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Alterations in GyrA and ParC Associated with Fluoroquinolone Resistance in *Enterococcus faecium*

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High-level quinolone resistance in *Enterococcus faecium* was associated with mutations in both *gyrA* and *parC* genes in 10 of 11 resistant strains. One low-level resistant strain without such mutations may instead possess an efflux mechanism or alterations in the other subunits of the gyrase or topoisomerase IV genes. These findings are similar to those for other gram-positive bacteria, such as *Enterococcus faecalis*.

Enterococci are important pathogens and a common cause of serious nosocomial infections such as bacteremia and endocarditis (21). *Enterococcus faecalis* is the most common isolate among enterococci. However, *Enterococcus faecium* has become increasingly important after the emergence of ampicillin and vancomycin resistance. Clinical isolates resistant to vancomycin are usually resistant to quinolones and almost all currently available antibiotics (1, 2, 10), but new fluoroquinolones may still have therapeutic value for enterococcal infections (12). At Huddinge Hospital, where this study was conducted, the frequency of quinolone-resistant enterococci has increased from 11% in 1994 to 25% in 1997.

Recent studies showed that fluoroquinolone resistance in gram-negative organisms is associated with mutations in the *gyrA* gene (subunit of DNA gyrase) or with a reduced accumulation of the drug (3, 9, 18, 22, 23, 29, 30). In gram-positive bacteria, fluoroquinolone resistance can be associated with mutations in the *gyrA* or *parC* (subunit of topoisomerase IV) gene (6, 7, 16, 17, 19, 26), and in some gram-positive organisms, such as *Staphylococcus aureus*, efflux pumps have been identified (6, 7, 13, 16, 17, 19, 26, 31).

The nucleotide sequence encoding 41 amino acids in the gyrase A protein, corresponding to *Escherichia coli* amino acids 67 to 106 in the GyrA protein (5, 8, 25, 27, 28), was identified in both gram-negative and gram-positive organisms as the quinolone resistance determining region.

In this study we have analyzed clinical quinolone-resistant *E. faecium* isolates for mutations in the genes encoding subunits of the quinolone target enzymes GyrA and ParC. Twelve clinical isolates of *E. faecium*, collected in different Swedish hospitals, were studied. The isolates were identified by using API 20 STREP (bioMérieux, Marcy l'Etoile, France). The MICs of ciprofloxacin and trovafloxacin were determined by using the E test (Biodisk, Solna, Sweden) on Iso-Sensitest agar (Oxoid) or, for those strains for which MICs of ciprofloxacin were ≥32 μg/ml, the agar dilution method on PDM-ASM agar (Biodisk) with 5% horse blood (4). The antibiotic susceptibilities are shown in Table 1. Of the 12 isolates, 10 were resistant (here defined as any strain for which the MIC of ciprofloxacin is >8 μg/ml), one was susceptible, and for another the MICs of both drugs were slightly higher than for the reference strain. The type strain *E. faecium* ATCC 19434 was used as a reference.

Genomic DNA was extracted from the 11 isolates and the type strain, by using the guanidium thiocyanate method (24). The target regions were amplified by using PCR with primers derived from *E. faecalis* sequences. A DNA fragment of 241 bp from the *gyrA* gene corresponding to the quinolone resistance determining region was amplified with the 23-mer oligonucleotide primers 5′-CGG GAT GAA CGA ATT GGG TGT GA-3′ and 5′-AAT TTT ACT CAT ACG TGC TTC GG-3′, equivalent to nucleotide positions 150 to 172 and 368 to 390 of the *E. coli gyrA* gene (15), respectively, and a 191-bp *parC* fragment was amplified with the 20-mer oligonucleotide primers 5′-AAT GAA TAA AGA TGG CAA TA-3′ and 5′-CGC CAT CCA TAC TTC CGT TG-3′ (positions 10 to 29 and 181 to 200 of the *E. faecalis parC* gene, respectively) (14). One-step PCR was performed for each gene in a 50-μl reaction mixture containing 1.25 U of *Taq* DNA polymerase, 200 μM concentrations of each deoxynucleoside triphosphate, 45 pmol of each primer, 1.5 mM $MgCl_2$, and 10× PCR buffer (Sigma). Each reaction was run for 30 cycles with the following temperature profile: denaturation at 94°C for 1 min, annealing at 55°C for *gyrA* and 48.7°C for *parC* for 1 min, and then extension at 72°C for 1 min. The amplified fragments were processed for cycle sequencing by using the ABI PRISM Big-dye terminator kit,

TABLE 1. Alterations in *gyrA* and *parC* genes in clinical isolates of *E. faceium*

ATCC strain	MIC (μg/ml)[a]		Amino acid (codon) at position[b]		
	CIP	TRV	GyrA 83	GyrA 87	ParC, 80
19434	4	1	Ser (AGT)	Glu (GAG)	Ser (AGC)
3797	1	0.125			
216	8	4			
214	32	8		Lys (AAG)	Ile (ATC)
211	32	8		Lys (AAG)	Ile (ATC)
209	32	16		Lys (AAG)	Ile (ATC)
210	256	8		Lys (AAG)	Ile (ATC)
3784	>256	4		Lys (AAG)	Ile (ATC)
208	64	16	Arg (CGT)		Ile (ATC)
221	256	32	Ile (ATT)		Ile (ATC)
215	256	8	Tyr (TAT)		Ile (ATC)
213	>256	16	Tyr (TAT)		Ile (ATC)
212	>256	32	Tyr (TAT)		Ile (ATC)

[a] CIP, ciprofloxacin; TRV, trovafloxacin.
[b] Positions correspond to *E. coli*. For those strains with no amino acid shown, the codon is identical to that of the type strain.

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A

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                                    *   *
Efem    PDKAHKKSARIVGDVMGKYHPHGDSAIYESMVRMAQPFSYRYMLVDGHGNFGSVDGDGAAA  61
Efecl   ...P.....................................A.................  65
Spn     ...P.......T.............S...A......WW..............M...S...  117
Ecoli   WN..Y.....V....I..........V.DTI........L.......Q.....I...S...  119
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B

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                                    *
Efem    YDKGFRKSAKSVGNIMGNYHPHGDSSIYEAMVRMSQDWKLREVLIEMHG  50
Efecl   F.............................L.............  66
Spn     F..SY............F.........D.......N..N..I.V....  62
Ecoli   ASAK.K...RT..DVL.K.......AC.....L.A.PFSY.YP.VDGQ.  104
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FIG. 1. *E. faecium* (Efem) GyrA (A) and ParC (B) partial gene sequences, compared with those of *E. faecalis* (Efecl), *S. pneumoniae* (Spn), and *E. coli*. The numbered amino acids start from the first amino acid in the complete sequence of *E. coli* and in partial sequences of the others. Only amino acids that differ from those of *E. faecium* are shown. Asterisks mark the mutation points.

and sequences were then analyzed by capillary electrophoresis in an ABI PRISM 310 automated sequencer. Analysis for alteration in target position was carried out as described by Jalal and Wretlind (11).

The nucleotide sequences of 195 bp of *gyrA* between the primers and 151 bp of *parC* between the primers were then determined and analyzed for alterations at the key amino acid positions. The *E. faecium* GyrA partial amino acid sequence showed 97, 88, and 63% identities with *E. faecalis*, *Streptococcus pneumoniae*, and *E. coli*, respectively. The corresponding identities for ParC were 92, 78, and 47% (Fig. 1).

In 10 of the strains GyrA showed single amino acid changes relative to strain ATCC 19434. The serine at position 83 (corresponding to *E. coli*) was changed to tyrosine in three strains, to isoleucine in one strain, and to arginine in another. At position 87, glutamate was changed to lysine in five isolates. In the *parC* gene, a single amino acid change was encoded, the serine at position 80 (corresponding to *E. coli*) to isoleucine in 10 strains (Table 1). One susceptible strain and one strain for which the MIC of ciprofloxacin was 8 μg/ml and that of trovafloxacin was 4 μg/ml had no alterations in *gyrA* or *parC*.

Two nucleotide substitutions were needed for the alteration of serine (AGT) to tyrosine (TAT). One possible explanation is that the parent strains had serine (TCT) in this position. Kanematsu et al. (14) found similar mutations with two nucleotide changes in *E. faecalis* at position 80 of the *parC* gene, where serine (AGT) was changed to arginine (CGC) in one strain and to isoleucine (ATC) in nine strains. Some of the strains in this study showed the same mutations in both *gyrA* and *parC* despite the fourfold difference in MICs of ciprofloxacin or trovafloxacin (Table 1). The most resistant strains may also have efflux systems and/or mutations in *gyrB* or *parE*.

Kanematsu et al. (14) found similar mutations in the *gyrA* and *parC* genes of *E. faecalis*, associated with resistance to fluoroquinolones. They detected one strain with a substitution in *parC* but not in *gyrA*. For this strain the MIC was between the MICs for those with no mutations and those with simultaneous mutations in both *gyrA* and *parC*; consequently they suggested that *parC* might be the primary target for quinolones as in other gram-positive bacteria such as *S. pneumoniae* and *S. aureus* (16, 17). In a previous study, Pan and Fisher (20) showed that the quinolone primary target can differ depending on the structure of the compound. They demonstrated that *parC* is the primary target for ciprofloxacin, whereas *gyrA* is the primary target for sparfloxacin in laboratory selected mutant strains of *S. pneumoniae*. However, in the present study we could not determine the quinolone primary target, since we found no isolates with mutations in only one of the genes. For *E. faecalis* and *S. pneumoniae* strains with substitutions only in either *parC* or *gyrA*, the MICs of at least one of the quinolones studied were eightfold higher or more (14, 20). For the low-level resistant strain in our study the MICs of quinolones were two- to fourfold higher than that for the type strain, and the strain may carry mutations in the other subunits of gyrase and topoisomerase IV or efflux system.

Nucleotide sequence accession numbers. The *gyrA* and *parC* partial sequences of *E. faecium* appear in GenBank under accession no. AF060881 and AB017811, respectively.

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