Plasmid-Borne *erm*(T) from Invasive, Macrolide-Resistant *Streptococcus pyogenes* Strains[†]

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Twenty-three isolates of group A streptococci (GAS) recovered from population-based invasive GAS surveillance in the United States were erythromycin resistant, inducibly clindamycin resistant, and lacked known macrolide resistance determinants. These 23 isolates, representing four different clones, contained a broad-host-range plasmid carrying the *erm*(T) methylase gene, which has not been detected in GAS previously.

Penicillins are the antibiotics of choice for treatment of pharyngitis caused by group A streptococci (GAS), with macrolides recommended for patients with penicillin allergy. Macrolide-resistant GAS are an increasing concern worldwide, with a correlation between increasing macrolide resistance and an increase in macrolide consumption (2, 6, 22).

In GAS, macrolide resistance is conferred by the 23S rRNA methylase genes *erm*(B) and *erm*(TR), as well as by the efflux determinant *mef*(A) (12, 15). The *erm*(B) and *erm*(TR) genes often confer inducible resistance to macrolides, lincosamides, and streptogramin B, but also may encode constitutive resistance to macrolides, lincosamides, and streptogramin B due to upstream attenuator sequence alterations. *mef*(A) encodes efflux pump-mediated resistance to erythromycin (and other 14- or 15-member ring macrolides), while the organism remains susceptible to clindamycin (and other lincosamides) and streptogramin B. Acquisition of macrolide resistance primarily occurs by horizontal gene transfer of *erm* or *mef* genes (4, 13), while more rarely resistance results from mutations within genes encoding ribosomal components (12). The *erm*(B) and *erm*(TR) genes often confer cross-resistance to both lincosamides and streptogramins and therefore limit the therapeutic possibilities available to clinicians for the treatment of GAS disease (12).

In this study, we identified the genetic basis of macrolide and inducible clindamycin resistance in 23 invasive GAS isolates that were negative for macrolide resistance determinants known to be disseminated among resistant GAS (5, 16, 18). These 23 isolates were collected among all invasive GAS infections reported during 1999, 2001, and 2003 (3,189 reported cases) from CDC's population-based Active Bacterial Core surveillance conducted in 10 sites throughout the United States (<http://www.cdc.gov/ncidod/dbmd/abcs/>).

Susceptibility to erythromycin was determined by the broth dilution technique with a standard panel of antibiotics, includ-

ing erythromycin and clindamycin. The double-disk diffusion (D) test (4, 16) confirmed erythromycin resistance and detected inducible clindamycin resistance. Mueller-Hinton agar plates supplemented with 5% sheep blood were inoculated with a suspension of GAS that met a McFarland 0.5 turbidity standard. An erythromycin disk containing 15 µg and a clindamycin disk containing 2 µg were placed 12 mm apart (edge to edge). Resistance to erythromycin with blunting of the zone of inhibition around the clindamycin disk facing the side of the erythromycin disk indicated inducible clindamycin resistance.

PCR was performed for detection of *erm*(A), *erm*(B), and *erm*(C) (18). Detection of *erm*(TR) and *mef* determinants employed primers designed at the Minnesota Department of Health (Table 1). PCRs for *erm*(TR) and *mef* contained 10 mM Tris HCl, 50 mM KCl, 1.5 mM MgCl₂, 1 µM each forward and reverse primers, and 2 µl of template DNA in a total volume of 50 µl. Annealing temperatures were 55°C for *erm*(TR) and 56°C for *mef*. When PCR assays were negative for the *erm* and *mef* determinants described above, *erm*(T)-specific primers (Table 1) were subsequently used to detect a PCR product of 478 bp from all 23 isolates after electrophoresis on 2% agarose gels.

Crude bacterial DNA templates were prepared as described previously (1; www.cdc.gov/ncidod/biotech/strep/protocol_erm-type.htm). Assuming a chromosomal location for *erm*(T) in the 23 isolates, single-primer PCR (10) was used to amplify the sequences adjacent to *erm*(T) as described previously for mapping the GAS *sof* locus (8). All sequencing data generated from the single-primer template were reconfirmed by conventional PCR amplification with appropriate primer pairs. DNA sequences were analyzed using the Wisconsin Package, version 10.3 (Accelrys, Inc., San Diego, CA). When a high level of homology to the *Lactobacillus* sp. plasmid p121BS (23) was found downstream and upstream of *erm*(T), we subsequently used appropriate reverse and forward primers for amplification of the putative plasmid as a linear PCR fragment. Plasmid DNA was purified from *Streptococcus pyogenes* isolates with the Qiagen Mini-Prep kit, with a 10-min incubation in buffer P1 containing lysozyme and mutanolysin at 37°C prior to the addition of reagent P2. Sequence analysis indicated that *erm*(T) was situated on a 4,962-bp replicative

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TABLE 1. PCR primers used in this study for detection of macrolide resistance genes

Gene(s)	Primer	Sequence
<i>erm</i> (TR)	TR-322U	5'-GGGTCAGGAAAAGGACAT-3'
	TR619L	5'-CCTAAAGCTCGTTGATT-3'
<i>mef</i> (A) and <i>mef</i> (E)	mef-3301U	5'-AGGGCAAGCAGTATCATTAA TCA-3'
	mef-3673L	5'-CTGCAAAGACTGACTATAGC CT-3'
<i>erm</i> (T)	<i>erm</i> (T) forward	5'-CCGCCATTGAAATAGATCC T-3'
	<i>erm</i> (T) reverse	5'-GCTTGATAAAATTGGTTTTT GGA-3'

plasmid, designated pRW35 (GenBank accession no. EU192194). The pRW35 sequence had a G+C content of 37% and three distinct open reading frames (ORFs) (Fig. 1A).

One ORF exhibited near identity ($\geq 98\%$) to the erythromycin resistance methylase gene *erm*(T) found on a transposon in *Streptococcus gallolyticus* subsp. *pasteurianus* (20) as well as

plasmid-borne *erm*(T) determinants from *Lactobacillus reuteri* (19), *Lactobacillus* sp. (23), and *Streptococcus bovis* (NCBI accession no. BAA75016). In addition to *erm*(T), pRW35 contains two ORFs highly similar to plasmid replication and transfer genes found in broad-host-range plasmids. ORF2 predicts a protein highly similar ($>60\%$) to mobilization proteins from *Streptococcus agalactiae*, *Lactococcus lactis*, and *Streptococcus bovis* (14, 21).

An attenuator region upstream of *erm*(T) in pRW35 nearly identical to its counterpart in *S. gallolyticus* subsp. *pasteurianus* was identified (Fig. 1B). This region shares features with the corresponding region upstream of the *S. aureus* plasmid-borne *erm*(C) (7) and is predicted to share the same mechanism of regulating translation of the *erm*(T) message (3, 7). During induction by erythromycin, sensitive ribosomes are stalled upon the upstream leader peptide favoring formation of hairpin structure 5-6 (active conformation). As the proportion of resistant ribosomes increases (originally present in relatively small numbers) to a critical level, translation of the *erm*(T) message greatly decreases due to a shift of the message to the inactive conformation

FIG. 1. (A) Diagram of pRW35. Three ORFs are indicated. (B) Nearly identical attenuator and translational start regions of *erm*(T) from *S. pyogenes* and *Streptococcus pasteurianus*. The sequences encompassing the leader peptide-encoding sequence and *erm*(T) translational start are shown. Perfect and imperfect inverted repeats are indicated with arrows. Pairing of inverted repeats 5 and 6 represents the putative active conformation according to the *erm*(C) model (10). Pairing of inverted repeats 1 and 2 and 3 and 4 represents the putative inactive conformation in which the *erm*(T) ribosome binding site (RBS) and translation initiation codon are sequestered by pairing of inverted repeats 3 and 4. The RBS, translational start codons, and leader peptide translational stop codons are in bold.

TABLE 2. Features of invasive GAS isolates containing *erm*(T)

<i>erm</i> type	Yr isolated (no. of isolates)	State(s) (no. of isolates)	T pattern (no. of isolates)	PFGE profile ^a
<i>erm</i> 92	1999 (3), 2001 (6), 2003 (10)	California (11), Oregon (7), Connecticut (1)	T/8/25/Imp19 ^b (17), T nontypeable (2)	1
<i>erm</i> 3	2003 (2)	Oregon (2)	T3	2
<i>erm</i> 9	2001 (1)	Georgia (1)	T14	3
<i>erm</i> 28	2001 (1)	Oregon (1)	T28	4

^a PFGE patterns within a given profile share identity or diverge from others within the profile by no more than three bands and differ from the other profiles by more than seven bands.

^b This pattern includes T/8/25/Imp19, T25/Imp19, T/Imp19, and T8/25.

(1-2 and 3-4 hairpins) that results in sequestration of the *erm*(T) translational start and ribosome binding site.

erm typing (2; www.cdc.gov/ncidod/biotech/strep/protocol_erm-type.htm) identified four unique types among the 23 *erm*(T)-positive isolates, including *erm*92 (19 isolates), *erm*3 (2 isolates), *erm*9, and *erm*28 (Table 2). The 19 *erm*92 isolates formed a distinct cluster of highly related pulsed-field gel electrophoresis (PFGE) patterns, and the two *erm*3 isolates displayed an additional distinct PFGE profile (data not shown). The *erm*9 and *erm*28 isolates represented two additional distinct patterns. The T types from all of the isolates and PFGE profiles from types *erm*92, *erm*3, and *erm*28 conformed to patterns typically associated with erythromycin-sensitive strains of the same *erm* types (9; unpublished data), indicating that the emergence of *erm*(T)-positive GAS is not associated with newly emerging clonal types.

We examined the ability of pRW35 to transfer resistance to another streptococcal species and to another *S. pyogenes* strain. Plasmid pRW35 was extracted from *S. pyogenes* isolates and electroporated into *S. agalactiae* (group B streptococci) and the *erm*49 *S. pyogenes* strain NZ131 by previously described methods (11, 17). Transformants of both species selected on sheep blood agar plates containing 1 µg/ml erythromycin were subsequently screened for the presence of the *erm*(T) gene by PCR and tested for inducible clindamycin resistance with the D test. All transformants had identical resistance phenotypes with the 23 GAS isolates in that they were resistant to erythromycin (≥32 µg/ml) and inducibly clindamycin resistant (12 µg/ml). All transformants were positive for *erm*(T) and the entire circular plasmid pRW35 by PCR analysis. These results indicated that pRW35 conferred erythromycin and inducible clindamycin resistance as a replicative plasmid in the distantly related group B species and in a heterologous *S. pyogenes* strain.

In summary, our data indicate that plasmid pRW25 is disseminated among multiple unrelated GAS strains, including classical opacity factor-positive (types *erm*28, *erm*9, and *erm*92) and -negative (*erm*3) strains. Transformation of pRW25 into *S. agalactiae* and an additional GAS strain as a replicative plasmid is consistent with the hypothesis that pRW35 is capable of dissemination to a broad range of hosts. *erm*(T)-specific primers may be useful in subsequent PCR screens for macrolide resistance determinants among GAS.

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