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# *mefE* Is Necessary for the Erythromycin-Resistant M Phenotype in *Streptococcus pneumoniae*

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**Recently, it was shown that a significant number of erythromycin-resistant *Streptococcus pneumoniae* and *Streptococcus pyogenes* strains contain a determinant that mediates resistance via a putative efflux pump. The gene encoding the erythromycin-resistant determinant was cloned and sequenced from three strains of *S. pneumoniae* bearing the M phenotype (macrolide resistant but clindamycin and streptogramin B susceptible). The DNA sequences of *mefE* were nearly identical, with only 2-nucleotide differences between genes from any two strains. When the *mefE* sequences were compared to the *mefA* sequence from *S. pyogenes*, the two genes were found to be closely related (90% identity). Strains of *S. pneumoniae* were constructed to confirm that *mefE* is necessary to confer erythromycin resistance and to explore the substrate specificity of the pump; no substrates other than 14- and 15-membered macrolides were identified.**

Erythromycin resistance in *Streptococcus pneumoniae* has increased considerably over the last 5 years (1, 5, 14, 20, 23, 38, 40). Currently, 15 to 20% of the pneumococci are resistant to 14- and 15-membered macrolides in the United States (5, 40).

Recent epidemiological surveys have shown that some erythromycin-resistant strains of pneumococci (23, 35, 36) and group A streptococci (10, 25, 29–31, 33, 36) are not coresistant to lincosamide and streptogramin B antibiotics, the $MLS_B$-resistant phenotype typical of strains carrying an *erm* methylase. Rather, many clinical strains have been shown to have the M phenotype, namely, resistant to macrolides but susceptible to lincosamide and streptogramin B antibiotics. Early studies with pneumococcal strains bearing the M phenotype revealed that they did not contain an *erm* methylase (32, 36) and did not inactivate radiolabeled erythromycin (36). These strains accumulated less erythromycin than susceptible strains, suggesting that erythromycin resistance might be due to an efflux pump (36).

The determinant in *Streptococcus pyogenes* strains with an M phenotype was cloned and sequenced (8). *mefA* encodes a novel hydrophobic 44.2-kDa protein with homology at the amino acid level to other efflux proteins. In this study, we cloned a *mef*-like gene from pneumococci and detected the presence of this gene in all pneumococcal strains with the M phenotype. Introduction of *mefE* into an erythromycin-susceptible pneumococcal strain rendered the strain erythromycin resistant, phenotypically M resistant, and able to efflux erythromycin.

## MATERIALS AND METHODS

**Strains and plasmids.** The relevant strains and plasmids used in this study are listed in Table 1.

**Chemicals, radiochemicals, and media.** Most reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.); the exceptions were X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) from Gibco-BRL (Gaithersburg, Md.) and tetraphenylphosphonium chloride (TPP) from Aldrich (Milwaukee, Wis.). Streptogramin A (M1 factor) and streptogramin B (S1 factor) were obtained from Francis Gossele (15), and azithromycin was prepared at Pfizer as described previously (6). Proteinase K was purchased from Amresco (Solon, Ohio). Brain heart infusion (BHI) medium, yeast extract, and agar were purchased from Remel Microbiology Products (Lenexa, Kans.). DNA primers were purchased from GenoSys Biotechnologies (The Woodlands, Tex.), and *Taq* DNA polymerase was supplied by Perkin-Elmer (Danbury, Conn.). All restriction and DNA-modifying enzymes were from New England Biolabs (Beverly, Mass.) or Gibco-BRL and were used according to the manufacturers' instructions. Hybridizations and labeling of probes with digoxigenin were performed as described by the manufacturer (The Genius System; Boehringer-Mannheim, Indianapolis, Ind.). Competence-stimulating peptide was purchased from AnaSpec Inc. (San Jose, Calif.). [*N-methyl*-$^{14}$C]erythromycin (55 mCi/mmol) was purchased from DuPont, NEN Research Products (Boston, Mass.). MicroScint 20 and organic solvents were purchased from Packard (Meriden, Conn.) and Fisher Scientific (Fair Lawn, N.J.), respectively.

**DNA isolation and cloning of the *mefE* gene.** Genomic DNA from *S. pneumoniae* was prepared by the method of Ausubel et al. (3). For maximal lysis of pneumococci, an initial incubation with 3 mg of lysozyme per ml in 7% sucrose–50 mM Tris–1 mM EDTA, pH 8.0, and increasing the period of proteinase K-sodium dodecyl sulfate digestion at 37°C to 3 h was helpful.

Initially, a *mef*-like sequence was identified in M-resistant pneumococcal strains with a probe, plasmid p53-6 containing *S. pyogenes mefA* (8). *Eco*RI-digested genomic DNA fragments (~5 kb) that hybridized to the probe were purified from 0.8% agarose gels with a SephaGlas Band Prep Kit (Pharmacia, Piscataway, N.J.). These fragments were ligated with *Apa*I-digested pACYC177, transformed into *Escherichia coli* DH5α (Gibco-BRL), and transformants were selected on BHI agar containing 50 μg of ampicillin per ml. Plasmid DNA was prepared from multiples of 10 transformants. The *mefA* probe (*Eco*RI fragment from p53-6) was used to identify the pool of 10 transformants containing the *mef*-like gene by Southern hybridization; the single transformant from the pool of 10 was identified by its ampicillin-resistant, erythromycin-resistant phenotype.

Synthetic oligonucleotide primers derived from the nucleotide sequences of the *mefA* and *mefE* genes (8) were used to generate full-length *mefE* sequences bounded by *Pst*I and *Nsi*I restriction sites from two other pneumococcal strains. A 29-mer, 5′-AAAACTGCAGGCGTTTAAGATAAGCTGGC-3′ was used as a forward primer, and a 29-mer, 5′-CCAATGCATCCTGCACCATTTGCTC CTAC-3′, was used as a reverse primer. All reactions were done in 10 mM Tris-HCl (pH 8.3)–50 mM KCl, in a 20-μl reaction volume (1.5 mM $MgCl_2$, with nucleotide triphosphates at 200 μM each, and 0.5 U of *Taq* polymerase). The polymerase reactions were amplified on a Perkin-Elmer Cetus GeneAmp PCR System 9600, with a cycle consisting of a melting step at 94°C for 1 min, a primer annealing step at 59°C for 1 min, and an extension step at 72°C for 1 min. The PCR products were digested with *Pst*I and *Nsi*I, gel purified, and ligated into plasmid pGEM-5Zf(+). Transformation was into *E. coli* DH5α with selection on BHI agar containing 100 μg of ampicillin per ml, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and 40 μg of X-Gal per ml.

**DNA sequencing.** The pneumococcal insert in pAT15-5 was sequenced on both strands by using TaqFS fluorescent cycle sequencing (Perkin-Elmer, Foster City, Calif.) and the ABI model 373A DNA sequencer (Applied Biosystems). Full-length sequencing was facilitated by using the AT-2 in vitro Artificial Transposon

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TABLE 1. Bacterial strains and plasmids

| Strain or plasmid | Description | Reference or source |
|---|---|---|
| *Escherichia coli* | | |
| DH5α | Commercial strain used for transformation | Gibco-BRL |
| DH10B | Commercial strain used for transformation | Gibco-BRL |
| *Streptococcus pneumoniae* | | |
| 02J1175 | Recent clinical isolate from Kentucky | 34 |
| 02J1048 | Recent clinical isolate from Kentucky | 36 |
| 02J1052 | Recent clinical isolate from Kentucky | 36 |
| 02J1046 | $MLS_B$-resistant clinical strain; Tn*1545*$^+$ | |
| R6 | Unencapsulated derivative of Avery's clinical strain | 4 |
| R6/1175-1 | R6 with *mefE* gene from 02J1175 | This work |
| R6/1175/16-1 | R6/1175-1 *mefE*::*cat* obtained by transformation with fragment from pAT16-1 | This work |
| R6/1175/16-2 | R6/1175-1 *mefE*::*cat* obtained by transformation with fragment from pAT16-2 | This work |
| Plasmids | | |
| pACYC177 | Low-copy-number cloning vector; ampicillin resistant, kanamycin resistant | 7 |
| pR326 | pBlueScribe SK$^+$ containing a synthetic choramphenicol acetyltransferase (*cat*) cassette for gene disruption in *S. pneumoniae* | 9 |
| pGEM-5Zf(+) | Commercial cloning vector | Promega |
| p53-6 | pACYC177 with 4.7-kbp *Pst*I fragment containing *mefA* from *S. pyogenes* | 8 |
| pAT15-5 | pACYC177 with 3.7 kb of *S. pneumoniae* 02J1175 genomic DNA containing the *mefE* gene | This work |
| pAT16-1 | pAT15-5 with the *cat* gene from pR326 inserted between two *Bsm*FI sites of pAT15-5 (promoters for *mefE* and *cat* are in the opposite orientation) | This work |
| pAT16-2 | pAT15-5 with the *cat* gene from pR326 inserted between two *Bsm*FI sites of pAT15-5 (promoters for *mefE* and *cat* are in the same orientation) | This work |
| pMR3 | 1.7-kbp PCR product containing the *mefE* gene of *S. pneumoniae* 02J1052 inserted into pGEM-5Zf(+) | This work |
| pMR4 | 1.7-kbp PCR product containing the *mefE* gene of *S. pneumoniae* 02J1048 inserted into pGEM-5Zf(+) | This work |

Kit (Perkin Elmer, Foster City, Calif.) (11). DNA and protein homologies were determined with the BlastX algorithm; DNA and protein sequence analyses were performed with Geneworks (Intelligenetics, Campbell, Calif.) or Lasergene (DNASTAR Inc., Madison, Wisc.) software.

**Transformation.** Transformation of *S. pneumoniae* R6 and derivatives was performed with synthetic competence-stimulating peptide I (CSP; H-Glu-Met-Arg-Leu-Ser-Lys-Phe-Phe-Arg-Asp-Phe-Ile-Leu-Gln-Arg-Lys-Lys-OH) by a modification of the method of Håverstein et al. (16). Briefly, cells were induced into the competent state by inoculating cells in early log phase (optical density at 625 nm [$OD_{625}$] ~0.05) into BHI containing 0.1% yeast extract and 100 ng of CSP per ml (BHI-YE-CSP); growth at 37°C continued until the $OD_{625}$ reached between 0.4 and 0.6. Glycerol was added to a final concentration of 20%, and competent cells were frozen in 20-μl aliquots at −80°C. For a typical transformation, a 20-μl aliquot of cells was added to 200 μl of BHI-YE-CSP. Total genomic DNA or purified DNA fragments containing the desired sequence (*mefE* or *mefE*::*cat*) were added at a final concentration of 1 μg/ml. The mixture was incubated at 30°C for 30 min and then transferred to 37°C for 4 h. The entire transformation mixture was transferred to an empty, sterile petri dish and overlaid with 15 ml of BHI agar plus 3 ml of sheep erythrocytes and a selecting agent.

**Construction of isogenic strains by transformation.** The *mefE* gene was introduced into strain R6 by transformation with total genomic DNA from strain 02J1175 and selection on BHI agar containing 0.5 μg of erythromycin per ml. Strain R6/1175-1 was one of the resultant erythromycin-resistant transformants bearing the M phenotype.

To construct a strain with a disruption in the reading frame of *mefE*, a vector containing *mefE* in which a *cat* gene cassette was introduced was prepared. First, pAT15-5 was digested to completion with *Bsm*FI to remove 706 bases from the *mefE* coding sequence; treatment with T4 DNA polymerase formed the blunt ends necessary for ligation of the *cat* gene cassette. pR326 (9) was digested to completion with *Sma*I and *Hin*dIII and treated with T4 DNA polymerase to liberate a blunt-ended 1.1-kb fragment containing the *cat* gene cassette. The fragment was purified and ligated with pAT15-5 overnight at 16°C. Ligation mixtures were transformed into DH5α competent cells (Gibco-BRL) and plated on BHI plates containing 35 μg of chloramphenicol per ml. Plasmids (pAT16-1 and pAT16-2) from chloramphenicol-resistant transformants were prepared with a Qiagen mini-prep kit according to the manufacturer's instructions and analyzed by restriction digestion with *Pst*I.

Plasmids pAT16-1 and pAT16-2 were digested to completion with *Xho*I and *Apa*LI. A 4,504-bp fragment containing the disrupted *mefE* gene was gel purified from the ampicillin-resistant gene on the original vector and used to transform strain R6/1175-1. Chloramphenicol-resistant transformants were selected with chloramphenicol (9 μg/ml), and multiple clones were screened for erythromycin susceptibility on blood plates spread with erythromycin (5 μg/ml). One erythromycin-susceptible transformant was selected from both R6/1175/16-1 and R6/1175/16-2 transformations for further analysis.

**MIC studies.** The MICs for a variety of antibacterial agents were determined in cation-supplemented Mueller-Hinton broth plus 2% lysed horse blood according to National Committee for Clinical Laboratory Standards guidelines (22).

**Macrolide efflux by *S. pneumoniae* R6 and transformants.** Freshly grown colonies from blood agar plates were used to inoculate 50 ml of prewarmed BHI to an $OD_{625}$ of ~0.05. The erythromycin-resistant transformant was grown on blood plates containing 8 μg of erythromycin per ml. The cultures were grown in 5% $CO_2$ at 37°C and carefully monitored until an $OD_{625}$ of ~0.15 to 0.3 (early log phase) was reached. [*N-methyl*-$^{14}$C]erythromycin was added at a final concentration of 0.2 μg/ml, and uptake was assessed as previously described (36).

**Nucleotide sequence accession number.** The *mefE* gene sequence has been deposited in GenBank under accession no. U83667.

## RESULTS

**Cloning of *mefE* in pneumococcal strains with the M phenotype.** Strains 02J1048, 02J1052, and 02J1175 had previously been found to be erythromycin and azithromycin resistant, but clindamycin and streptogramin B susceptible (36). The resistance did not appear to be mediated at the level of the ribosome (36) or conferred by other known macrolide-resistant determinants (*msrA*, *ereA*, *ereB*, *mphA*, *ermA*, *ermB*, and *ermC*) (34). Because we had previously shown that both *S. pyogenes* and *S. pneumoniae* strains with the M phenotype appeared to efflux erythromycin (36), we tested the hypothesis that a *mef*-like gene was present in 02J1175. Initially, we found that DNA from this strain (and other pneumococcal strains with the M phenotype) hybridized to a *mefA* probe under conditions of high stringency (65°C) (data not shown). Macrolide-susceptible pneumococcal strains or strains with other known erythromycin-resistant determinants did not hybridize to the probe. By using the strategy outlined in Materials and Methods, a 3.7-kb fragment of 02J1175 DNA was identified that, when transformed into *E. coli* DH5α with the vector pACYC177, conferred an erythromycin-resistant phenotype on the host.

TABLE 2. MICs of *mefE* isogenic strains

| Strain | MIC (μg/ml) of test agent[a] | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ery | Azi | Clar | Clind | Josa | StrepA | StrepB | Tet | Acr | Rhod | Nor | TPP | Ars | PenG | CCCP | Eth |
| R6 | 0.156 | 0.156 | 0.156 | 0.039 | 0.078 | 3.13 | 3.13 | 0.078 | 12.5 | 10 | 12.5 | 100 | 2,500 | 0.0156 | 25 | 2.5 |
| R6/1175/1 | 12.5 | 12.5 | 12.5 | 0.039 | 0.078 | 6.25 | 3.13 | 0.078 | 6.25 | 10 | 25 | 50 | 5,000 | 0.0156 | 25 | 2.5 |
| R6/1175/16-1 | 0.313 | 0.156 | 0.313 | ≤0.02 | 0.039 | 6.25 | 1.56 | 0.156 | 3.13 | 10 | 6.25 | 25 | 1,250 | 0.0078 | 6.25 | 2.5 |
| R6/1175/16-2 | 0.156 | 0.156 | 0.156 | ≤0.02 | 0.078 | 6.25 | 3.13 | 0.078 | 12.5 | 10 | 25 | 50 | 2,500 | 0.0313 | 25 | 5 |

[a] Test agent: Ery, erythromycin; Azi, azithromycin; Clar, clarithromycin; Clind, clindamycin; Josa, josamycin; StrepA, streptogramin A; StrepB, streptogramin B; Tet, tetracycline; Acr, acridine orange; Rhod, rhodamine; Nor, norfloxacin; Ars, arsenate; PenG, penicillin G; Eth, ethidium bromide.

The fragment was sequenced, and through conventional subcloning, the coding region responsible for erythromycin resistance was identified and found to be sufficient to confer an erythromycin-resistant phenotype in *E. coli* and *S. pneumoniae* (see below). Since the gene was 90% identical to *mefA* from *S. pyogenes* (8), we designated the pneumococcal gene *mefE.*

**Heterogeneity of *mefE* and comparison to *mefA*.** To determine if *mefE* alleles were heterogeneous, we cloned *mefE* from two other clinical isolates of *S. pneumoniae*, 02J1048 and 02J1052, with primers specific for *mefA* and *mefE* (8). The coding sequences of the three *mefE* alleles were nearly identical, differing in only 2 of 1,218 nucleotides: position 528 in strain 02J1048 is A, while in strains 02J1052 and 02J1175 it is G. At position 850, strain 02J1052 has a T while the other two strains have a C. The base change at position 528 is silent with respect to its translation product, while the change at position 850 results in a Y (Tyr) in position 284 in strain 02J1048 and an H (His) in strains 02J1052 and 02J1175.

**MefE appears responsible for erythromycin efflux.** To determine if *mefE* encoded the erythromycin determinant necessary for macrolide efflux in pneumococci, we constructed isogenic strains of R6 that were *mefE*$^+$ and *mefE*. Genomic DNA from 02J1175 was used as a source of DNA to transform R6; clones (i.e., R6/1175-1) containing *mefE* were selected on BHI plates containing 8 μg of erythromycin per ml. Replacement of *mefE* by a null *mefE* cassette was accomplished with a fragment containing *mefE* disrupted with *cat* (fragment from either pAT16-1 or pAT16-2) as the source of DNA to transform R6/1175-1. From each transformation, 16 chloramphenicol-resistant transformants were picked onto blood plates spread with 5 μg of erythromycin per ml. All 16 transformants resulting from the integration of the *mefE*::*cat* fragment from pAT16-2 were susceptible to erythromycin. From the pat16-1 fragment transformation, 12 were susceptible and 4 were resistant to erythromycin. Hybridization analysis showed that the erythromycin-resistant, chloramphenicol-resistant transformants contain a larger *mef*-hybridizing band than the parental strain or the erythromycin-susceptible, chloramphenicol-resistant transformants (data not shown), implying that a gene duplication event, rather than a simple gene replacement, had occurred. Clones that were chloramphenicol resistant and erythromycin susceptible were confirmed by PCR analysis and hybridization to contain the replacement; restriction analysis distinguished that transformants resulting from incorporation of the pAT16-2 fragment had the *cat* gene inserted in the same orientation as that of *mefE*, while clones from the other transformation were shown to have *cat* and *mefE* in opposite orientations.

Analysis of the strains by a traditional microtiter-based in vitro susceptibility assay revealed that disruption of *mefE* in strain R6/1175-1 reduced the erythromycin MIC of the resultant strain (R6/1175/16-1) to within twofold of the MIC observed for R6 (Table 2). Further, when the uptake of erythromycin in the three strains was compared, only R6/1175-1 had a reduced accumulation of $^{14}$C-labeled erythromycin (Fig. 1). Thus, *mefE* appears to be required for expression of erythromycin efflux in strains bearing the M phenotype.

**MefE appears to efflux only 14- and 15-membered macrolides.** The substrate specificity of the MefE pump was explored with the isogenic strain constructs. Table 2 shows that substrates that are typical of other multidrug-resistant pumps (i.e., NorA and Bmr) (19, 24) like acridine orange, TPP, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), chloramphenicol, tetracycline, and norfloxacin did not appear to be substrates of the MefE pump. Although strain R6/1175-1/16-1 was slightly more susceptible to norfloxacin, acridine orange, and CCCP than strain R6/1175-1/16-2, this result may have been influenced by the difference in orientation of the *cat* insertion in *mefE* in the two strains or just indicative of the two- to fourfold dilution error observed in typical MIC dilution schemes. Other compounds like penicillin G, josamycin, and clindamycin failed to show significant differences in MICs when the three strains were compared, suggesting that these compounds are not substrates. Only 14-membered (erythromycin and clarithromycin) and 15-membered macrolides (azithromycin) appeared to be recognized by the MefE efflux pump.

## DISCUSSION

The presence of an rRNA methylase in pneumococci was recognized early as being responsible for erythromycin resistance (2, 18). The *ermB* (*ermAM*) gene in pneumococci is often part of the Tn*1545* transposon that carries determinants that confer resistance to tetracycline and streptomycin (39). However, recent epidemiological studies have revealed that a wide-

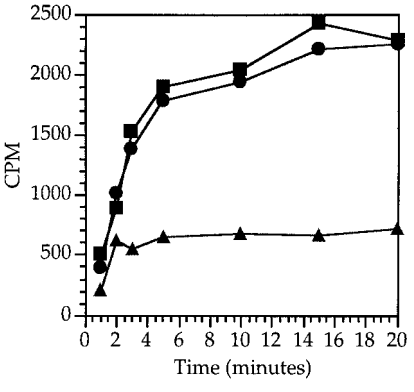


FIG. 1. Efflux experiments with *S. pneumoniae* strains isogenic for *mefE*. Strains R6 (■), R6/1175/1, a *mefE*$^+$ derivative (▲), and R6/1175/16-2, a *mefE*::*cat* derivative (●), were used.

spread form of erythromycin resistance in clinical strains of streptococci is encoded by an element that yields a macrolide-resistant but clindamycin- and streptogramin B-susceptible or M phenotype (32, 36). The results of this study reveal that the determinant responsible for the M phenotype is *mefE*, an efflux gene that is ~90% identical to the efflux gene *mefA* (8) found in clinical strains of *S. pyogenes* that also have the M phenotype (10, 25, 29–31, 33, 36).

The 405-amino-acid protein encoded by *mefE* has many of the same characteristics recently described for the protein encoded by *mefA* (8). It is a hydrophobic protein with 12 putative membrane-spanning regions and no recognizable signal sequence. BlastX analysis reveals homologies to other transporters or efflux proteins (8), including a putative transporter of ethambutol from a high-level ethambutol-resistant mutant of *Mycobacterium smegmatis* (37) and *cmr*, a transmembrane protein with homology to the major facilitator family from *Corynebacterium glutamicum* that confers resistance to erythromycin, tetracycline, puromycin, and bleomycin when cloned in *Escherichia coli* (17). The homologies cover extensive portions of both the N- and C-terminal halves of the proteins.

The construction of isogenic strains of *S. pneumoniae* (*mefE*$^+$ and *mefE*::*cat*) confirms that *mefE* has a role in mediating the efflux of erythromycin in pneumococcal strains with the M phenotype. Based on MIC comparisons, MefE appears to pump 14- and 15-membered macrolides and not a diversity of other compounds. *mefE*, unlike *msrA* and *msrB* in staphylococci (21, 26–28), is homologous to transporters that use proton motive force rather than ATP as part of the pump mechanism. We have previously shown that *mefE*$^+$ strains of pneumococci accumulate significantly greater levels of erythromycin if the pump is inhibited by arsenate, an agent used to disrupt proton motive force (36).

Using PCR primers specific for *mefA* and *mefE* (34), we have confirmed that all previously examined pneumococcal and group A streptococcal strains (36) with the M phenotype contain *mefE* or *mefA*, respectively. Recently, we found that other erythromycin-resistant streptococci (group B and viridans group) with the M phenotype also contain *mefE* (35). Colleagues at The Graduate Hospital (Philadelphia, Pa.) have found a *mef* PCR product in *Enterococcus faecium* clinical strains that are moderately resistant to erythromycin and susceptible to clindamycin (13). Neither group has obtained any evidence that *mefE* is a normally silent resident in pneumococci (or enterococci), as PCRs with *mef*-specific primers do not yield a PCR product in macrolide-susceptible strains of pneumococci or enterococci (13, 34). The *E. faecium* gene is identical to *mefE* and, subsequently, ~90% identical to *mefA*. The finding of *mefE* in enterococci suggests that the prevalence of the *mef* determinant and its role in conferring macrolide resistance may be underestimated in the clinic, leading to potentially missed opportunities for therapeutic intervention with clindamycin (12, 20, 23).

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