Characterization of \( \text{emeA} \), a \( \text{norA} \) Homolog and Multidrug Resistance Efflux Pump, in \( \text{Enterococcus faecalis} \)

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We hypothesized that multidrug resistance efflux pumps (MDRs) may be contributing to the drug resistance of enterococci. We recently identified potential MDR-encoding genes in the \( \text{Enterococcus faecalis} \) V583 genome. Among the putative MDRs, we found a gene that encodes a NorA homolog and have characterized this enterococcal MDR in the present study. A mutant from which the enterococcal NorA homolog has been deleted has reduced resistance to several NorA substrates. Complementation of the deletion mutant with the wild-type gene verified the involvement of this enterococcal gene in resistance to ethidium bromide (EtBr) and norfloxacin. Known MDR inhibitors (reserpine, lansoprazole, and verapamil) inhibit the efflux of EtBr and norfloxacin in wild-type strain OG1RF. A fluorescence assay with EtBr allowed us to quantitate the efflux capability of the enterococcal NorA pump. On the basis of these results, we have named this enterococcal gene \( \text{emeA} \) (enterococcal multidrug resistance efflux).

Many enterococcal isolates are resistant to the majority of antimicrobial agents that are in clinical use. This causes therapeutic problems, especially for patients with serious, multidrug-resistant enterococcal infections. Enterococci are equipped with a variety of antibiotic resistance genes, some of which are inherent and some of which are acquired. The intrinsic resistance of enterococci to certain antibiotics may be partially due to genes that encode multidrug resistance efflux pumps (MDRs).

MDRs are found in a variety of organisms ranging from bacteria to humans, suggesting their universal importance. Although the primary functions and mechanisms of action for most MDRs remain unclear, these proteins are known to actively transport toxic compounds out of the cell. Antibiotic-specific efflux pumps (e.g., Tet(K) and CmlA) are often encoded on transmissible plasmids and transposons in bacteria. However, most genes for MDRs are encoded on the bacterial chromosome (6).

In a number of other bacteria, MDRs have also been implicated as important contributors to multidrug resistance (3, 4, 5, 7). Because enterococci are intrinsically resistant to a variety of antibiotics, it seems reasonable to assume that MDRs could be contributing to the drug resistance. However, there has been only one study on MDRs in enterococci prior to the present study. Enterococci were shown to extrude chloramphenicol, tetracycline, and norfloxacin; but the efflux pump(s) responsible for extruding these drugs was not identified (9). The need to identify drug efflux pumps, to find suitable drug targets, and to understand drug resistance has led to our investigation of MDRs in enterococci. Previously, we used a genomics approach to identify 34 potential enterococcal MDR-encoding genes (2). We have now characterized an enterococcal MDR, EmeA, which was identified because of its sequence homology to \( \text{Staphylococcus aureus} \) NorA, and have demonstrated that EmeA is involved in the resistance of enterococci to various unrelated compounds. Known MDR inhibitors were also effective at inhibiting drug efflux in enterococci. Our analysis demonstrates that EmeA is an MDR that belongs to the major facilitator superfamily (MFS).

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and culture conditions.** The bacterial strains and plasmids used in the study are listed in Table 1. All \( \text{Escherichia coli} \) strains were grown by using standard conditions (15). All \( \text{Enterococcus faecalis} \) strains were grown with aeration at \( 37^\circ \text{C} \) in brain heart infusion (BHI), Todd-Hewitt (TH; Difco Laboratories, Detroit, Mich.), or Mueller-Hinton cation-adjusted (MH II; Becton Dickinson) broth or agar supplemented with appropriate antibiotics. The antibiotics used for \( \text{E. coli} \) included ampicillin at 100 \( \mu \text{g/ml}, \) kanamycin (KAN) at 25 \( \mu \text{g/ml}, \) chloramphenicol at 25 \( \mu \text{g/ml}, \) and erythromycin (ERY) at 300 \( \mu \text{g/ml}. \) The antibiotics used for \( \text{E. faecalis} \) included KAN at 1 to 2 \( \mu \text{g/ml} \) and ERY at 10 \( \mu \text{g/ml}. \)

**Chemicals and antibiotics.** Antibiotics, carbonyl cyanide \( m \)-chlorophenylhydrazone (CCCP), ethidium bromide (EtBr), verapamil, lansoprazole, and reserpine were purchased from Sigma-Aldrich Chemical Co. (St. Louis, Mo.). CCCP was dissolved in dimethyl sulfoxide (100 mM); verapamil was dissolved in distilled \( \text{H}_2\text{O} \) (10 mg/ml); reserpine was dissolved in \( \text{CH}_3\text{OH} \) (10 mg/ml) and then diluted in MH II broth, as needed; lansoprazole (125 mg) was first dissolved in 5 ml of methanol and was then diluted to 5 mg/ml in \( \text{H}_2\text{O} \); and norfloxacin was suspended in \( \text{H}_2\text{O} \) (one-half of the total volume needed), and 100 mM NaOH was added (in 100-\( \mu \text{l} \) aliquots) until the solution became clear and then the solution was diluted to the desired concentration with \( \text{H}_2\text{O} \).

**MIC testing.** MIC testing was performed with MH II medium in microtiter plates and on agar dilution plates by the protocols provided by NCCLS (11). The compounds tested were diluted and tested at several different concentrations in order to determine more precise MICs. MICs were recorded as the lowest concentration of antibiotic that totally inhibited growth after 20 h of incubation at \( 35^\circ \text{C} \). The compounds tested included EtBr, norfloxacin, verapamil, lansoprazole, and reserpine. Each test was performed at least in triplicate. As a control, \( \text{E. faecalis} \) ATCC 29212 was included in tests for MICs. Strains tested on E-test strips (PDM Epsilometer test; AB Biodisk North America, Inc., Piscataway, N.J.) were suspended in 0.8% saline to a 0.5 McFarland standard (~10\(^3\) CFU/ml); the bacteria were then swabbed onto Mueller-Hinton agar plates and allowed to dry for about 10 min. After the plates were dry, the E-test strips were placed in the middle of the plate with the labeled side up. The plates were incubated overnight.
at 37°C. The MICs were determined as the lowest concentration of drug that totally inhibited growth.

EtBr efflux assay. Cultures of bacteria were grown overnight in BHI broth at 37°C. The cells were then pelleted and washed twice with 20 mM HEPES buffer (pH 7.0). The cells were resuspended in HEPES buffer to 30 Klett units (2 × 10^9 CFU/ml). The cells were then loaded with EtBr by shaking of the cells at 37°C and the addition of CCCP (final concentration, 40 μM) to dissipate the membrane potential and EtBr (final concentration, 2.5 μM). The cells were incubated for 1 h. The cells were then washed three times with HEPES buffer containing EtBr (2.5 μM) and were resuspended in the same buffer to 15 Klett units (2 × 10^9 CFU/ml). The cells were then stored on ice until initiation of efflux. Efflux was initiated by the addition of 80 μl of TH broth to 3 ml of the cell suspension. In some cases, efflux pump inhibitors (reserpine at 20 μg/ml, verapamil at 100 μg/mL, lansoprazole at 100 μg/ml) were added before the addition of TH broth. The concentrations of the efflux pump inhibitors were chosen on the basis of previous research (1). Fluorescence was measured with a spectrophotometer (Photon Technology International, Lawrenceville, N.J.).

DNA preparations and transformation. DNA preparation, purification, restriction digestion, agarose gel electrophoresis, and ligation were performed by standard methods or following the manufacturer’s instructions (15). E. coli transformations and cloning and transformations with the TA and TOPO cloning vectors were performed by the protocol provided by Invitrogen (Carlsbad, Calif.). Electroporation of E. faecalis was performed as described previously (15). PCR and sequence analysis. Sequencing reactions were performed by the Taq dye-deoxy terminator method with a 373 DNA sequencing system (PE Applied Biosystems, Foster City, Calif.). The sequences of the primers used in the PCRs are shown in Table 2. PCR reagents and enzymes were purchased from Perkin-Elmer. A disruption mutant of emeA was obtained by standard methods (15). An internal fragment of emeA (653 bp) was amplified with primers 455 and 456. The PCR product was cloned between the BamHI and EcoRI sites of the pTEX4577 vector. This construct (pGW4427) was electroporated into E. faecalis OG1RF competent cells.

Deletion of emeA from chromosome of OG1RF and complementation. The 1.6-kb insert of the flanking regions was released from pGW4475 with EcoRI and was ligated into the EcoRI site of pTEX4577 (resulting in plasmid pGW4476), transformed into E. coli DH5α cells, and plated onto Luria-Bertani agar containing KAN (25 μg/ml), isopropyl-β-D-thiogalactopyranoside, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Plasmid DNA from transformant colonies was verified by digestion with EcoRI, agarose gel electrophoresis, and DNA sequencing. The pGW4476 construct was then electroporated into OG1RF competent cells, and the cells were plated onto TH agar with KAN (2 mg/ml). After overnight incubation, several colonies were obtained and restreaked. Integration of pGW4476 into the chromosome of OG1RF was verified by colony PCR with primers 513 and 516. Transformant pGW4477 (OG1RF:pGW4476) was used to obtain the deletion mutant. First, an overnight culture was diluted 100-fold and was grown in BHI broth at 37°C for 90 min. Aliquots were plated onto TH agar plates, and colonies were then replica plated onto TH agar plates with and without KAN (2 mg/ml). Colonies that were no longer resistant to KAN were sequenced. The pGW4477 construct was then electroporated into OG1RF competent cells, and the cells were plated onto TH agar plates. The correct transformant strain, pGW4477, which contained the construct pGW4475, was verified by restriction enzyme analysis and DNA sequencing.
then tested for possible deletion of emeA by colony PCR with primers 500 and 511. For complementation of OG1RFΔemeA, primers 500 and 511 were used to amplify the emeA open reading frame (ORF) from strains V583, OG1RF, and the OG1RFΔemeA mutant (only flanking regions of emeA were amplified). The PCR products were cloned separately into the pAT18 enterococcal shuttle vector (Table 1). The pAT18 constructs were then transformed into E. coli cells, plasmid DNA was extracted, and the constructs were confirmed by sequencing and restriction enzyme digestion. Next, the pAT18 constructs were electroporated into OG1RFΔemeA competent cells, and transformants were selected on TH agar plates containing ERY (10 μg/ml). After overnight incubation, complemented transformants were restreaked onto selective plates. The presence of the pAT18 constructs was verified by colony PCR with primers M13F and M13R (Table 2).

## RESULTS AND DISCUSSION

### Characterization of emeA

EmeA was identified by searching the *E. faecalis* V583 database with the sequence of the *S. aureus* NorA protein (accession number M80252). Once the ORF was identified, the enterococcal protein sequence was searched against the nonredundant protein database (NCBI website). EmeA shows 32% identity to NorA from *S. aureus* and 39 to 40% identities to Bmr2 and Bmr1 efflux pumps, respectively, from *Bacillus subtilis*. EmeA consists of 380 amino acids and has 12 predicted transmembrane regions and a predicted signal peptide. These characteristics are similar to those of other MFS MDRs (13).

Figure 1 shows the alignments between the sequences of the EmeA, Bmr2, Bmr1, and NorA proteins. The translocase consensus sequence (GXXD<sub>R</sub>Y<sub>K</sub>XGR<sub>R</sub>Y<sub>K</sub>) and the drug extrusion (DE) consensus sequence (GXhhyhGPXXGG [where h represents some hydrophobic residue and X represents any residue]), which are found in most MFS MDRs, are conserved in all four sequences (13). MDRs and specific efflux pumps from the same species share the same DE consensus sequence. Therefore, this sequence apparently codes for a domain that determines the direction of transport of a ligand, which demonstrates the similarity between MDRs and conventional translocases (7). The sequences of MDRs do not point to any particular multidrug consensus sequence.

### Mutagenesis of emeA in *E. faecalis* and MIC testing

In order to determine if the emeA ORF encodes a product involved in resistance to toxic compounds, we initially used insertional mutagenesis to inactivate the gene. Following electroporation of strain OG1RF with pGW4427, 14 KAN-resistant colonies were obtained and their structure was verified by long-range colony PCR with primers 479 and 463. All 14 colonies produced 9.0-kb products, indicating that integration of pGW4427 into the chromosome of OG1RF had occurred. Therefore, the enterococcal emeA had been disrupted. Southern hybridization also confirmed the integration (data not shown).

The MICs for wild-type strain OG1RF and the disruption mutant were determined in triplicate with E-test strips. The mutant strain was approximately twofold more sensitive than the wild type to ciprofloxacin and norfloxacin (data not shown). These results suggested that emeA or a downstream gene is involved in resistance to toxic compounds.

### Deletion of emeA from OG1RF chromosome, complementation, and susceptibility testing of resulting strains

In order to eliminate the effects of truncated products or the possibility of polar effects on other genes, we sought to delete the gene from the chromosome. Colonies obtained through screening of GW4477 for the loss of KAN resistance were tested for deletion of emeA by PCR. Colony PCR yielded a 2.5-kb product for OG1RF and a 1.6-kb product for GW4481, suggesting deletion of emeA from the OG1RF chromosome. Sequencing showed that emeA was deleted from base pairs 7 to 108 bp in strain GW4481.

The OG1RFΔemeA mutant was complemented with the wild-type emeA from OG1RF(pGW4661) or V583(pGW4575) or the PCR product from the OG1RFΔemeA mutant itself (pGW4662, negative control); complementation was verified by colony PCR (data not shown). It was observed that, with primers 500 and 511, the PCR product from V583 was ~3.2 kb

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FIG. 1. Multiple sequence alignment. ClustalW was used to analyze sequences from the NCBI website. The translocase consensus sequence (GXXXD$^H_x$XGR$^H_x$) and the DE consensus sequence (GXhyhyGPXXGG [where hy represents hydrophobic residues and X is any residue]) are labeled and highlighted. Hyphens indicate no residues are present, residues on a black background indicate conserved residues, and residues on a shaded background indicate related residues.
and the products from OG1RF and BO1C6(2) were 2.6 kb. Sequence analysis revealed that strain V583 has an additional 630 bp compared to strain OG1RF directly after the stop codon of emeA; this region is not homologous to any known genes.

In S. aureus, EtBr and norfloxacin are two substrates of the NorA efflux pump. Also, reserpine, verapamil, and lansoprazole are inhibitors of many bacterial MDRs including NorA (1). Therefore, we tested these compounds with the emeA deletion mutant, wild-type strain OG1RF, and the complemented strains (Table 3). Strain OG1RFΔemeA was twofold more sensitive to EtBr and norfloxacin than wild-type strain OG1RF was. Also, the norfloxacin MICs for the strains complemented with emeA from either V583 or OG1RF were increased eightfold compared to those for OG1RFΔemeA. Reserpine (a competitive pump blocker), verapamil (a calcium channel blocker), and lansoprazole (a H+ and K+ ATPase pump inhibitor) decreased the level of resistance of OG1RF to norfloxacin twofold. Resistance to EtBr was affected only slightly by reserpine, but the level of resistance was decreased twofold in the presence of lansoprazole or verapamil. For the complemented strains, there was also a twofold decrease in the norfloxacin MIC in the presence of reserpine and lansoprazole; verapamil decreased the EtBr MIC twofold, and lansoprazole decreased the EtBr MIC fourfold. The MICs of the pump inhibitors were all above the concentrations used in this assay. Benzalkonium chloride and sodium dodecyl sulfate were also tested, but they showed no effect on growth even at very high concentrations.

OG1RFΔemeA has also been shown to be twofold more sensitive to acriflavine, ciprofloxacin, clindamycin, erythromycin, and novobiocin (2 [in reference 2, ΔemeA was referred to as ΔnorA]). These results verify that emeA in E. faecalis encodes an MDR protein involved in resistance to many unrelated toxic compounds and that EmeA is affected by several MDR inhibitors.

EmEa seems to have a modest but consistent effect on the susceptibility of E. faecalis to diverse compounds such as norfloxacin and EtBr. The reason for this modest effect could be that there are other pumps and/or mechanisms that confer resistance to these MDR substrates in the absence of EmeA. Also, it is possible that high-level resistance to many toxic compounds may depend on the combined effects of many resistance mechanisms, in which case, the elimination of one mechanism would not totally abolish resistance.

**EtBr efflux assay.** EtBr is highly fluorescent when it is bound to DNA inside the cell. When the EtBr is effluxed from the cell by way of an efflux pump, the fluorescence will decrease and this can be measured with a fluorescence spectrophotometer. The fluorescence of EtBr was used to measure the efflux of the compound from bacterial cells. The bacterial cells were starved and loaded with EtBr after dissipation of the membrane potential with the protonophore CCCP. Without an energy source the MDRs cannot function; therefore, the EtBr cannot be effluxed and the cells become loaded. After the cells were loaded, they were washed to remove the CCCP and were resuspended in buffer containing EtBr. Efflux of EtBr was initiated by the addition of a carbon source that allows reconstitution of the membrane potential. Figure 2A shows that in the presence of TH broth, wild-type strain OG1RF was able to efflux EtBr. However, without TH broth or the EmeA MDR, EtBr was not pumped out of the bacterial cells. After 35 min there was a 30% difference in fluorescence between wild-type OG1RF (with TH broth) and OG1RFΔemeA (with TH broth). No decrease in fluorescence was seen in the mutant or the wild type suspended in buffer only. These results indicate that EmeA is an energy-dependent MDR.

MDR inhibitors were also tested for their ability to inhibit the efflux of EtBr in wild-type strain OG1RF (Fig. 2B). Efflux was initiated with TH broth, and fluorescence was measured for 30 min. Reserpine was the least inhibitory, while lansoprazole inhibited efflux by about 30% compared to that for the wild type. Verapamil was the most effective inhibitor, with fluorescence actually increasing for wild-type strain OG1RF by almost 30%. This indicated that EtBr is still able to enter the bacterial cells but is not extruded efficiently in the presence of verapamil. These results, along with the MIC data, verify that EmeA is an energy-dependent MDR, is inhibited by several known MDR inhibitors, and is the main pump for EtBr efflux. The differences in levels of inhibition seen between the inhibitors are probably due to different mechanisms of inhibition for each compound.

In summary, we have determined that emeA encodes an MDR efflux pump that is similar to MFS MDRs in terms of

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<tr>
<th>Strain</th>
<th>MIC (µg/ml)</th>
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<tr>
<td></td>
<td>EtBr</td>
</tr>
<tr>
<td>OG1RF</td>
<td>20</td>
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<tr>
<td>OG1RFΔemeA</td>
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<tr>
<td>OG1RFΔemeA complemented with emeA from OG1RF</td>
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<tr>
<td>OG1RFΔemeA complemented with emeA from V583</td>
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<tr>
<td>OG1RFΔemeA complemented with pCR product from OG1RFΔemeA</td>
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<tr>
<td>E. faecalis 29212</td>
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a EtBr (with or without inhibitor) was tested at least in triplicate, with consistent results at 25, 20, 15, 10, 5, and 1 µg/ml.

b Norfloxacin (with or without inhibitor) was tested at least in triplicate, with consistent results at 36, 28, 24, 20, 16, 12, 8, 4, 2, and 1 µg/ml.
sequence similarities, energy dependence, inhibition by several known MDR inhibitors, and resistance to unrelated compounds. EmE contributes to the intrinsic resistance of strain OG1RF to some fluoroquinolones; whether it has a role in the higher levels of acquired resistance to fluoroquinolones often seen in clinical isolates remains to be determined. Also remaining to be studied are other identified enterococcal MDRs, including ABC7 (which is involved in resistance to doxorubicin, daunorubicin, EtBr, and ofloxacin), ABC11 (which is involved in resistance to pentamidine and chlorhexidine), ABC16 (which is involved in resistance to erythromycin, azithromycin, and clarithromycin), and ABC23 (which is involved in resistance to virginiamycin and quinupristin-dalfopristin), which appear to contribute to the intrinsic drug resistance of E. faecalis (2).

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REFERENCES


