Role of a qnr-Like Gene in the Intrinsic Resistance of Enterococcus faecalis to Fluoroquinolones

Stéphanie Arsène and Roland Leclercq*

Service de Microbiologie and EA 2128 Interactions hôte et microorganismes des épithéliums, Hôpital Côte de Nacre, Université de Caen Basse-Normandie, 14033 Caen cedex, France

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Fluoroquinolones are poorly active against enterococci. Recently, plasmid-borne resistance to fluoroquinolones due to the qnr gene was reported in members of the Enterobacteriaceae family. The gene encodes a pentapeptide repeat protein that protects DNA gyrase from inhibition by fluoroquinolones. We have identified in the genome of Enterococcus faecalis V583 a qnr-like gene, named E. faecalis qnr (qnrE. faecalis), encoding a putative pentapeptide repeat protein that shares 25% identity with Qnr. To assess its potential role in the intrinsic resistance of E. faecalis to fluoroquinolones, qnrE. faecalis was inactivated in E. faecalis JH12-2 by insertion of the thermosensitive vector pG1KT. This strain was then complemented with qnrE. faecalis, cloned in the multicopy plasmid pORI23. The effects of its overexpression were also studied. Inactivation of the qnrE. faecalis gene resulted in twofold decreases in the MICs of ofloxacin and ciprofloxacin. When the gene was complemented or overexpressed, MICs of fluoroquinolones increased four- to nine-fold, leading to MICs of ofloxacin and ciprofloxacin equal to 32 μg/ml and 8 μg/ml, respectively. The E. faecalis Qnr (QnrE. faecalis) protein was produced and purified. QnrE. faecalis protein protected Escherichia coli DNA gyrase from inhibition by ofloxacin. The qnrE. faecalis gene was then introduced into E. coli DH10B, Staphylococcus aureus RN4220, and Lactococcus lactis IL-1419 to study its heterologous expression. MICs of the various fluoroquinolones tested increased 4- to 16-fold, showing that QnrE. faecalis conferred resistance to fluoroquinolones in various bacterial backgrounds. Overexpression of qnrE. faecalis in enterococci or mobilization of the gene to other bacterial species may be anticipated as a possible new mechanism for fluoroquinolone resistance.

Fluoroquinolones are synthetic antibacterial agents that show potent activity against gram-negative bacteria, including members of the Enterobacteriaceae family and staphylococci. More recently, fluoroquinolones such as gatifloxacin, levofloxacin, and moxifloxacin that have increased activity against Streptococcus pneumoniae have been developed. As a result of their wide-spectrum activity, fluoroquinolones are used to treat a great variety of infections, including urinary tract infections, osteomyelitis, enteric infections, and respiratory tract infections; however, they are of limited interest in the treatment of enterococcal infections. Enterococci are important nosocomial pathogens that cause serious life-threatening infections, including bacteremia, endocarditis, and meningitis (15). Fluoroquinolones show poor or moderate activity against this bacterial genus, as the MIC of ofloxacin for the reference strain Enterococcus faecalis ATCC 29212 is 2 to 4 μg/ml, in contrast to 0.03 to 0.06 μg/ml for the reference strain Escherichia coli ATCC 25922 (8).

Targets of quinolones are DNA gyrase and topoisomerase IV that are tetrameric A2B2 enzymes encoded by the gyrA and gyrB and the parC and parE genes, respectively. DNA gyrase catalyzes the negative supercoiling of DNA essential for transcription initiation and chromosome condensation. Topoisomerase IV is implicated in DNA decatenation. The fluoroquinolone targets in gram-negative bacteria are different than those in gram-positive bacteria. In gram-negative organisms, the primary target is DNA gyrase, whereas in gram-positive organisms, it seems to be topoisomerase IV, as reported for Staphylococcus aureus (4). However, in S. pneumoniae, the primary target seems to depend on a specific fluoroquinolone (17). In enterococci, the primary target is still unclear. Some studies have shown that a single mutation in the gyrA gene was associated with fluoroquinolone resistance (11, 23). Data from Kanematsu et al. (10) were in favor of topoisomerase IV as the primary target, since a single mutation in the parC gene was sufficient to obtain a low level of E. faecalis resistance to fluoroquinolones. According to a recent study by Oyamada et al. (16), the primary target of fluoroquinolones in Enterococcus faecium would depend on the structure of the compound tested. The weak activity of quinolones against enterococci has not been clearly explained and might hypothetically be due to a low affinity of the gyrase of this bacterial genus for quinolones. The extensive use of fluoroquinolones has lead to a rapid development of bacterial resistance. This resistance is due mainly to chromosomal mutations in genes encoding quinolone targets, DNA gyrase, and topoisomerase IV, located predominantly in the quinolone resistance-determining region. Decreased accumulation into the bacteria by mutations in the regulatory genes of outer-membrane proteins or efflux pumps was also reported. The first plasmid-borne resistance was described in 1998 in a strain of Klebsiella pneumoniae isolated in Alabama (14). This strain harbored a plasmid, pMG252, which conferred resistance when transferred to a recipient strain. This plasmid contained a new gene, qnr, responsible for the resistance (24). Since then, several proteins belonging to the Qnr protein family have been described in enterobacteria: QnrA, QnrB (9), and QnrS (5). These proteins are characterized by tandem pentapeptide repeats organized in the consen-
sus sequence (A/C) (D/N) (L/F) (S/R) (G/R) (2, 19, 24). QnrA is a 218-amino-acid protein that protects DNA gyrase (24, 25) and topoiso merase IV (26) from inhibition by fluoroquinolones by decreasing their ability to bind DNA. Proteins similar to Qnr seem to be present in the genome of various microorganisms, and some are implicated in the resistance to DNA gyrase inhibitors, such as MecB or MfpA. MecB is a component of the system that protects bacteria synthesizing microcin B17 from self-inhibition. This microcin is a peptide that blocks DNA replication and can inhibit DNA gyrase supercoiling by stabilizing the cleavage complex (6, 27). MfpA is a Mycobacterium smegmatis protein that interacts with DNA gyrase in a dimeric form (7). The inactivation and the overexpression of mfpA gene are implicated in variations of ciprofloxacin MICs (27).

We detected the presence of a homologue of the qnr gene in the genome of E. faecalis V583 (18), and we assessed its potential role in the intrinsic resistance of enterococci to fluoroquinolones.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains were routinely grown at 37°C in tryptone soy (TS) broth or agar (Bio-Rad, Marnes-la-Coquette, France), except when noted. TS agar medium was supplemented with 10% horse blood for Lactococcus lactis. Media for the selection of transformants contained kanamycin (20 and 500 μg/ml) and/or erythromycin (5 and 500 μg/ml) and/or chloramphenicol (5 and 20 μg/ml). E. faecalis JH2-2, E. coli DH10B and BL21(DE3) L. lactis IL-1419, and S. aureus RN4220 were used as recipient strains in transformation experiments.

Antimicrobial susceptibility. MICs of fluoroquinolones for the different strains were determined in three independent experiments by the broth microdilution method in Mueller-Hinton broth (Bio-Rad), as recommended by the CA-SFM (http://www.sfm.aso.fr; last release, January 2007). For E. faecalis JH2-2, the dilutions tested were 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 8, 16, 32, 64, and 128 μg/ml for ofloxacin and 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.25, 2.5, 3, 4, 8, 16, 32, and 64 μg/ml for ciprofloxacin. For heterologous expression experiments, MICs of chloramphenicol, levofloxacin, sparfloxacin, and moxifloxacin were determined by a standard twofold dilution method.

PCR conditions. Oligonucleotide primers used in this study are listed in Table 1. The PCR consisted of 30 cycles of denaturation (94°C, 30 s), annealing (50°C, 30 s), and extension (72°C, 30 s to 1 min). E. faecalis JH2-2 DNA was extracted by an Instagen Matrix kit (Bio-Rad) as recommended by the manufacturer.

Identification of a homologue of the qnr gene in E. faecalis JH2-2. A BLAST analysis of the genomic sequence of E. faecalis V583 was performed (http://www.ncbi.nlm.nih.gov/BLAST; accession number AE016830). A homologue of the qnr gene was identified at the locus EF9005 and named E. faecalis qnr (qnrE. faecalis). This gene was amplified with specific primers qnr-1F and qnr-1R (Table 1) from E. faecalis JH2-2 DNA and sequenced (Ceq 8000; Beckmann Coulter, Villepinte, France) for comparison with the E. faecalis V583 qnrE. faecalis gene.

Inactivation of qnrE. faecalis. A fragment internal to the qnrE. faecalis gene was amplified from E. faecalis JH2-2 DNA with specific primers qnr-2F and qnr-2R (Table 1). After purification on Microspin S400 columns (Amersham Biosciences, Orsay, France), this fragment was digested with the BamHI and SalI enzymes (Amersham), cloned into the thermosequential vector pG1KT, and introduced into the electrocompetent E. coli DH10B strain. Plasmid pG1KT was a derivative of the thermosensitive shuttle plasmid pG’host5 (conferring resistance to erythromycin) containing a promoterless and terminatorless kanamycin resistance cassette ApH A-3 (12). The transformants were selected on medium containing 500 μg/ml erythromycin. Proper qnrE. faecalis gene placement in the recombinant plasmid was confirmed by specific PCR. This plasmid was introduced into electrocompetent E. faecalis JH2-2. The transformants were selected on medium containing kanamycin, 500 μg/ml and erythromycin, 5 μg/ml, after incubation at 42°C, a temperature that promotes plasmid integration in the chromosomal qnrE. faecalis gene by homologous recombination. The spontaneous loss of pG1KT was obtained by daily subculture in TS broth at 42°C without any antimicrobial agent. The derivatives susceptible to erythromycin and kanamycin were tested for their susceptibility to tetracycline and ciprofloxacin.

Overexpression of the qnrE. faecalis gene and complementation of the qnr gene-inactivated strain. The entire gene was amplified with the qnr-1F and qnr-1R primers from E. faecalis JH2-2 and digested with SalI enzyme. The promoterless pC194 chloramphenicol-acetyl-transferase (cat) gene was then amplified from the plasmid pBT1 (3) with specific primers CAT-F1 and CAT-R3 and digested with SalI. The two fragments were ligated with the T4 DNA ligase (New England Biolabs, Ipswich, MA), and the fusion was amplified with the qnr-1F and CAT-R3 primers. The resulting 1,444-bp fragment was digested with BamHI and PstI and cloned into the electrocompetent E. coli JH2-2. The transformants were selected on medium containing kanamycin, 500 μg/ml, and chloramphenicol, 20 μg/ml. The recombinant plasmid was then introduced into the electrocompetent E. coli JH2-2 strain to study the effects of qnrE. faecalis overexpression. The transformants were selected on medium containing erythromycin, 5 μg/ml, and chloramphenicol, 20 μg/ml.

Production and purification of QnrE. faecalis protein. The entire qnrE. faecalis gene was amplified with specific primers qnrX-F and qnrX-R2 from E. faecalis JH2-2 DNA. After it was digested with NcoI and XhoI, the DNA segment was introduced into the electrocompetent E. coli BL21(DE3) strain that synthesizes a T7 phage RNA polymerase, and the transformants were selected on medium containing kanamycin, 20 μg/ml. The recombinant plasmid was then introduced into electrocompetent E. coli BL21(DE3) strain to study the effects of qnrE. faecalis overexpression. The transformants were selected on medium containing kanamycin, 500 μg/ml, and chloramphenicol, 20 μg/ml.

TABLE 1. Oligonucleotide primers

<table>
<thead>
<tr>
<th>Target</th>
<th>Target size (bp)</th>
<th>Primer name</th>
<th>Nucleotide sequence (5′-3′)</th>
<th>Restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>qnrE. faecalis</td>
<td>731</td>
<td>qnr-1F</td>
<td>AATTGATGGATCTTTGGAACATC</td>
<td>BamHI</td>
</tr>
<tr>
<td>Internal fragment of qnrE. faecalis</td>
<td>459</td>
<td>qnr-2F</td>
<td>AAATCATTTTGCCAGATTTG</td>
<td>BamHI</td>
</tr>
<tr>
<td>Chloramphenicol acetyltransferase</td>
<td>713</td>
<td>CAT-R3</td>
<td>AAAATCTGACAGTGGCATTATCTC</td>
<td>PstI</td>
</tr>
<tr>
<td>qnrE. faecalis</td>
<td>696</td>
<td>qnrX-F</td>
<td>CCTACTTCCATGAGTTAGAAACCTTTG</td>
<td>NcoI</td>
</tr>
<tr>
<td>qnrE. faecalis</td>
<td>696</td>
<td>qnrX-R2</td>
<td>AAAATCTGAGTTAATCCCACAACC</td>
<td>XhoI</td>
</tr>
</tbody>
</table>

* Restriction sites are underlined.
buffer (50 mM NaH2PO4, 300 mM NaNCl, 20 mM imidazole [pH 8.0]). Four elutions followed, two elutions with 250 mM imidazole and then two elutions with 325 mM imidazole (25). All centrifugations were performed at 700 × g for 2 min at 4°C. Samples were dialyzed immediately in Slide-A-Lyzer 3.5 K (Pierce, Rockford, IL) for 18 h at 4°C in 50 mM Tris-HCl (pH 7.5). After dialysis, 10% glycerol was added, and the samples were stored at −20°C. Protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained with Coomassie blue, and protein final concentration was determined by using a protein assay kit (Bio-Rad).

DNA gyrase assay. The DNA supercoiling assay was realized in the presence of the relaxed plasmid pBR322 (Topogen, Marne la Vallée, France) with purified E. coli DNA gyrase (John Innes Enterprises, Norwich, United Kingdom), as recommended by the manufacturer. One unit of DNA gyrase was incubated with 0.5 μg of relaxed pBR322 in a reaction volume of 30 μl at 37°C for 30 min in incubation buffer (Tris-HCl 35 mM [pH 7.5], 24 mM KCl, 4 mM MgCl2, 2 mM dithiothreitol, 1.8 mM spermidine, 1 mM ATP, 6.5% glycerol, and 0.1 mg/ml bovine serum albumin). The different concentrations of ofloxacin tested (0, 0.25, 0.5, 1, 5, and 10 μg/ml) were incubated with the reaction mixture for 1 h at 25°C and for 30 min at 37°C. The resulting topoisomers of pBR322 were resolved by running a 1% agarose gel stained with ethidium bromide for 1 h at 25°C, before staining under UV light. In experiments using QnrV583, the dialyzed protein extract was first preincubated with DNA gyrase for 1 h at 25°C, before ofloxacin was added to the reaction mixture.

Heterologous expression. To assess the effects of the qnrE. faecalis gene expression in other bacterial species, the recombinant plasmid pORI23::qnr-cat was introduced into electrocompetent strains E. coli DH10B, S. aureus RN4240, and L. lactis IL-1419. The transformants were selected on medium containing erythromycin, 5 μg/ml, and chloramphenicol, 10 μg/ml, for the E. coli DH10B strain and on medium containing erythromycin, 5 μg/ml, and chloramphenicol, 10 μg/ml, for the S. aureus RN4240 and L. lactis IL-1419 strains. The MICs of ofloxacin, ciprofloxacin, sparfloxacin, levofloxacin, and moxifloxacin were determined as expected for the Qnr proteins, including QnrE. faecalis.

Role of the qnrE. faecalis gene. The chromosomal qnrE. faecalis gene was inactivated in the E. faecalis JH2-2 strain by homologous recombination using an internal fragment of this gene cloned into the pG1KT thermosensitive vector. The resistance of the transformants to kanamycin at a nonpermissive temperature was the indicator of the qnrE. faecalis gene inactivation. Gene inactivation resulted in a twofold decrease in MICs of ofloxacin and ciprofloxacin for E. faecalis JH2-2. This weak difference was repeatedly observed in three independent experiments.

RESULTS AND DISCUSSION

Characterization of the QnrE. faecalis protein. The BLAST analysis of the complete genome of E. faecalis V583 revealed a homologue of the qnrA gene at locus EF0905 (positions 871,088 to 871,723). This gene putatively encoded a 211-amino-acid pentapeptide repeat protein that shared 25% identity and 40% similarity with that of QnrA. Homology was also found with other Qnr proteins and with other proteins implicated in resistance to DNA gyrase inhibitors, such as McbG and MfpA (Table 2). No sequence characteristic of the presence of a mobile element was found in the close vicinity of the qnrE. faecalis gene in E. faecalis V583.

The homologue of the qnrA gene, named qnrE. faecalis, was amplified from E. faecalis JH2-2 with specific primers and sequenced. The analysis of the deduced amino acid sequence revealed a putative protein that shared 99% identity with the E. faecalis V583 protein. Only two amino acids differed: the proline at position 40 of the E. faecalis V583 sequence was replaced by a serine in the E. faecalis JH2-2 sequence, and the valine at position 44 was replaced by an isoleucine.

The QnrE. faecalis protein had a calculated size of 24.25 kDa and could be organized in 42 pentapeptide repeats that formed two distinct domains of 9 and 33 pentapeptides each, separated by a single asparagine (Fig. 1). In this hypothetical configuration, 21% of the residues in the first position were alanine or cysteine, 20% in the second position were aspartate or asparagine, and 64% in the third position were leucine or phenylalanine. This observation is consistent with the description of the consensus sequence made by Tran and Jacoby (24): (A/C) (D/N) (L/F) (S/R) (G/R). Taken together, these observations confirmed that the QnrE. faecalis protein belonged to the Qnr family. In addition, the C terminus of QnrE. faecalis presented more similarity than the N terminus with members of the pentapeptide repeat family, which was mentioned by Bateman et al. (2) as a general characteristic of this family.

Among proteins that interact with DNA gyrase, MfpA is the only one that has been crystallized (7). Structure studies showed that this protein formed a right-handed beta-helical structure and displayed size, shape, and electrostatic similarities to DNA. This capacity to mimic DNA explains both the inhibitory effect of the protein on DNA gyrase and the fluoroquinolone resistance. A similar mechanism could be suspected for the Qnr proteins, including QnrE. faecalis.

TABLE 2. Percentage of identity and similarity between QnrE. faecalis and homologous proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>GenBank accession no.</th>
<th>% of protein identity (similarity) relative to QnrE. faecalis</th>
</tr>
</thead>
<tbody>
<tr>
<td>QnrA</td>
<td>AAL60061</td>
<td>43 (59)</td>
</tr>
<tr>
<td>QnrB</td>
<td>ABC86904</td>
<td>44 (63)</td>
</tr>
<tr>
<td>QnrS</td>
<td>BAD88776</td>
<td>21 (35)</td>
</tr>
<tr>
<td>McbG</td>
<td>CAAP0724</td>
<td>25 (42)</td>
</tr>
<tr>
<td>MfpA</td>
<td>2BM7_74</td>
<td>22 (38)</td>
</tr>
</tbody>
</table>

Percentages of similarity are in parentheses.

The QnrE. faecalis gene was inactivated in the E. faecalis JH2-2 strain by homologous recombination using an internal fragment of this gene cloned into the pG1KT thermosensitive vector. The resistance of the transformants to kanamycin at a nonpermissive temperature was the indicator of the qnrE. faecalis gene inactivation. Gene inactivation resulted in a twofold decrease in MICs of ofloxacin and ciprofloxacin for E. faecalis JH2-2. This weak difference was repeatedly observed in three independent experiments.

FIG. 1. Hypothetical structure of the QnrE. faecalis protein. The amino acid sequence of QnrE. faecalis was divided into pentapeptide repeats organized in two domains of 9 and 33 units each and connected by a single asparagine. The conserved amino acid residues according to the consensus sequence (A/C) (D/N) (L/F) (S/R) (G/R) are in bold, and the most characteristic pentapeptide units are underlined.
Qnr\textsubscript{E. faecalis} protein was purified to electrophoretic homogeneity using 325 mM imidazole elutions (data not shown). Protein concentration was measured at 1.9 \textmu M in the third elution fraction and at 0.9 \textmu M in the fourth elution fraction.

The supercoiling activity of DNA gyrase was evaluated by its ability to form supercoiled isomers from relaxed pBR322 DNA. A series of topoisomers was visualized by agarose gel electrophoresis, with the most supercoiled isomer having the greatest mobility (Fig. 2, lanes 1 and 2). The presence of ofloxacin in the reaction mixture inhibited DNA gyrase supercoiling activity: supercoiled isomers disappeared proportionally to ofloxacin concentration (Fig. 2, lanes 3 to 7). Surprisingly, the electrophoretic pattern of supercoiled isomers was different when DNA gyrase was incubated with the Qnr\textsubscript{E. faecalis} protein, suggesting that the enzyme activity might be modified by the presence of the Qnr protein (Fig. 2, lane 8). When a 0.9 \textmu M Qnr\textsubscript{E. faecalis}-His\textsubscript{6} tag was preincubated with DNA gyrase and then 1 \mu M ofloxacin added, the migration profile was similar to that with Qnr alone, showing that ofloxacin no longer inhibited supercoiling (Fig. 2, lane 9). This effect was not observed when ofloxacin was replaced by buffer or in the absence of a pre-incubation step (data not shown). In conclusion, the effect of Qnr\textsubscript{E. faecalis} on quinolone susceptibility could be explained by a mechanism of DNA gyrase protection similar to that proposed for QnrA.

**Heterologous expression of the qnr\textsubscript{E. faecalis} gene.** The effect of qnr\textsubscript{E. faecalis} gene expression on fluoroquinolones MICs was evaluated in various genetic backgrounds, using two gram-positive organisms, \textit{S. aureus} and \textit{L. lactis}, and one gram-negative organism, \textit{E. coli}, by introducing the recombinant plasmid pORI23::qnr-cat into electrocompetent cells. The control strains contained pORI23 alone. The expression of the qnr\textsubscript{E. faecalis} gene in \textit{S. aureus} RN4220 led to fourfold increases in the MICs of ofloxacin, ciprofloxacin, levofloxacin, and moxifloxacin and an eightfold increase for sparfloxacin, compared to that of the control strain \textit{S. aureus} RN4220/pORI23 (Table 4). The effects were similar in \textit{L. lactis} IL-1419: fourfold in-

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{E. faecalis derivative} & \textbf{Characteristic} & \textbf{MIC (\mu M)} & \textbf{Ofloxacin} & \textbf{Ciprofloxacin} \\
\hline
JH2–2 & Control & 3 & 1.5 \\
JH2–2pG1KT & qnr inactivation & 1.5 & 0.75 \\
JH2–2\Delta pG1KT & Spontaneous loss of pG1KT & 3 & 1.75 \\
JH2–2pORI23 & Control & 3.5 & 2 \\
JH2–2pG1KT/qori23::qnr cat & qnr complementation & 32 & 8 \\
JH2–2pORI23::qnr cat & qnr overexpression & 32 & 8 \\
\hline
\end{tabular}
\caption{Activities of ofloxacin and ciprofloxacin for derivatives of \textit{E. faecalis} JH2–2}
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\section*{Protection of DNA gyrase by the Qnr\textsubscript{E. faecalis} protein.}
The qnr\textsubscript{E. faecalis} gene was inserted into the pET28a+ expression vector, adding a C-terminal His\textsubscript{6} tag that allowed purification of the complex with nickel nitriloacetate columns. The 25-kDa Qnr\textsubscript{E. faecalis} protein was purified to electrophoretic homogeneity using 325 mM imidazole elutions (data not shown). Protein concentration was measured at 1.9 \textmu M in the third elution fraction and at 0.9 \textmu M in the fourth elution fraction.

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\caption{Activities of ofloxacin and ciprofloxacin for derivatives of \textit{E. faecalis} JH2–2}
\end{table}
creases occurred for the different fluoroquinolones, except for an eightfold increase for sparfloxacin. In E. coli DH10B, for which the fluoroquinolones MICs are very low (from 0.002 to 0.008 μg/ml), the expression of the qnrE. faecalis gene also led to marked increases in MICs, 8-fold for ofloxacin, levofloxacin, and moxifloxacin and 16-fold for ciprofloxacin and sparfloxacin, compared to that of the control strain E. coli DH10B/pORI23. Therefore, the effect of the QnrE. faecalis protein on quinoline susceptibility was not confined to the original E. faecalis species but extended to other studied gram-positive and gram-negative organisms. Therefore, mobilization of the gene to other bacterial genera might be an efficient way for the acquisition of quinoline resistance, as previously reported for the naturally occurring qnrA gene of Shewanella algae, which was the ancestor of the plasmid-borne qnrA4 gene in enterobacteria (20).

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REFERENCES


