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Drug Efflux and *parC* Mutations Are Involved in Fluoroquinolone Resistance in Viridans Group Streptococci

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Nine ciprofloxacin-resistant viridans group streptococci isolated from asymptomatic carriers were analyzed. Identification to the species level by using three different commercial systems and a PCR-based approach was inconsistent. The nucleotide sequences of fragments of the *parC*, *parE*, *gyrA*, and *gyrB* genes showed considerable intra- and interspecies variations, and these variations mainly involved silent mutations. Three isolates had changes in Ser-79 of ParC (to Phe or Tyr). Phenotypic characterization indicated that eight of the nine isolates had a putative efflux mechanism that would confer low-level resistance to ciprofloxacin.

Although viridans group streptococci (VGS) form part of the normal flora of the human oral cavity, they cause infective endocarditis (9, 25, 27) and are a major cause of bacteremia in neutropenic cancer patients (4, 6, 8, 10). Increasing levels of resistance to penicillin and macrolide antibiotics have been observed in these bacteria (2, 3, 6, 8). Fluoroquinolone (Fq) resistance (Fq^r) has been reported in VGS isolates from the blood of neutropenic cancer patients who received quinolone prophylaxis (14, 29). In general, there are two major mechanisms of Fq^r including alterations in DNA topoisomerase IV (topo IV) and DNA gyrase (gyrase) and reduced levels of drug accumulation as a result of enhanced efflux. Efflux mechanisms as a cause of low-level Fq^r have been described in *Streptococcus pneumoniae* (5, 7, 31) and other gram-positive bacteria (1, 16, 20–23, 30). In a recent study, the *pmrA* gene has been identified as a gene that codes for a pneumococcal Fq efflux pump (13). Genetic disruption of the transporter genes or inhibition of the transporter activity by the alkaloid reserpine decreased the MICs of Fqs and other toxins for those gram-positive bacteria (1, 13, 18, 21).

Fluoroquinolone susceptibility and classification of VGS isolates. The VGS strains were isolated from asymptomatic patients during 1998 at the Hospital of Móstoles and were identified by standard methods (11, 26). The strains were first screened for decreased ciprofloxacin (Cp) susceptibility by disk diffusion (5 μg/disk), and those that yielded inhibition zone diameters of <12 mm were interpreted as resistant. By these criteria, a sample of 50 Cp-resistant (Cp^r) VGS isolates was selected. These isolates were preliminarily classified at the species level by using three different standard phenotypic systems: API 20-Strep and ID 32-Strep (Biomerieux, Marcy L'Etoile, France) and BBL Crystal (Becton-Dickinson Europe, Meylan, France). The API 20-Strep and ID 32-Strep systems identified 48 and 49 of the 50 strains, respectively, while the BBL Crystal system identified only 34 of the 50 strains. The three methods allowed the coincidental identification of only 3 of the 50 isolates analyzed, while the two systems from the same manufacturer showed concordance for the identification of about half of the isolates (26 strains).

Further characterization of Fq susceptibility among the 50 Cp^r VGS isolates was performed by E-test analysis on Mueller-Hinton agar plates (Difco) supplemented with 5% defibrinated sheep blood. This analysis revealed that nine strains had high-level Cp^r (≥16 μg/ml) (Table 1). Their susceptibilities to Cp, sparfloxacin, and clinafloxacin were determined by the agar dilution method as described previously (14). Among the nine strains, for seven strains the Cp MICs by the agar dilution method were 4 μg/ml (a value fourfold lower than that observed by the E test), and the seven strains were then classified as having low-level Cp^r. However, two strains had high-level Cp^r by both the E-test and the agar dilution methods. Similar differences between the E-test and the agar dilution methods were observed when the susceptibilities to sparfloxacin were considered (Table 1).

Although these nine isolates were coincidentally identified by both the API 20-Strep and the ID 32-Strep systems (Table 1), they were also subjected to an additional identification by the PCR method described by Garnier et al. (12). Three pairs of specific oligonucleotides, each one being specific for one species, were used in PCRs with DNA from each isolate obtained as described previously (14). This method allowed the identification of *Streptococcus mitis* NCTC 12261 and *Streptococcus oralis* ATCC 10557. However, *S. oralis* NCTC 11427 was amplified with the pairs of primers specific for both *S. mitis* and *Streptococcus sanguis*, with the size of the PCR fragment obtained with the *S. mitis* pair being larger than expected (Fig. 1). Among the nine clinical isolates, only four, phenotypically identified as *S. mitis*, were amplified with any pair of oligonucleotides. By this PCR method, strains V2 and V10 were identified as *S. sanguis* and *S. oralis*, respectively. Isolate V3 was amplified with the *S. oralis*-specific primers, but it also showed two bands with the *S. sanguis*-specific pair of primers. Likewise, isolate V1 also showed two PCR products with the *S. mitis*-specific pair of primers; one of the products corresponded to *S. mitis* and the other corresponded to *S. oralis*.

Sequencing of the *parC*, *parE*, *gyrA*, and *gyrB* QRDRs. Because topo IV is a primary target for Cp in VGS, the quinolone resistance-determining regions (QRDRs) of the *parC* and *parE* genes were amplified with pneumococcus-specific oligonucleotides and the sequences of both DNA strands were deter-

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TABLE 1. Susceptibilities of strains to selected Fqs and mutations in the *parC*, *parE*, *gyrA*, and *gyrB* genes[a]

Strain	MIC (μg/ml)					Amino acid change (codon change)[b]			
	Cp		SPA		CLX, agar dilution				
	E test	Agar dilution	E test	Agar dilution		ParC[c]	ParE	GyrA[d]	GyrB
S. pneumoniae R6	0.5	0.5	0.25	0.25	0.125	—	—	—	—
S. oralis NCTC11427	4	2	0.5	0.5	0.125	—	—	—	ND
S. oralis ATCC10557	2	2	0.5	0.5	0.125	—	—	—	ND
S. mitis NCTC12261	1	1	0.25	0.25	0.06	—	—	ND	—
S. mitis V1[e]	16	4	1	0.25	0.125	—	—	ND	ND
S. mitis V2	16	4	1	0.5	0.125	—	—	ND	ND
S. mitis V3	16	4	1	0.25	0.125	—	—	ND	ND
S. mitis V4	16	4	1	0.5	0.125	—	—	ND	ND
S. sanguis V5	16	4	1	0.5	0.125	—	—	ND	^{425}A→G (GCT→GGT)
S. mitis V6	16	4	2	0.5	0.125	—	—	—	^{425}A→G (GCT→GGT)
S. mitis V10	16	4	1	0.5	0.125	^{79}S→F (TCT→TTT)	—	—	ND
S. mitis V8	>32	16	2	1	0.25	^{79}S→F (TCT→TTT)	—	—	—
S. oralis V9	>32	32	2	1	0.5	^{79}S→Y (TCT→TAT)	—	—	—

[a] SPA, sparfloxacin; CLX, clinafloxacin; ND, not determined.
[b] Positions of substitutions are according to the coordinates for *S. pneumoniae* R6. —, no change.
[c] The sequenced strains had an additional ^{91}N→D (AAC→GAC) change compared to the sequence of *S. pneumoniae* R6.
[d] The sequenced strains had an additional ^{114}S→G (AGT→GGT) change compared to the sequence of *S. pneumoniae* R6.
[e] Classification of the VGS clinical isolates to the species level was done with the API 20-Strep and ID 32-Strep systems.

mined as described previously (14). The differences in the 185-nucleotide (nt) *parC* sequences among the VGS (excluding those mutations involved in Cp^r) were 1.1 to 13.0%, and those in the 210-nt *parE* sequences were 1.1 to 15.7%. Likewise, the differences in the VGS nt sequences compared with the sequence of *S. pneumoniae* R6 were 4.9 to 10.8% for *parC* and 4.3 to 14.6% for *parE*. Comparisons of the amino acid sequences of the VGS with that of *S. pneumoniae* R6 showed a single change in residue 91 of ParC: Asn in R6 and Asp in the VGS. Point mutations that affect Ser-79 of *parC* (change to Tyr or Phe) were found in the two high-level-Cp^r strains (strains V8 and V9) and also in the low-level-Cp^r strain (strain V10) (Table 1). This residue position has been found to be involved in Cp^r in pneumococci (19) as well as in VGS (14).

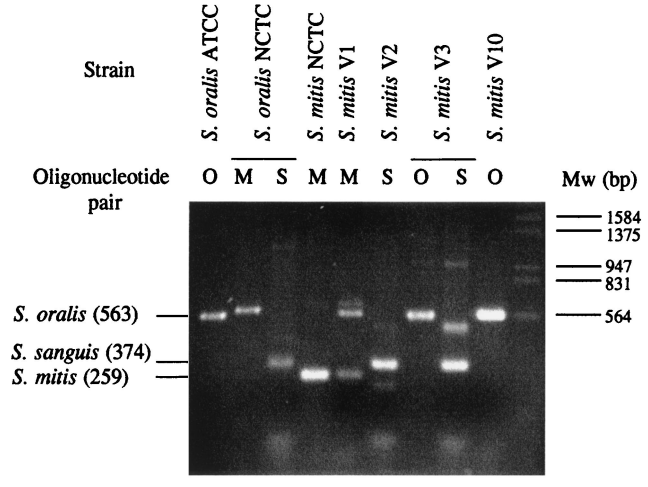

FIG. 1. Identification of Cp^r VGS isolates by the PCR method described by Garnier et al. (12). The DNAs of *S. oralis* ATCC 10557, *S. oralis* NCTC 11427, *S. mitis* NCTC 12261, and clinical isolates (*S. mitis* V1, V2, V3, and V10) were amplified with the oligonucleotide pairs indicated. O, M, and S, the *S. oralis*-, *S. mitis*-, and *S. sanguis*-specific pairs, respectively; Mw, bacteriophage λ DNA digested with *Eco*RI and *Hin*dIII. PCR products were resolved by electrophoresis on a 1.5% agarose-Tris-acetate-EDTA gel that was stained with a 0.5-μg/ml ethidium bromide solution. The sizes (in base pairs) of the PCR products expected for the different species are indicated on the left.

The sequences of the *gyrA* and *gyrB* QRDRs were also determined for the two high-level-Cp^r isolates and a low-level-Cp^r isolate (isolate V6). Additionally, the *gyrB* QRDR sequence of isolate V5 was also determined. The differences in a 280-nt fragment of *gyrA* were 2.1 to 12.6% (among the VGS) and 4.6 to 12.5% (compared with the sequence of *S. pneumoniae* R6). Similar differences were found for a 311-nt fragment of *gyrB*: 1.6 to 11.2% (among the VGS) and 2.2 to 7.1% (compared with the sequence of *S. pneumoniae* R6). A single change at residue 114 of GyrA was found: Ser in R6 and Gly in the VGS. The change observed in GyrB was Ala-425-Gly in isolates V5 and V6. The significance of this change will be discussed below.

The high rate of variation observed between the nt sequences of identical genes from VGS (type strains and clinical isolates) is an indication of the poor classification of the group, as suggested by several investigators (17, 24, 28), and possibly reflects an interchange of genetic material between these bacteria. Thus, sequence comparisons cannot be used to classify VGS to the species level.

Characterization of efflux phenotype of Fq resistance. The susceptibilities of the strains to two hydrophilic fluoroquinolones in the presence or absence of reserpine and known efflux pump substrates were determined by the validated agar dilution method of Brenwald et al. (7). The results are shown in Table 2. For these studies, *S. pneumoniae* R6 was used as a control strain since no differences in the Cp or norfloxacin MICs in the presence or absence of reserpine were found. However, for the VGS type strains, twofold reductions in the Cp MICs and fourfold reductions in the norfloxacin MICs were found in the presence of reserpine. For eight of the nine Cp^r VGS isolates, fourfold or greater reductions in the Cp MICs and eightfold or greater reductions in the norfloxacin MICs were found in the presence of reserpine. In contrast, no change in the Cp MIC and a twofold reduction in the norfloxacin MIC were found for isolate V10 in the presence of reserpine.

From these results, we could assume that no efflux mechanism is involved in the Cp resistance of isolate V10, probably

TABLE 2. Susceptibilities of strains to Fqs and efflux pump substrates

Strain	MIC (μg/ml)[a]											
	Cp	Cp + RS	CP/CIP + RS	NOR	NOR + RS	NOR/NOR + RS	EBR	EBR + RS	EBR/EBR + RS	ACR	ACR + RS	ACR/ACR + RS
S. pneumoniae R6	0.5	0.5	1	4	4	1	2	0.5	4	4	2	2
S. oralis NCTC 11427	2	1	2	16	4	4	16	1	16	8	1	8
S. oralis ATCC 10557	2	1	2	16	4	4	16	1	16	8	2	4
S. mitis NCTC 12261	1	0.5	2	8	2	4	16	1	16	16	2	8
S. mitis V1[b]	4	1	4	16	2	8	32	0.25	128	8	0.5	16
S. mitis V2	4	1	4	32	4	8	32	1	32	8	1	8
S. mitis V3	4	1	4	32	4	8	32	1	32	16	2	8
S. mitis V4	4	1	4	32	4	8	32	1	32	16	0.5	32
S. sanguis V5	4	1	4	32	4	8	32	0.5	64	8	0.5	16
S. mitis V6	4	1	4	32	4	8	32	0.5	64	16	0.5	32
S. mitis V10	4	4	1	32	16	2	8	2	4	4	2	2
S. mitis V8	16	4	4	128	16	8	32	1	32	16	1	16
S. oralis V9	32	4	8	256	16	16	64	1	64	16	1	16

[a] RS, reserpine; NOR, norfloxacin; EBR, ethidium bromide; ACR, acriflavine. MICs are the averages of at least two determinations, which did not differ by more than a twofold dilution.

[b] Classification of the VGS clinical isolates to the species level was done with the API 20-Strep and ID 32-Strep systems.

due to some mutation that abolishes the functionality of the pump, as has been observed in the *Staphylococcus aureus* Smr protein (15). We consider that if an isolate has an efflux mechanism, its (drug MIC)/(drug MIC in the presence of reserpine) ratio should be at least fourfold greater that those for *S. pneumoniae* R6 and isolate V10. Given this assumption, it can be presumed that all the VGS clinical isolates studied (with exception of isolate V10) have an Fq efflux mechanism (Table 2). These included isolates with low-level Cp^r (MICs, 4 μg/ml) in which the only resistance mechanism was the efflux pump, such as isolates V1, V2, V3, V4, V5, and V6. Since isolates V5 and V6 have low-level Cp^r and an efflux mechanism and gyrase is a secondary target for Cp in VGS (14), the mutations in their *gyrB* genes (Table 1) would not be involved in resistance. Isolates with high-level Cp^r (MICs, ≥16 μg/ml), isolates V8 and V9, had both active efflux mechanisms and mutations in *parC*. Isolate V10 had a *parC* mutation and no efflux mechanism and the Cp MIC for the isolate was 4 μg/ml. These results suggest that the Cp efflux mechanism would confer a fourfold increase in Cp^r, while a mutation in *parC* would also confer a fourfold increase. The Cp MIC for strain V9 was 32 μg/ml. This represents a 16-fold increase (compared with the MICs for the type strains), suggesting a synergistic effect of the putative efflux mechanism (which confers a 4-fold increase in the MIC) and the *parC* mutation (which confers a 4-fold increase in the MIC).

When the ethidium bromide and acriflavine susceptibilities are considered, 32- to 128-fold and 8- to 32-fold reductions in the ethidium bromide and acriflavine MICs, respectively, in the presence of reserpine were found for the eighth Cp^r VGS with a putative efflux mechanism. A 16-fold reduction in ethidium bromide MICs and an 8-fold reduction in acriflavine MICs (with the exception of that for *S. oralis* ATCC 10557) were also observed for the VGS type strains in the presence of reserpine. In the presence or absence of reserpine small differences (two- to fourfold) were observed for *S. pneumoniae* R6 and isolate V10 with ethidium bromide and acriflavine. These results suggest the activity of an efflux pump(s) for these drugs in the VGS type strains studied (with the exception of *S. oralis* ATCC 10557 and acriflavine) and clinical isolates (with the exception of strain V10). The differences observed with Fqs, ethidium bromide, and acriflavine in the strains studied suggest that the efflux pump(s) in the strains studied would differ in their substrate specificities.

Nucleotide sequence accession number. The new DNA sequences reported in this paper have been assigned the following GenBank accession numbers: AF144766 to AF144774 (*parC* regions), AF144784 to AF144787 (*gyrA* regions), AF144775 to AF144783 (*parE* regions), and AF144788 to AF144791 (*gyrB* regions).

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