Plasmid-borne *erm*(T) from invasive, macrolide resistant *Streptococcus pyogenes* strains

Running title: new GAS macrolide-resistance determinant.

Key words: erythromycin and inducible clindamycin resistance, streptococcal plasmid, *erm*(T), methylase

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Abstract

Twenty-three 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 4 different clones, contained a broad host-range plasmid carrying the $erm(T)$ methylase gene which has not been previously 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 \textit{erm}(B) and \textit{erm}(TR), as well as by the efflux determinant \textit{mef}(A) [12,15]. The \textit{erm}(B) and \textit{erm}(TR) genes often confer inducible resistance to macrolides, lincosamides, and streptogramin B (iMLS\textsubscript{B} resistance), but also may encode constitutive MLS\textsubscript{B} (cMLS\textsubscript{B}) resistance due to upstream attenuator sequence alterations. \textit{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 or \textit{erm} of \textit{mef} genes (4,13), while more rarely resistance results through mutations within genes encoding ribosomal components (12). The \textit{erm}(B) and \textit{erm}(TR) genes often confer cross-resistance to both lincosamides and streptogramins, and therefore limit the therapeutic possibilities available to clinicians in 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 (3189 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, including 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 \( \text{erm}(A) \), \( \text{erm}(B) \), and \( \text{erm}(C) \) (18). Detection of \( \text{erm}(\text{TR}) \) and \( \text{mef} \) determinants employed primers designed at MDH (Table 1). PCR reactions for \( \text{erm}(\text{TR}) \) and \( \text{mef} \) contained 10 mM Tris HCl, 50 mM KCl, 1.5 mM MgCl2, 1 µM forward and reverse primers, and 2 µl of template DNA in a total volume of 50 µl. Annealing temperatures were 55°C (\( \text{erm}(\text{TR}) \)) and 56°C (\( \text{mef} \)). When PCR assays were negative for the \( \text{erm} \) and \( \text{mef} \) determinants above, \( \text{erm}(\text{T}) \)- specific primers (Table 1) were subsequently used to detect a PCR product of 478bp from all 23 isolates after electrophoresis on 2% agarose gels.

Crude bacterial DNA templates were prepared as described (1, www.cdc.gov/ncidod/biotech/strep/protocol_emm-type.htm). Assuming a chromosomal location for \( \text{erm}(\text{T}) \) in the 23 isolates, single-primer PCR (10) was used to amplify the sequences adjacent to \( \text{erm}(\text{T}) \) as described previously for mapping the GAS sof locus (8). All sequencing data generated from single primer template was reconfirmed by using 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 \( \text{Lactobacillus} \) sp. plasmid p121BS (23) was found downstream and upstream of \( \text{erm}(\text{T}) \) we
subsequently used appropriate reverse and forward primers for amplification of the putative plasmid as a linear PCR fragment. Plasmid DNA was purified from *S. pyogenes* isolates using the Qiagen Mini-Prep kit with a 10 minute 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 base pair replicative plasmid, designated pRW35 (Genbank accession 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 (≥ 98%) to the erythromycin resistance methylase gene *erm*(T) found on a transposon in *Streptococcus galolyticus* subsp. *pasteurianus* (20) as well as plasmid-borne *erm*(T) determinants from *Lactobacillus reuteri* (19), *Lactobacillus* sp. (23), and *Streptococcus bovis* (NCBI accession BAA75016). In addition to *erm*(T), pRW35 contains two ORFs highly similar to plasmid replication and transfer genes found in broad host 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. galolyticus* 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 number) to a critical level, translation of the *erm*(T) message greatly decreases due to a shift of the message to the inactive
conformation (1-2, 3-4 hairpins) that results in sequestration of the \textit{erm}(T) translational start and ribosome binding site.

\textit{emm} typing (2, \url{www.cdc.gov/ncidod/biotech/strep/protocol_emm-type.htm}) identified 4 unique types among the 23 \textit{erm}(T)-positive isolates, including \textit{emm}92 (19 isolates), \textit{emm}3 (2 isolates), \textit{emm}9, and \textit{emm}28 (Table 1). The 19 \textit{emm}92 isolates formed a distinct cluster of highly related PFGE patterns and the two \textit{emm}3 isolates displayed an additional distinct PFGE profile (data not shown). The \textit{emm}9 and \textit{emm}28 isolates represented 2 additional distinct patterns. The \textit{T} types from all of the isolates and PFGE profiles from types \textit{emm}92, \textit{emm}3, and \textit{emm}28 conformed to patterns typically associated with erythromycin-sensitive strains of the same \textit{emm} types (13, unpublished data), indicating that the emergence of \textit{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 \textit{S. pyogenes} strain. Plasmid pRW35 was extracted from \textit{S. pyogenes} isolates and electroporated into \textit{S. agalactiae} (group B streptococci) and the \textit{emm}49 \textit{S. pyogenes} strain NZ131 using 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 \textit{erm}(T) gene by PCR, and tested for inducible clindamycin resistance using 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 resistance (12 µg/ml). All transformants were positive for \textit{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 \textit{S. pyogenes} strain.
In summary, our data indicates that plasmid pRW25 is disseminated among multiple unrelated GAS strains, including classical opacity factor positive (types emm28, emm9, and emm92) and negative (emm3) 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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References


Figure Legends.

Figure 1. A. Diagram of pRW35. Three open reading frames are indicated.

B. Near 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 repeat 5-6 represents the putative active conformation according to the  erm(C) model (10). Pairing of inverted repeats 1-2 and 3-4 represents the putative inactive conformation where the  erm(T) ribosome binding site and translation initiation codon is sequestered by pairing of 3 and 4.

Table 1.

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

<table>
<thead>
<tr>
<th>gene</th>
<th>Primer</th>
<th>sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>erm(TR)</td>
<td>TR-322U</td>
<td>5’-GGGTCAGGAAAGGACAT-3’</td>
</tr>
<tr>
<td></td>
<td>TR619L</td>
<td>5’-CCTAAAGCTCGTTGATT-3</td>
</tr>
<tr>
<td>mef(A) and</td>
<td>mef-3301U</td>
<td>5’-AGGGCAAGCAGTATCATTAATCA-3’</td>
</tr>
<tr>
<td>mef(E)</td>
<td>mef-3673L</td>
<td>5’-CTGCAAAGACTGACTATAGCCT-3’</td>
</tr>
<tr>
<td>erm(T)</td>
<td>erm(T) forward</td>
<td>5’ –CCGCCATTGAAATAGATCCT-3’</td>
</tr>
<tr>
<td></td>
<td>erm(T) reverse</td>
<td>5’ –GCTTGATAAAAATTGTTTTTGA-3’</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>emm type</th>
<th>Year isolated (no. of isolates)</th>
<th>States</th>
<th>T pattern (no. of isolates)</th>
<th>PFGE profile**</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>2003 (2)</td>
<td>Oregon (2)</td>
<td>T3</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>2001 (1)</td>
<td>Georgia (1)</td>
<td>T14</td>
<td>3</td>
</tr>
<tr>
<td>28</td>
<td>2001 (1)</td>
<td>Oregon (1)</td>
<td>T28</td>
<td>4</td>
</tr>
</tbody>
</table>

*This pattern includes T/8/25/Imp19, T25/Imp19, T/Imp19, and T8/25

** PFGE patterns within a given profile share identity or diverge from others within the profile by no more than 3 bands, and differ from the other profiles by more than 7 bands.
Fig. 1.

A.

B.
1) *S. pyogenes*
2) *S. pasteurianus*

1. ACTTTGCAACAGAACCAAAAGGAGAATTAATATTGGCATTTTTTAGTATTTTTGTAA
2. TTATTCTAATTTCATAAAAAGGAGAATTAATATTGGCATTTTTTAGTATTTTTGTAA

RBS

TCAACACAGTTTATACACCAACAAACAAAAAATTAAAGAGGATTGGATATGATGTTATGATA
TCAACACAGTTTATACACCAACAAACAAAAAATTAAAGAGGATTGGATATGATGTTATGATA

1           2           3           4

erm(T) start

TAACATTTCTATACGCGGTATTACGATCAAAAAATATAAAAG
TAACATTTCTATACGCGGTATTACGATCAAAAAATATAAAAG

1          2          3          4

Stop

Leader peptide

1. ACTTTGCAACAGAACCAAAAGGAGAATTAATATTGGCATTTTTTAGTATTTTTGTAA
2. TTATTCTAATTTCATAAAAAGGAGAATTAATATTGGCATTTTTTAGTATTTTTGTAA

ORF2 (mob, 1500 bp)

ORF1 (rep, 663 bp)

ermT (726 bp)

pRW35, 4,962 bp