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Role of a *qnr*-Like Gene in the Intrinsic Resistance of *Enterococcus faecalis* to Fluoroquinolones[▽]

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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* ($qnr_{E.\ faecalis}$), 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, $qnr_{E.\ faecalis}$ was inactivated in *E. faecalis* JH2-2 by insertion of the thermosensitive vector pG1KT. This strain was then complemented with $qnr_{E.\ faecalis}$ cloned in the multicopy plasmid pORI23. The effects of its overexpression were also studied. Inactivation of the $qnr_{E.\ faecalis}$ 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 ($Qnr_{E.\ faecalis}$) protein was produced and purified. $Qnr_{E.\ faecalis}$ protein protected *Escherichia coli* DNA gyrase from inhibition by ofloxacin. The $qnr_{E.\ faecalis}$ 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 $Qnr_{E.\ faecalis}$ conferred resistance to fluoroquinolones in various bacterial backgrounds. Overexpression of $qnr_{E.\ faecalis}$ 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 meningitidis (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 A_2B_2 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-

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TABLE 1. Oligonucleotide primers

Target	Target size (bp)	Primer name	Nucleotide sequence (5′–3′)[a]	Restriction site
$qnr_{E.\ faecalis}$	731	qnr-1F	AATTGATGGATCCTTTGAAAATC	BamHI
	731	qnr-1R	TAAGAAAGAGTCGACAGAACCAG	SalI
Internal fragment of $qnr_{E.\ faecalis}$	459	qnr-2F	AAATCATTTGGATCCGCAGATTG	BamHI
	459	qnr-2R	GTCAAGCCTGCAGAGGTGTTTTG	PstI
Chloramphenicol acetyltransferase	713	CAT-F1	AATTCACTGTCGACAAAAATTTAGG	SalI
	713	CAT-R3	AAAACTGCAGAGTCGGCATTATCTC	PstI
$qnr_{E.\ faecalis}$	696	qnrX-F	CCTACTCCATGGATGAAACCTTTG	NcoI
	696	qnrX-R2	AAAATCTCGAGTGTAATCACCAAACC	XhoI

[a] Restriction sites are underlined.

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 topoisomerase 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 McbG or MfpA. McbG 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.asso.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 ofloxacin, ciprofloxacin, 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.nih.gov/BLAST; accession number AE016830). A homologue of the *qnr* gene was identified at the locus EF0905 and named *E. faecalis qnr* ($qnr_{E.\ faecalis}$). 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 $qnr_{E.\ faecalis}$ gene.

Inactivation of $qnr_{E.\ faecalis}$. A fragment internal to the $qnr_{E.\ faecalis}$ 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 thermosensitive 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 AphA-3 (12). The transformants were selected on medium containing 500 μg/ml erythromycin. Proper $qnr_{E.\ faecalis}$ 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 $qnr_{E.\ faecalis}$ 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 susceptibilities to ofloxacin and ciprofloxacin.

Overexpression of the $qnr_{E.\ faecalis}$ 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 by 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 enzymes and cloned into the multicopy plasmid pORI23 under the control of a *L. lactis* strong promoter (21). The chloramphenicol resistance of the transformants was therefore the indicator of the fusion expression. The recombinant plasmid was introduced into the electrocompetent *E. faecalis* JH2-2 strain inactivated for the $qnr_{E.\ faecalis}$ gene in order to complement this derivative. The transformants were selected on medium containing kanamycin, 500 μg/ml, erythromycin, 5 μg/ml, and chloramphenicol, 20 μg/ml. The recombinant plasmid was also introduced into the electrocompetent *E. faecalis* JH2-2 strain to study the effects of $qnr_{E.\ faecalis}$ overexpression. The transformants were selected on medium containing erythromycin, 5 μg/ml, and chloramphenicol, 20 μg/ml.

Production and purification of $Qnr_{E.\ faecalis}$ protein. The entire $qnr_{E.\ faecalis}$ 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 ligated into expression vector pET28a+ (Apha-3) (Novagen, Nottingham, United Kingdom), placing the $qnr_{E.\ faecalis}$ gene under the control of a phage T7 promoter associated with a *lac* gene operator, and adding coding sequence for a C-terminal His_6 tag. The construct was verified by gel electrophoresis of the recombinant plasmid and PCR specific of the insert. This plasmid was introduced into the *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 production and the purification of $Qnr_{E.\ faecalis}$ protein were realized as described previously (1, 24). Briefly, the strain was incubated in 50 ml of Mueller-Hinton broth with kanamycin, 20 μg/ml, at 30°C until the optical density at 600 nm reached 0.6. Then, isopropyl-β-D-thiogalactopyranoside was added at a final concentration of 1 mM, and the culture was incubated 4 h further, until cells were harvested by centrifugation for 20 min at 10,000 × *g* and stored at −20°C before protein isolation. The purification was realized on nickel nitriloacetate columns (Ni-NTA; QIAGEN, Courtaboeuf, France), as recommended by the manufacturer. The next steps were carried out at 4°C. The pellet was resuspended in 1 ml of lysis buffer (50 mM NaH_2PO_4-300 mM NaCl-10 mM imidazole [pH 8.0]), sonicated, and centrifuged for 20 min at 10,000 × *g*. The supernatant (600 μl) was charged on Ni-NTA columns and washed four times with 600 μl of wash

TABLE 2. Percentage of identity and similarity between $Qnr_{E.\ faecalis}$ and homologous proteins

Protein	GenBank accession no.	% of protein identity (similarity) relative to $Qnr_{E.\ faecalis}$[a]				
		QnrB	QnrS	McbG	MfpA	$Qnr_{E.\ faecalis}$
QnrA	AAL60061	43 (59)	59 (74)	21 (40)	24 (36)	25 (40)
QnrB	ABC86904		44 (63)	21 (43)	25 (34)	25 (39)
QnrS	BAD88776			21 (35)	25 (36)	20 (36)
McbG	CAA30724				25 (42)	23 (40)
MfpA	2BM7_C					22 (38)

[a] Percentages of similarity are in parentheses.

buffer (50 mM NaH_2PO_4-300 mM NaCl-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 $MgCl_2$, 2 mM dithiothreitol, 1.8 mM spermidine, 1 mM ATP, 6.5% glycerol, and 0.1 mg/ml 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 16 h at 3.5 V/cm that was visualized under UV light. In experiments using $Qnr_{E.\ faecalis}$, 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 $qnr_{E.\ faecalis}$ gene expression in other bacterial species, the recombinant plasmid pORI23::*qnr-cat* was introduced into electrocompetent strains *E. coli* DH10B, *S. aureus* RN4220, and *L. lactis* IL-1419. The transformants were selected on medium containing erythromycin, 500 μg/ml, and chloramphenicol, 20 μg/ml, for the *E. coli* DH10B strain and on medium containing erythromycin, 5 μg/ml, and chloramphenicol, 5 μg/ml, for the *S. aureus* RN4220 and *L. lactis* IL-1419 strains. The MICs of ofloxacin, ciprofloxacin, sparfloxacin, levofloxacin, and moxifloxacin were determined as described above.

RESULTS AND DISCUSSION

Characterization of the $Qnr_{E.\ faecalis}$ 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 the 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 $qnr_{E.\ faecalis}$ gene in *E. faecalis* V583.

The homologue of the *qnrA* gene, named $qnr_{E.\ faecalis}$, 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 $Qnr_{E.\ faecalis}$ 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) X X, where X could be any amino acid. Moreover, 17% of the residues in the fourth position were serine or arginine, and 14% in the fifth position were glycine or arginine, which corresponds to the consensus sequence proposed later by Poirel et al. (19): (A/C) (D/N) (L/F) (S/R) (G/R). Taken together, these observations confirmed that the $Qnr_{E.\ faecalis}$ protein belonged to the Qnr family. In addition, the C terminus of $Qnr_{E.\ faecalis}$ 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 $Qnr_{E.\ faecalis}$.

Role of the $qnr_{E.\ faecalis}$ gene. The chromosomal $qnr_{E.\ faecalis}$ 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 $qnr_{E.\ faecalis}$ 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 ex-

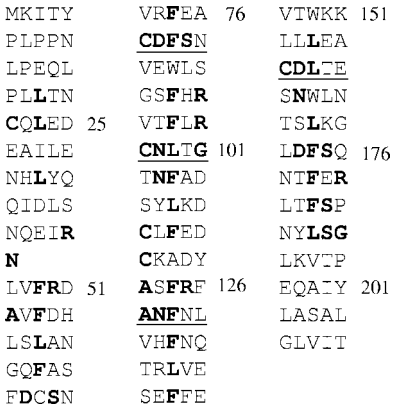

FIG. 1. Hypothetical structure of the $Qnr_{E.\ faecalis}$ protein. The amino acid sequence of $Qnr_{E.\ faecalis}$ was divided into pentapeptide repeats organized in two domains of 9 and 33 units each and connected by a single asparagine. The conserved amino acid residues according to the consensus sequence (A/C) (D/N) (L/F) (S/R) (G/R) are in bold, and the most characteristic pentapeptide units are underlined.

TABLE 3. Activities of ofloxacin and ciprofloxacin for derivatives of *E. faecalis* JH2–2

E. faecalis derivative	Characteristic	MIC (μg/ml)	
		Ofloxacin	Ciprofloxacin
JH2–2	Control	3	1.5
JH2–2ΩpG1KT	*qnr* inactivation	1.5	0.75
JH2–2ΔpG1KT	Spontaneous loss of pG1KT	3	1.75
JH2–2/pORI23	Control	3.5	2
JH2–2ΩpG1KT/pORI23::*qnr-cat*	*qnr* complementation	32	8
JH2–2/pORI23::*qnr-cat*	*qnr* overexpression	32	8

periments and also at 42°C in the presence of kanamycin (500 μg/ml) incorporated into the agar to prevent spontaneous plasmid excision (Table 3). However, given the crossing-over technique that we used, we cannot fully exclude the occurrence of spontaneous excision of the plasmid in a subpopulation with an intact *qnr* gene that could lead to an underestimation of the effect of the *qnr* inactivation. After the loss of pG1KT by spontaneous excision and plasmid curing at a nonpermissive temperature, the MICs returned to their original values.

The complementation experiment was realized by cloning the $qnr_{E.\ faecalis}$ gene under the control of the strong promoter of pORI23 and by introducing the recombinant plasmid into the *E. faecalis* JH2-2 strain inactivated for this gene. A promoterless gene, *cat*, was cloned as a reporter just downstream from the $qnr_{E.\ faecalis}$ gene, allowing expression of chloramphenicol resistance by the recombinant strains. The MICs of ofloxacin and ciprofloxacin for the control *E. faecalis* JH2-2 strain containing the plasmid pORI23 alone were 3.5 μg/ml and 2 μg/ml, respectively. When the recombinant plasmid pORI23::*qnr-cat* was introduced in the JH2-2ΩpG1KT strain, the MICs of ofloxacin and ciprofloxacin increased to 32 and 8 μg/ml, respectively, which corresponds to a 4- to 9-fold increase (Table 3). The introduction of the recombinant plasmid pORI23::*qnr-cat* in *E. faecalis* JH2-2 led to a similar increase in MICs of fluoroquinolones (ofloxacin MIC of 32 μg/ml and ciprofloxacin MIC of 8 μg/ml). The multiplication factor of quinolone MICs was similar to that resulting from the expression of the *qnrA* gene in *E. coli* J53 (14). However, in this case, the MIC increase was not sufficient to confer resistance in *E. coli* (MIC of ciprofloxacin, 0.25 μg/ml), since the wild-type strain displayed low quinolone MICs. Although the role of QnrA alone seemed to be marginal to conferring resistance, it can supplement resistance due to mutated quinolone target enzymes, efflux pump activation, or deficiencies in outer-membrane porin channels (13) and facilitate selection of higher resistance by mutation (22). In the case of *E. faecalis* JH2-2, the overexpression of the $qnr_{E.\ faecalis}$ gene was sufficient to confer resistance to ciprofloxacin and ofloxacin.

Overall, these experiments indicate a role for the $qnr_{E.\ faecalis}$ gene in the intrinsic resistance of *E. faecalis* JH2-2 to fluoroquinolones.

Protection of DNA gyrase by the $Qnr_{E.\ faecalis}$ protein. The $qnr_{E.\ faecalis}$ gene was inserted into the pET28a+ expression vector, adding a C-terminal His_6 tag that allowed purification of the complex with nickel nitriloacetate columns. The 25-kDa $Qnr_{E.\ faecalis}$ protein was purified to electrophoretic homogeneity using 325 mM imidazole elutions (data not shown). Protein concentration was measured at 1.9 μM in the third elution fraction and at 0.9 μM 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 $Qnr_{E.\ faecalis}$ protein, suggesting that the enzyme activity might be modified by the presence of the Qnr protein (Fig. 2, lane 8). When a 0.9 μM $Qnr_{E.\ faecalis}$-His_6 tag was preincubated with DNA gyrase and then 1 μg/ml 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 preincubation step (data not shown). In conclusion, the effect of $Qnr_{E.\ faecalis}$ on quinolone susceptibility could be explained by a mechanism of DNA gyrase protection similar to that proposed for QnrA.

Heterologous expression of the $qnr_{E.\ faecalis}$ gene. The effect of $qnr_{E.\ faecalis}$ gene expression on fluoroquinolones MICs was evaluated in various genetic backgrounds, using two gram-positive organisms, *S. aureus* and *L. lactis*, and one gram-negative organism, *E. coli*, by introducing the recombinant plasmid pORI23::*qnr-cat* into electrocompetent cells. The control strains contained pORI23 alone. The expression of the $qnr_{E.\ faecalis}$ gene in *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 *S. aureus* RN4220/pORI23 (Table 4). The effects were similar in *L. lactis* IL-1419: fourfold in-

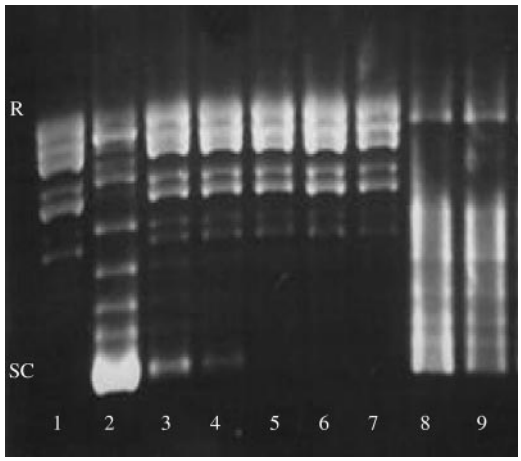

FIG. 2. $Qnr_{E.\ faecalis}$ protein protects *E. coli* DNA gyrase from inhibition by ofloxacin. Reaction mixtures contained 0.5 μg of pBR322 (lanes 1 to 9), 1 U of *E. coli* DNA gyrase (lanes 2 to 9), ofloxacin (lane 3 contained 0.25 μg/ml; lane 4, 0.5 μg/ml; lane 5, 1 μg/ml; lane 6, 5 μg/ml; lane 7, 10 μg/ml; and lane 9, 1 μg/ml), and a $Qnr_{E.\ faecalis}$-His_6 tag (lanes 8 and 9, 0.9 μM). SC, supercoiled form; R, relaxed form.

TABLE 4. Heterologous expression of $qnr_{E.\ faecalis}$

Strain	MIC (μg/ml)[a]				
	OFX	CIP	LVX	SPX	MOX
Staphylococcus aureus RN4220	0.5	0.5	0.25	0.125	0.064
S. aureus RN4220/pORI23	0.5	0.5	0.25	0.125	0.064
S. aureus RN4220/pORI23::*qnr-cat*	2	2	1	1	0.25
Lactococcus lactis IL-1419	1	2	0.5	0.5	0.25
L. lactis IL-1419/pORI23	1	2	0.5	0.5	0.25
L. lactis IL-1419/pORI23::*qnr-cat*	4	8	2	4	1
Escherichia coli DH10B	0.008	0.002	0.008	0.002	0.002
E. coli DH10B/pORI23	0.016	0.002	0.008	0.004	0.002
E. coli DH10B/pORI23::*qnr-cat*	0.125	0.032	0.125	0.064	0.032

[a] OFX, ofloxacin; CIP, ciprofloxacin; LVX, levofloxacin; SPX, sparfloxacin; MOX, moxifloxacin.

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 $qnr_{E.\ faecalis}$ 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 $Qnr_{E.\ faecalis}$ protein on quinolone 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 quinolone resistance, as previously reported for the naturally occurring *qnrA* gene of *Shewanella algae*, which was the ancestor of the plasmid-borne *qnrA* gene in enterobacteria (20).

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REFERENCES

1. **Aubry, A., X. S. Pan, L. M. Fisher, V. Jarlier, and E. Cambau.** 2004. *Mycobacterium tuberculosis* DNA gyrase: interaction with quinolones and correlation with antimycobacterial drug activity. Antimicrob. Agents Chemother. **48:**1281–1288.
2. **Bateman, A., A. G. Murzin, and S. A. Teichmann.** 1998. Structure and distribution of pentapeptide repeats in bacteria. Protein Sci. **7:**1477–1480.
3. **Bruckner, R.** 1997. Gene replacement in *Staphylococcus carnosus* and *Staphylococcus xylosus*. Mol. Microbiol. **151:**1–8.
4. **Ferrero, L., B. Cameron, B. Manse, D. Lagneaux, J. Crouzet, A. Famechon, and F. Blanche.** 1994. Cloning and primary structure of *Staphylococcus aureus* DNA topoisomerase IV: a primary target of fluoroquinolones. Mol. Microbiol. **13:**641–653.
5. **Hata, M., M. Suzuki, M. Matsumoto, M. Takahashi, K. Sato, S. Ibe, and K. Sakae.** 2005. Cloning of a novel gene for quinolone resistance from a transferable plasmid in *Shigella flexneri* 2b. Antimicrob. Agents Chemother. **49:**801–803.
6. **Heddle, J. G., S. J. Blance, D. B. Zamble, F. Hollfelder, D. A. Miller, L. M. Wentzell, C. T. Walsh, and A. Maxwell.** 2001. The antibiotic microcin B17 is a DNA gyrase poison: characterisation of the mode of inhibition. J. Mol. Biol. **307:**1223–1234.
7. **Hegde, S. S., M. W. Vetting, S. L. Roderick, L. A. Mitchenall, A. Maxwell, H. E. Takiff, and J. S. Blanchard.** 2005. A fluoroquinolone resistance protein from *Mycobacterium tuberculosis* that mimics DNA. Science **308:**1480–1483.
8. **Hoogkamp-Korstanje, J. A.** 1997. In-vitro activities of ciprofloxacin, levofloxacin, lomefloxacin, ofloxacin, pefloxacin, sparfloxacin and trovafloxacin against gram-positive and gram-negative pathogens from respiratory tract infections. J. Antimicrob. Chemother. **40:**427–431.
9. **Jacoby, G. A., K. E. Walsh, D. M. Mills, V. J. Walker, H. Oh, A. Robicsek, and D. C. Hooper.** 2006. *qnrB*, another plasmid-mediated gene for quinolone resistance. Antimicrob. Agents Chemother. **50:**1178–1182.
10. **Kanematsu, E., T. Deguchi, M. Yasuda, T. Kawamura, Y. Nishino, and Y. Kawada.** 1998. Alterations in the GyrA subunit of DNA gyrase and the ParC subunit of DNA topoisomerase IV associated with quinolone resistance in *Enterococcus faecalis*. Antimicrob. Agents Chemother. **42:**433–435.
11. **Korten, V., W. M. Huang, and B. E. Murray.** 1994. Analysis by PCR and direct DNA sequencing of *gyrA* mutations associated with fluoroquinolone resistance in *Enterococcus faecalis*. Antimicrob. Agents Chemother. **38:**2091–2094.
12. **Lalioui, L., E. Pellegrini, S. Dramsi, M. Baptista, N. Bourgeois, F. Doucet-Populaire, C. Rusniok, M. Zouine, P. Glaser, F. Kunst, C. Poyart, and P. Trieu-Cuot.** 2005. The SrtA sortase of *Streptococcus agalactiae* is required for cell wall anchoring of proteins containing the LPXTG motif, for adhesion to epithelial cells, and for colonization of the mouse intestine. Infect. Immun. **73:**3342–3350.
13. **Martínez-Martínez, L., A. Pascual, I. Garcia, J. Tran, and G. A. Jacoby.** 2003. Interaction of plasmid and host quinolone resistance. J. Antimicrob. Chemother. **51:**1037–1039.
14. **Martínez-Martínez, L., A. Pascual, and G. A. Jacoby.** 1998. Quinolone resistance from a transferable plasmid. Lancet **351:**797–799.
15. **Murray, B. E.** 1990. The life and times of the *Enterococcus*. Clin. Microbiol. Rev. **3:**46–65.
16. **Oyamada, Y., H. Ito, K. Fujimoto, R. Asada, T. Niga, R. Okamoto, M. Inoue, and J. Yamagishi.** 2006. Combination of known and unknown mechanisms confers high-level resistance to fluoroquinolones in *Enterococcus faecium*. J. Med. Microbiol. **55:**729–736.
17. **Pan, X. S., and L. M. Fisher.** 1999. *Streptococcus pneumoniae* DNA gyrase and topoisomerase IV: overexpression, purification, and differential inhibition by fluoroquinolones. Antimicrob. Agents Chemother. **43:**1129–1136.
18. **Paulsen, I. T., L. Banerjei, G. S. Myers, K. E. Nelson, R. Seshadri, T. D. Read, D. E. Fouts, J. A. Eisen, S. R. Gill, J. F. Heidelberg, H. Tettelin, R. J. Dodson, L. Umayam, L. Brinkac, M. Beanan, S. Daugherty, R. T. DeBoy, S. Durkin, J. Kolonay, R. Madupu, W. Nelson, J. Vamathevan, B. Tran, J. Upton, T. Hansen, J. Shetty, H. Khouri, T. Utterback, D. Radune, K. A. Ketchum, B. A. Dougherty, and C. M. Fraser.** 2003. Role of mobile DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. Science **299:**2071–2074.
19. **Poirel, L., A. Liard, J. M. Rodriguez-Martinez, and P. Nordmann.** 2005. *Vibrionaceae* as a possible source of *qnr*-like quinolone resistance determinants. J. Antimicrob. Chemother. **56:**1118–1121.
20. **Poirel, L., J. M. Rodriguez-Martinez, H. Mammeri, A. Liard, and P. Nordmann.** 2005. Origin of plasmid-mediated quinolone resistance determinant *qnrA*. Antimicrob. Agents Chemother. **49:**3523–3525.
21. **Que, Y. A., J. A. Haefliger, P. Francioli, and P. Moreillon.** 2000. Expression of *Staphylococcus aureus* clumping factor A in *Lactococcus lactis* subsp. *cremoris* using a new shuttle vector. Infect. Immun. **68:**3516–3522.
22. **Rodriguez-Martinez, J. M., C. Velasco, A. Pascual, I. Garcia, and L. Martinez-Martinez.** 2006. Correlation of quinolone resistance levels and differences in basal and quinolone-induced expression from three *qnrA*-containing plasmids. Clin. Microbiol. Infect. **12:**440–445.
23. **Tankovic, J., F. Mahjoubi, P. Courvalin, J. Duval, and R. Leclercq.** 1996. Development of fluoroquinolone resistance in *Enterococcus faecalis* and role of mutations in the DNA gyrase *gyrA* gene. Antimicrob. Agents Chemother. **40:**2558–2561.
24. **Tran, J. H., and G. A. Jacoby.** 2002. Mechanism of plasmid-mediated quinolone resistance. Proc. Natl. Acad. Sci. USA **99:**5638–5642.
25. **Tran, J. H., G. A. Jacoby, and D. C. Hooper.** 2005. Interaction of the plasmid-encoded quinolone resistance protein Qnr with *Escherichia coli* DNA gyrase. Antimicrob. Agents Chemother. **49:**118–125.
26. **Tran, J. H., G. A. Jacoby, and D. C. Hooper.** 2005. Interaction of the plasmid-encoded quinolone resistance protein QnrA with *Escherichia coli* topoisomerase IV. Antimicrob. Agents Chemother. **49:**3050–3052.
27. **Zamble, D. B., D. A. Miller, J. G. Heddle, A. Maxwell, C. T. Walsh, and F. Hollfelder.** 2001. In vitro characterization of DNA gyrase inhibition by microcin B17 analogs with altered bisheterocyclic sites. Proc. Natl. Acad. Sci. USA **98:**7712–7717.