase in the expression of mefE and a 15-fold increase in the expression of mel. However, when GA16638 was grown with a concentration of 1.2 µg/ml Em (10-fold less than the MIC), expression of both mefE and mel was increased more than 300-fold. These results suggest a regulatory mechanism of mefE and mel in S. pneumoniae that is inducible by Em. No significant effect on mef or mel expression was observed when cultures were grown with the nonmacrolide antibiotic kanamycin (data not shown).

Accumulation and efflux of [14C]erythromycin. Cell-associated [14C]erythromycin was increased in the mefE and mel mutant strains compared to the level in the parent strain (Fig. 4). The mefE and mel mutants and the double mutants consistently accumulated more erythromycin than the erythromycin-resistant parent strains. Differences between the mutants in accumulation were not demonstrated. A wild-type-susceptible strain showed increased accumulation of erythromycin (data not shown). The accumulation in the mutants indicated decreased efflux of [14C]erythromycin at all time points.

DISCUSSION

Macrolide resistance mediated by efflux emerged as a major global problem in the 1990s (14, 20, 39, 44) in S. pneumoniae and is now one of the major mechanisms of macrolide resistance worldwide. Efflux-mediated macrolide resistance is me-

FIG. 2. Expression of mefE (A) and mel (B). RNA was isolated from mid-exponential cultures using the QIAGEN RNasey minipreps. Three replicates were performed for each strain on duplicate and independent RNA samples. The amount of target is normalized to a control target gene, fabK, relative to an internal ribosomal calibrator. Data are expressed as percentages of the amount in the parent GA16638. Statistical analyses were done using the unpaired Student t test (***, P < 0.005).
mediated by the macrolide efflux genetic element, mega (15), and larger genetic elements, such as Tn1207.1, that contain mega or closely related homologs (1, 11, 35, 36). Two adjacent genes, mef and mel (msr), have been identified in these mega-containing elements. To determine the molecular basis of macrolide efflux in S. pneumoniae, independent deletion mutations in mefE and mel were constructed in S. pneumoniae. Mutations in either mefE or mel in GA16638, a serotype 14 type 2 mega insertion strain, resulted in significant (P < 0.0001) decreases in resistance to erythromycin. Levels of resistance to erythromycin were reduced up to 22-fold by independent mefE and mel deletion mutants. The mef and mel double mutant further decreased erythromycin resistance an additional threefold to fourfold. Similar results were obtained with the serotype 14 strain containing an allelic variant of mega that differs by 99 bp in the intergenic region between mefE and mel (15).

Interestingly, the expression of mefE in the Δmel mutants was increased more than 10-fold, but the increased expression of mefE in the Δmel mutants did not restore resistance to erythromycin. The increase observed in transcription due to the mutation in mel located downstream and in the same transcriptional unit suggests a regulatory role of Mel on mef and mel expression. Alternatively, mRNA stability of mefE is increased in the mel mutant. Either would be predicted to increase levels of MeF in a mel deletion background, but the predicted increase in MeF does not influence levels of resistance to erythromycin.

Previous studies have shown the requirement of mefE in S. pyogenes and pneumococcal resistance to erythromycin (7, 40). However, the genetic elements harboring mefE- or mefA-resistant determinants in S. pneumoniae and more recently, S. pyogenes, all reveal a similar genetic organization, with mel located downstream of mefE (1, 10, 11, 15, 35, 36). Because sequence analyses of these elements have become available only recently, it is predicted that mel was present in the original mefA isolates of S. pyogenes and a mefE isolate of S. pneumoniae (7).

In view of the structure of the genetic elements, the erythromycin-susceptible isolates that were transformed to erythromycin-resistant isolates with genomic DNA of clinical isolates harboring mefE would have also contain mel. Thus, mel along with mefE is predicted to be present in mefE-containing, gram-positive clinical isolates that are erythromycin resistant. This hypothesis is supported by sequence data of the efflux erythromycin resistance elements (1, 11, 15).

The requirement of both mefE and mel in resistance to erythromycin supports a dual efflux pump model; however, the exact mechanism by which the two gene products function in mediating efflux remains unclear. Because the levels of resistance to erythromycin in ΔmefE and Δmel mutants are similar, and the expression of each gene in the mutant strains is either unaffected or increased, both MeF and Mel appear to be necessary for erythromycin resistance and are predicted to interact to drive the efflux of macrolides. The lower MICs in the Δmel mutant and ΔmefE Δmel double mutant may suggest that mel has some residual pump activity independent of mef.
but this is not likely of clinical importance. The data also suggest that macrolide resistance (2, 4, or 16 μg or higher) requires mef and mel but is not sufficient to explain the range of MICs seen for MefE/Mel-containing isolates. These differences may depend on factors (e.g., expression of the operon, chromosomal location, posttranslational modification, or other phenotypes in the strain) other than the presence of the genes.

In staphylococci, the mel homolog msrA encodes an ABC transporter protein which results in an energy-dependent efflux of erythromycin (34). Previous studies have suggested that msrA interacts with another protein since it lacks the membrane-spanning domains characteristic of ABC transporter pumps; however, this putative protein has not been identified in Staphylococcus aureus (29, 32, 33). While both Msra and Mel lack hydrophobic membrane-spanning domains of classical ABC transporters and have considerable homology at the predicted amino acid level, the question of whether they are functional homologs remains unclear. Mel confers an M phenotype in S. pneumoniae, while Msra confers an MS phenotype in staphylococci (29), suggesting differences in the mechanisms of these proteins. More recently, mel [designated msr(D)] alone was found to be capable of conferring macrolide resistance in a susceptible pneumococcal strain by transformation (9) but did not fully restore the MIC resistance of the donor strain. The Mel transformants also had slightly increased resistance to ketolides. The strain used in that study, CP1250, is a derivative of the highly competent unencapsulated Rx that was chemically mutagenized using 1-methyl-3-nitro-1-nitrosoguanidine (25, 27). Our data also indicate a role for MefE/Mel in ketolide export. Efflux of telithromycin was recently demonstrated for S. pyogenes (4). In our studies, both Mef and Mel are required for maximal mef-mediated efflux of erythromycin. In support of this model, deletions of mefE and/or mel resulted in increased accumulation of radiolabeled [14C]erythromycin, suggesting a decrease in efflux. In additional studies of the parent strains, the accumulation of erythromycin was increased when inhibitors of both proton motive force pumps and ABC transporters, such as carbonyl cyanide m-chlorophenylhydrazone, sodium arsenate, and sodium orthovanadate, were added (K. D. Ambrose et al., unpublished). Further, the increased expression of mefE in the mel mutants did not restore erythromycin resistance. Thus, Mel is required for macrolide resistance in S. pneumoniae and functions with MefE as part of the efflux pump. In S. pneumoniae, Mef could be the membrane-spanning protein necessary for ABC transporters like Mel that lack hydrophobic membrane-spanning domains. This would represent a novel model of efflux in bacteria.

The Mel/Mef efflux pump is inducible by erythromycin. Complicating the emergence of pneumococcal macrolide resistance, the MICs of erythromycin for mefE mel-containing strains having drastically increased in invasive S. pneumoniae, with 88% of strains now having MICs of ≥8 μg/ml and 63.5% having MICs of ≥16 μg/ml (38). While several factors (e.g., encapsulated [serotype] background) may have contributed to this trend, levels of Mef and Mel expressed in isolates by erythromycin induction may contribute to this phenomenon or may have led to higher levels of constitutive expression in some isolates. Inducible efflux pumps have been described to occur in pathogenic bacteria, such as the MexXY efflux pump in Pseudomonas aeruginosa, which is inducible by erythromycin, tetracycline, or gentamicin (23). Msra is inducible in staphylococci (24), and Daly et al. (9) recently showed that Mel (Msra) is inducible by erythromycin.

In conclusion, efflux mechanisms of macrolide resistance associated with the mega element have emerged as a major resistance mechanism in S. pneumoniae and other gram-positi-
tive pathogens. Macrolide-resistant S. pneumoniae harboring the 5.5- or 5.4-kb mega genetic element requires the presence of both of the mefE and mel gene products to confer high-level macrolide resistance.

ACKNOWLEDGMENTS

We thank Lane Pucko and surveillance personnel of Georgia Emerging Infections Program, Active Bacterial Core Surveillance (ABCs), for technical and helpful assistance and William M. Shafer for helpful comments. We also thank Bernard Beall and Delois Jackson at the CDC and Larry Martin and Susu Zughaier for help with the antimicrobial susceptibility testing.

This work was supported by a Fellowships in Research Science and Teaching (FIRST) Award (to K.D.A.), Emory University, and a VA Merit Award (to D.S.S.).

REFERENCES


