Disruption of an *Enterococcus faecium* Species-Specific Gene, a Homologue of Acquired Macrolide Resistance Genes of Staphylococci, Is Associated with an Increase in Macrolide Susceptibility

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The complete sequence (1,479 nucleotides) of msc, part of which was recently reported by others using a different strain, was determined. This gene was found in 233 of 233 isolates of *Enterococcus faecium* but in none of 265 other enterococci. Disruption of *msc* was associated with a two- to eightfold decrease in MICs of erythromycin azithromycin, tylosin, and quinupristin, suggesting that it may explain in part the apparent greater intrinsic resistance to macrolides of isolates of *E. faecium* relative to many streptococci. This endogenous, species-specific gene of *E. faecium* is 53% identical to *msrA*, suggesting that it may be a remote progenitor of the acquired macrolide resistance gene found in some isolates of staphylococci.

We have recently screened a number of gram-positive cocci for macrolide susceptibility (12) and subsequently for some of the acquired resistance genes, *erm*(A), *erm*(B), *erm*(C), *ere*(A), *ere*(B), *meR*(A), and *msr*(A) (24, 29), that are known to effect macrolide, streptogramin, and/or lincosamide (MS/L) susceptibility (unpublished data). We noted that some of the *Enterococcus faecium* isolates for which the MICs (2 to 16 μg/ml) of erythromycin (ERY) were elevated failed to hybridize to any of the aforementioned resistance gene probes. We next performed PCR amplification using DNA from macrolide non-susceptible, probe-negative *E. faecium* strains and primers for *msr*(A/B) (29); a fragment with homology to *msr*(A) and *msr*(B), the acquired macrolide resistance genes found in staphylococci, was recovered. In the current work, the complete sequence (1,479 nucleotides [nt]) of the gene encompassing this fragment along with ~450 bp upstream was determined. This gene was found to contain the 405-bp fragment previously deposited in GenBank (accession no. AJ243209) and recently reported as *msc*, a species-specific gene of *E. faecium* (22). An insertion disruption mutation of this gene has now been generated and the *E. faecium* mutant was found to be more susceptible to ERY, azithromycin, tylosin, and quinupristin, suggesting that this *msr*-like gene can confer some protection to isolates of *E. faecium* against these antimicrobials.

**MATERIALS AND METHODS**

**Bacterial strains and MIC studies.** The microorganisms used in this study were obtained from the collection of our laboratory over the past several years. A total of 498 isolates of enterococci, 56 streptococcal isolates (some of which were previously described) (5, 12), and two staphylococcal isolates (as controls for the gene described) were used in the various studies. The majority of these clinical isolates came from the United States but some were from Thailand, Argentina, Belgium, and Spain. The enterococcal isolates included 246 *Enterococcus faecalis*, 233 *E. faecium*, 6 *E. bireu*, 5 *E. durans*, 2 *E. casseliflavus*, 2 *E. munditi*, 2 *Staphylococcus aureus*, 1 *E. gallinarum*, 2 *E. solitarius*, and 1 *E. raffinosus* isolate. ERY MICs were also determined by agar dilution (19, 20) for a group of 90 *E. faecalis*, 64 *E. faecium*, 29 *Streptococcus pneumoniae*, 10 group B streptococci, and 17 *Streptococcus pyogenes* isolates. ERY, kanamycin (KAN), and tylosin were purchased from Sigma Chemical Co., St. Louis, Mo., and quinupristin was provided by Rhone-Poulenc Rorer Pharmaceuticals, Inc., Collegeville, Pa.

**DNA extraction, PCR, sequencing, and cloning.** *E. faecium* isolate SE34 (TX1330) (MIC of ERY, 0.25 to 0.75 μg/ml) was used as a recipient strain; it was recovered from the feces of a healthy community volunteer (5) and has been used in our lab because it lacks resistance to most agents tested and is transformable by electroporation. DNA extraction (32) and PCR were done using the PCR Optimizer kit (Invitrogen, San Diego, Calif.); PCR products were analyzed by automated DNA sequencing at the Microbiology and Molecular Genetics core facility, University of Texas Medical School, Houston, Tex. Parts of the sequence described in this study were generated using *msr*(A/B) primers (29) (primer I, 5′-GCA AAT GGT GTA GGT AAG ACA ACT-3′ and primer II, 5′-ATG TGA TGT AAA CAA AAT-3′) and other sequence parts were generated by inverse PCR and octamer primer of the RaD Prime labeling kit (Gibco BRL, Grand Island, N.Y.) using DNA from TX2465 (16), TX2597, and TX2046 (15). These three *E. faecium* clinical isolates (ERY MIC, 2 to 16 μg/ml) were chosen arbitrarily as examples of nonsusceptible isolates that were negative with *erm*(A), *erm*(B), *erm*(C), *ere*(A), *ere*(B), *mef*(A), and *msr*(A) probes (henceforth referred to as MS/L probe-negative isolates). The sequence of the *msc* coding region using DNA from TX1330 was also determined in later experiments using specific primers designed from the other sequences. Sequence analysis was done using the BLAST network service of the National Center for Biotechnology Information. The GCG software package (Genetics Computer Group, Madison, Wis.) was used to compare similarities among other sequences. Filter settings were performed using *E. faecium* GE-1 (7), which is tetracycline (TET) resistant, as a recipient strain. Cloning was done with standard methods (26) by using Sau3A-digested genomic DNA from TX2465, TX2597, and TX2046 *E. faecium* isolates and using BlueScript vector and Escherichia coli DH5α cells.

**Disruption mutation in *msc* of *E. faecium*.** In order to construct the disruption mutation in the *msc* gene, we generated a 628-bp intragenic DNA fragment (nt 1251 to 1879; see Fig. 1) by PCR from TX2465, one of the macrolide nonsusceptible, MS/L probe-negative *E. faecium* strains, and cloned it into the pCR2.1 vector of the TA Cloning kit (Invitrogen), resulting in pTEX5259. The fragment was recloned into the previously published pBluescript derivative pTEX4577, containing aph(3′)-Ia (8, 28), resulting in pTEX5259.03. Plasmid
RESULTS AND DISCUSSION

PCR amplification using the msr(A/B) primers and DNA from TX2465, TX2597, and TX2046 generated an ~350-bp DNA fragment from each of these three strains. Then, using inverse PCR and also the octamer primer, we generated an ~2.4-kb sequence (Fig. 1) from the TX2465 E. faecium isolate. When this sequence was used to search GenBank, it showed the highest homology score with a 405-bp fragment (accession no. AJ243209), recently named msrC (22). The 405-bp fragment is 95% identical to nt 1488 to 1890 of the coding region of the sequence shown in Fig. 1; based on this identity, we consider the current sequence to be the complete sequence of an msrC gene. Since this gene appears to be an endogenous chromosomally encoded gene, we have maintained the format of the gene name as msrC rather than adopt the recent recommendations for acquired macrolide resistance genes [e.g., mrs(A)] (24). Analysis of the 2.4-kb sequence of strain TX2465 (Fig. 1) revealed an open reading frame (1,479 bp) with an ATG potential start codon at nt 496, preceded by a putative Shine-Dalgarno (SD) sequence and a TAA stop codon at nt 1972 to 1974. The coding sequence of this msrC gene (1,479 bp) showed 53% identity to mrs(A) (1,467 bp), 57% identity to mrs(B) (531 bp) over the corresponding region, and 47% identity to vga(A) (1,569 bp) and vga(B) (1,659 bp) (1, 2). The predicted MsrC protein (492 amino acids [aa]) showed similarities to ABC proteins of other gram-positive bacteria (54% similarity to Msr(A) (488 aa) of Staphylococcus epidermidis; 59% similarity over aa 301 to 492 of MsrC, compared to the 176-aa C-terminal region of Msr(B) of Staphylococcus xylosus; 50% similarity to Vga(A) (522 aa); and 46% similarity to Vga(B) (552 aa)) (2, 13, 14, 25). As reported in these references, Msr(A), Vga(A), and Vga(B) contain two ATP-binding domains, each of which in turn contains the two ATP-binding motifs, WA and WB, described by Walker et al. (2, 13, 14, 25, 30). The predicted amino acid sequence of MsrC also contains two homologous ATP-binding domains and, in the region corresponding to these domains of Msr(A), Vga(A), and Vga(B), we detected the presence of the highly conserved SGG sequence found between the WA and WB ATP-binding motifs of the previously investigated proteins (2, 3, 10). The interdomain sequence, called the Q-linker, which separates the two ATP-binding domains of Msr(A) and Vga(B), has been described as

FIG. 1. Complete nucleotide sequence from strain TX2465 of msrC and its upstream region. Shown are the putative promoter sequences (~10 and ~35, underlined), a possible polypeptide (15 aa) and five inverted repeat regions in the upstream region (indicated by arrows), the predicted amino acids (with one-letter code), SD sequence (in bold letters), and stop codons (indicated by asterisks). Possible WA and WB regions are shown in boxes with bold letters, and the possible Q-linker region is in brackets. Conserved SGG sequences are shown with bold letters and underlined.
TABLE 1. MICs of macrolides (14-, 15-, and 16-membered) for wild-type E. faecium TX1330 and two msrC disruption mutants

<table>
<thead>
<tr>
<th>Organism</th>
<th>Erythromycin</th>
<th>Azithromycin</th>
<th>Tyllosin</th>
<th>Quinupristin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>E-test MIC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>MIC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>E-test MIC&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>TX1330</td>
<td>0.75</td>
<td>0.25</td>
<td>1.56</td>
<td>16</td>
</tr>
<tr>
<td>TX1330</td>
<td>0.5</td>
<td>0.38</td>
<td>1.56</td>
<td>16</td>
</tr>
<tr>
<td>msrC mutant 1</td>
<td>0.09</td>
<td>0.094</td>
<td>0.38</td>
<td>8</td>
</tr>
<tr>
<td>msrC mutant 2</td>
<td>0.09</td>
<td>0.094</td>
<td>0.38</td>
<td>8</td>
</tr>
<tr>
<td>msrC mutant 3</td>
<td>0.06</td>
<td>0.094</td>
<td>0.38</td>
<td>8</td>
</tr>
<tr>
<td>msrC mutant 4</td>
<td>0.06</td>
<td>0.094</td>
<td>0.38</td>
<td>8</td>
</tr>
</tbody>
</table>

<sup>a</sup> By broth microdilution method. The ERY concentrations tested were 0.04, 0.06, 0.09, 0.125, 0.187, 0.25, 0.37, 0.5, and 0.75 μg/mL; the quinupristin concentrations tested were 32, 48, 64, and 96 μg/mL.

<sup>b</sup> Performed using inocula as recommended by the manufacturer.

<sup>c</sup> TX1330 and msrC mutant 1 were evaluated in triplicate, and mutant 2 was evaluated in duplicate.

were shown to have aph(3′)-IIIa; all 5 were interrupted in msrC, which was confirmed by pulsed-field gel electrophoresis and hybridization (data not shown). TX1330 and one of the mutant colonies showed almost identical growth curves by hourly determinations of optical density at 600 nm and CFU. There was little to no loss of Kan<sup>R</sup> by the cultures, indicating that the insertion was stable during the 24-h incubation period. Mutant colonies showed a decrease in broth microdilution MICs (Table 1) of ERY (from 0.5 to 0.75 μg/mL for TX1330 to 0.06 to 0.09 μg/mL for mutants), of tyllosin (from 16 μg/mL for TX1330 to 8 μg/mL for mutants), and of quinupristin (from 96 μg/mL for TX1330 to 48 to 64 μg/mL for mutants), suggesting that this gene provides some protection against these agents. Because of the small difference for quinupristin, we also tested a lower inoculum of 10<sup>3</sup> CFU/mL. TX1330 (tested in triplicate) grew in medium with 32 μg/mL but not at 48 μg/mL (MIC, 48 μg/mL), while mutants 1 (in triplicate) and 2 (in duplicate) all grew on 12 μg/mL but not on 16 μg/mL (MIC, 16 μg/mL), further verifying that there is a small but true difference. Mutant colonies also showed a decrease in E-test MICs (Table 1) of azithromycin (from 1.56 μg/mL for TX1330 to 0.38 μg/mL for mutants). E-test MICs of clindamycin and norfloxacin for TX1330 and mutant colonies were almost identical (data not shown).

Neu (21) previously pointed out that isolates of E. faecium often tend to be more resistant to 14- and 16-membered ring macrolides with MICs at which 50% of strains are inhibited of 8 to 16 μg/mL. Among our clinical isolates, many of the E. faecalis (61 of 90) and most of the E. faecium (55 of 64) isolates hybridized with one of the macrolide resistance gene probes tested (unpublished data). Table 2 shows the distribution of ERY MICs found among MS/L probe-negative clinical isolates of E. faecalis, E. faecium, and streptococci. While 20 of 29 MS/L probe-negative E. faecalis isolates required MICs of ERY of ≤1 μg/mL, none of the 9 clinical isolates of MS/L probe-negative E. faecium required MICs of <1 μg/mL (MICs ranged from 2 to 16 μg/mL). Almost all S. pyogenes and group B streptococcal isolates required MICs of ERY of ≤0.125 μg/mL. The 17 S. pneumoniae isolates negative for MS/L probes showed MICs of ≤0.125 μg/mL. While more susceptible isolates of E. faecium do exist, such as the recipient strain used in this study, which was isolated from the feces of a healthy nonhospitalized volunteer (5), the above results indicate that clinical isolates of E. faecium are less susceptible to ERY than are isolates of E. faecalis or of streptococcal species. Whether the higher ERY MICs for MS/L probe-negative clinical iso-

<table>
<thead>
<tr>
<th>Organism (n)</th>
<th>Total no. of strains inhibited at each concn (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤0.03 0.06 0.125 0.25 0.5 1 2 4 8 16 32</td>
</tr>
<tr>
<td>E. faecalis (29)</td>
<td>2 1 3 5 9 8 1</td>
</tr>
<tr>
<td>E. faecium (9)</td>
<td>3 2 2 2</td>
</tr>
<tr>
<td>S. pyogenes (29)</td>
<td>7 4 17 1</td>
</tr>
<tr>
<td>Group B streptococci (10)</td>
<td>10</td>
</tr>
<tr>
<td>S. pneumoniae (17)</td>
<td>1 8 8</td>
</tr>
</tbody>
</table>

<sup>a</sup> Probes for erm(A), erm(B), erm(C), ermA, erMB, mef(A), and msr(A).
lates of *E. faecium* are related to changes in the structure or expression of Mrsc has not been determined, in part due to the difficulty in generating and selecting targeted mutations in these organisms. We did not determine the role of amino acid changes in the Mrsc of TX1330 relative to other *E. faecium* isolates.

In conclusion, we have determined the complete sequence of *msrC*, a species-specific gene of *E. faecium*, from two strains and have shown that the presence of *msrC*, or possibly a downstream gene, results in some protection of an isolate of *E. faecium* against ERY by increasing efflux (2), we have not determined the exact function encoded by the endogenous *msrC* gene in *E. faecium*; however, the similarity of Mrsc to Mrsa(A) (54%) suggests that it also mediates efflux. Based on the hybridization results showing species specificity, *msrC* also appears useful as a means of identifying *E. faecium* isolates.

**REFERENCES**


