Fluoroquinolone Resistance in Atypical Pneumococci and Oral Streptococci: Evidence of Horizontal Gene Transfer of Fluoroquinolone Resistance Determinants from *Streptococcus pneumoniae*[▽][†]

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Atypical strains, presumed to be pneumococcus, with ciprofloxacin MICs of ≥4.0 μg/ml and unique sequence variations within the quinolone resistance-determining regions (QRDRs) of the gyrase and topoisomerase genes in comparison with the *Streptococcus pneumoniae* R6 strain, were examined. These strains were reidentified using phenotypic methods, including detection of optochin susceptibility, bile solubility, and agglutination by serotype-specific antisera, and genotypic methods, including detection of pneumolysin and autolysin genes by PCR, 16S rRNA sequencing, and multilocus sequence typing (MLST). The analysis based on concatenated sequences of the six MLST loci distinguished the "atypical" strains from pneumococci, and these strains clustered closely with *S. mitis*. However, all these strains and five of nine strains from the viridans streptococcal group possessed one to three *gyrA*, *gyrB*, *parC*, and *parE* genes whose QRDR sequences clustered with those of *S. pneumoniae*, providing evidence of horizontal transfer of the QRDRs of the gyrase and topoisomerase genes from pneumococci into viridans streptococci. These genes also conferred fluoroquinolone resistance to viridans streptococci. In addition, the fluoroquinolone resistance determinants of 32 well-characterized *Streptococcus mitis* and *Streptococcus oralis* strains from bacteremic patients were also compared. These strains have unique amino acid substitutions in GyrA and ParC that were distinguishable from those in fluoroquinolone-resistant pneumococci and the "atypical" isolates. Both recombinational events and de novo mutations play an important role in the development of fluoroquinolone resistance.

It has been recognized that an overestimation of antimicrobial resistance may result from the misidentification of oral streptococci. Misleading macrolide and penicillin resistance rates in *Streptococcus pneumoniae* had resulted from the use of one or more phenotypic/genotypic characteristics in the identification of pneumococcus (28, 38). In a study by Neeleman et al. (28), the identities of 141 macrolide-resistant presumptive pneumococcal isolates from 38 laboratories were reexamined. Only 65% (91/141) of the strains were confirmed as *S. pneumoniae*, while 23% (32/141) were *Streptococcus mitis* and the remaining streptococcal species. This led to significant overreporting of macrolide-resistant *S. pneumoniae*. Increasingly, "atypical" pneumococcal isolates that do not have the defining characteristics of *S. pneumoniae*, such as optochin susceptibility and deoxycholate (bile) solubility, have been identified, while other closely related oral streptococci, such as *S. mitis* and *Streptococcus oralis*, are found to harbor the pneumolysin (*ply*) and/or the autolysin (*lytA*) genes (13, 14, 39), causing confusion in the presumptive identification of *S. pneumoniae* in a diagnostic laboratory. Likewise, those strains which are dis-

crepant in phenotype(s) and/or genotype(s) have also been identified from surveillance studies in Asian countries (22).

The development of fluoroquinolone resistance in *S. pneumoniae* is a global concern, as it is an option for use in the era of drug-resistant *S. pneumoniae* (24, 25). In most countries, fluoroquinolone resistance remained low, in the range of 0% to 1.8% (1, 33, 35) and the strains were genetically diverse. An exception was seen in Hong Kong, in which a high percentage of levofloxacin (LEV) resistance, ranging from 3.8% to 13.3%, was reported, and the strains were related to the Spanish [23F]-1 clone (15, 16). The prevalence of resistance to fluoroquinolone would be misrepresented if *S. pneumoniae* and other oral streptococci were not correctly identified. A high prevalence of fluoroquinolone resistance in the closely related species *S. mitis* and *S. oralis* has been reported, with up to one-third of hospitalized patients having carried fluoroquinolone-resistant viridans streptococci in their oropharynges (12). These viridans streptococci have been demonstrated in vitro as potential donors of the gyrase, topoisomerase, and efflux genes to *S. pneumoniae* in transformation (3, 12, 19). However, so far, there is little evidence to support the notion that interspecies recombination of fluoroquinolone resistance determinants is a common phenomenon in pneumococci (31, 36), except for a few ciprofloxacin (CIP)-resistant strains that had been described previously (9). On the contrary, recent data are supporting the notion that the reverse is more plausible, with transfer of these genes from pneumococci to other viridans streptococci (36).

Previously, we utilized a rapid screening method using PCR-

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restriction fragment (RF)-single-strand conformation polymorphism (SSCP) (17) to screen for variants/mutations at the quinolone resistance-determining regions (QRDRs) of the gyrase and topoisomerase genes in CIP-resistant *S. pneumoniae* (MIC ≥ 4.0 μg/ml). Using PCR-RF-SSCP, we identified a collection of atypical strains, presumed to be pneumococcus, with CIP MICs of ≥4.0 μg/ml and unique patterns suggesting sequence variations within the QRDR in comparison with *S. pneumoniae* R6 and possible recombinational events with other streptococci. We thus characterized these strains by phenotypic methods, including detection of optochin susceptibility, bile solubility, and agglutination by serotype specific antisera, and genotypic methods, such as detection of the pneumolysin and autolysin genes, 16S rRNA sequencing, and multilocus sequence typing (MLST). In addition, we sought to compare the fluoroquinolone susceptibilities and respective resistance determinants of 32 well-characterized clinical isolates of *S. mitis* and *S. oralis* to those of the "atypical" pneumococcal strains.

MATERIALS AND METHODS

Bacterial isolates. Two groups of nonduplicate clinical isolates were studied: 26 strains, presumptively identified as pneumococci, with MICs of ≥4.0 μg/ml, from respiratory specimens of patients between 2000 and 2003 and 32 isolates of *Streptococcus mitis* (12) and *Streptococcus oralis* (20) from blood, cerebrospinal fluid, and body fluid samples of patients isolated between 1994 and 2005. All pneumococcal strains were isolated from patients admitted to the Prince of Wales Hospital, a 1,350-bed teaching hospital in Hong Kong. These strains had been identified based on a standard methodology with hemolysis on blood agar, Gram stain morphology, optochin susceptibility detection, and (where indicated) the ABI32 Strept system (BioMerieux, Marcy d' etoile, France). *S. mitis* and *S. oralis* had also been characterized by MLST (7, 18). Optochin susceptibility detection was performed with a 5-μg optochin disk (Oxoid, Basingstoke, Hampshire, England) on horse blood agar plates. The plates were incubated for 18 to 24 h at 35°C in both 5% CO_2 and ambient air. A zone of inhibition of ≥14 mm was defined as sensitive. Bile solubility detection was performed according to a standard protocol. *S. pneumoniae* was suspended in two tubes containing 1 ml 0.85% NaCl saline solution. A few drops of 10% sodium deoxycholate solution were added to one tube while saline was added to the control tube, and the suspensions were incubated at 35°C for 15 min. A positive test was indicated by visible clearing of the suspension. Reference strains used included *S. pneumoniae* R6 and ATCC 49619, *S. mitis* ATCC 49456, and *S. oralis* ATCC 10557.

Antibiotic susceptibility testing, detection of efflux phenotype, and serotypes. The MICs of CIP and LEV were determined using a broth dilution method according to the Clinical Laboratory Standards Institute (CLSI, formerly NCCLS) (8). *S. pneumoniae* ATCC 6303 and ATCC 49619 were included as controls. Reduced drug accumulation due to efflux phenotype was examined for isolates with CIP MICs of ≥2 μg/ml using agar dilution in the presence of CIP with or without reserpine (10 μg/ml). The isolates which had fourfold or greater decreases in CIP MIC in the presence of reserpine were considered to have a positive efflux phenotype (6).

DNA isolation and amplification of pneumolysin (*ply*) and autolysin (*lytA*) genes by PCR. DNA was extracted using a Wizard genomic DNA purification kit (Promega, Madison, WI). PCR on the autolysin (*lytA*) and pneumolysin (*ply*) genes was performed with a modification of the method of Rudolph et al. (34). DNA amplification was carried out in 10 pmol of each primer, 1× PCR buffer (GE Healthcare), a 200 μM final concentration of each nucleotide (GE Healthcare), 1 unit of *Taq* DNA Polymerase (GE Healthcare), and approximately 10 ng DNA extract in a 25-μl total volume. The reaction condition used was 96°C for 3 min, followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 50 s, and a final extension at 72°C for 5 min. One microliter of the amplified mixture was added for the second-round PCR. The same reaction condition was used, except with 25 cycles. The specificities of the PCRs were examined using DNA extracted from clinical isolates of *Streptococcus milleri*, *S. bovis*, *S. agalactiae*, *S. mitis*, *S. salivarius*, *S. equinus*, *S. anginosus*, *S. constellatus*, a group G *Streptococcus*, and *S. pyogenes* ATCC 19615.

Analysis of fluoroquinolone resistance determinants by PCR-RF-SSCP. The mutations and the amino acid substitutions at the QRDRs in the *gyrA*, *gyrB*, *parC*, and *parE* genes were analyzed by PCR-RF-SSCP as previously described (17). Briefly, for each isolate, PCR amplicons of QRDRs of the *gyrA* and *-B* and *parC* and *-E* genes were digested with AluI, HinFI, Sau3AI, and MspI, respectively, and analyzed by SSCP. SSCP patterns were correlated with mutations characterized from sequence analyses of PCR amplicons, and these were aligned and compared with the corresponding region in the R6 strain, obtained from the NCBI database.

MLST and sequencing. An approximately 500-bp internal fragment of each of the six MLST genes (13) was amplified from chromosomal DNA of the isolates by PCR and then sequenced based on the pneumococcal MLST scheme recommended (http://spneumoniae.mlst.net/). Two self-designed primers were used for *spi* when the recommended primers did not work, and they were spi-up2 (5′-ACG CTT AGA AAG GTA AGT TAT G-3′) and spi-dn2 (5′-GGT TTA TAA AAT GTT TCC GAT AC-3′) (7). A 1,300-bp 16S rRNA gene fragment was amplified by PCR and sequenced. The primer pairs were 16srRNA-8F (5′-AGA GTT TGA TCC TGG (C/T)TC AG-3′) and 16srRNA-R (5′-CGG GAT CCC AGG CCC GGG AAC GTA TTC AC-3′). Sequencing was performed using two additional primers, 16srRNA-247F (5′-GTT GGT GAG GTA ACG GC-3′) and 16srRNA-1088R (5′-CTA GGG CGG TCA TCG GG-3′). All the sequencing was performed with an ABI310 sequencer and an ABI Prism D-rhodamine terminator cycle sequencing kit (Applied Biosystems, Foster City, CA).

MLST and phylogenetic analysis. The sequences of the six of the seven relevant MLST loci (*ddl* sequences were omitted from analysis) were concatenated to obtain contigs 2,758 bp in length. A few isolates failed to yield amplification products in each locus. In this case, shorter contigs with gaps marking those gene fragments not available were constructed. Minimum evolution trees were constructed using MEGA 3.1 (23), with bootstrapping assessed by 1,000 replications.

RESULTS

Characteristics of the "atypical pneumococcal" isolates. The phenotypic and genotypic characteristics of the presumed pneumococcal isolates with CIP MICs of ≥4.0 μg/ml are listed in Table 1. Three groups of isolates were included: those typical of *S. pneumoniae*, the majority belonging to serotypes 19F and 23F; those which were resistant to optochin, bile insoluble, and negative for pneumolysin and autolysin genes, belonging to the viridans streptococcal group; and those in the "atypical" pneumococcal group. The isolates in the last group were all positive for the *ply* gene, and all but one were positive for the *lytA* gene. Two subgroups were identified: six strains resistant to optochin (group "a") and three susceptible to optochin (group "b"). The respective amino substitutions for these isolates at the QRDR regions of the gyrase and topoisomerase genes are also included in Table 1.

MLST and 16S rRNA sequence analysis. Among the typical *S. pneumoniae* isolates, only one gave a known allelic profile (L14, *spi*) from the MLST database (http://spneumoniae.mlst .net/). For the viridans streptococcal and "atypical" isolates, unique sequences were obtained for nearly all loci, except for five known allelic profiles available in the MLST database (http://spneumoniae.mlst.net/). Some of the isolates failed to produce the desired fragment, despite the use of the modified primer set. The analysis based on concatenated sequences of the six housekeeping genes from all the isolates is shown in Fig. 1. Three clusters were identified; isolates in cluster A were all typical *S. pneumoniae*, and isolates in clusters B and C fell into groups related to *S. mitis* and *S. oralis*, respectively. In contrast, there was no clustering with these groups in a phylogenetic tree generated from the 16S rRNA sequences (tree not shown).

Sequence analyses of the QRDRs of gyrase and topoisomerase genes. Among *S. pneumoniae* isolates, the major amino acid substitutions were Ser-81-Phe in GyrA; Ser-79-Phe, Asp-

TABLE 1. Phenotypic and genotypic characteristics of typical and atypical pneumococci and of viridans streptococci, their MICs to fluoroquinolones, and respective amino acid substitutions at the QRDRs of the gyrase and topoisomerase genes

Group and isolate no.	OPT	Bile	lytA	ply	Sero-type	gyrA Ser 81	gyrA Ser 114	gyrA Asp 142	gyrB Ser 494	parC Ser 52	parC Phe 55	parC Ser 58	parC Ser 79	parC Asp 83	parC Asn 91	parC Glu 96	parC Glu 135	parC Lys 137	parE Ile 460	CIP	LEV
Typical S. pneumoniae																					
L5	S	P	P	P	NT														Val	4	2
L6	S	P	P	P	23F	Phe												Asn	Val	8	16
L12	S	P	P	P	19F	Phe						Phe							Val	64	16
L13	S	P	P	P	19F									Tyr					Val	4	2
L14	S	P	P	P	NT													Asn	Val	4	2
L15	S	P	P	P	23F									Asn				Asn	Val	8	2
L22	S	P	P	P	23F													Asn	Val	4	2
L24	S	P	P	P	23F	Phe			**Thr**					Asn				Asn	Val	≥16	8
Viridans streptococci																					
L2	R	N	N	N	Rough		**Gly**		**Thr**	**Gly**					**Asp**				–	4	2
L7	R	N	N	N	Rough		**Gly**		**Thr**	**Gly**					**Asp**				–	4	2
L16	R	N	N	N	Rough		**Gly**		**Thr**	**Gly**					**Asp**				–	4	1
L17	R	N	N	N	Rough		**Gly**		**Thr**	**Gly**					**Asp**				–	4	1
L18	R	N	N	N	Rough		**Gly**	Asn	**Thr**										Val	4	2
L19	R	N	N	N	Rough		**Gly**		**Thr**	**Gly**					**Asp**				Val	4	2
L20	R	N	N	N	Rough				**Thr**	**Gly**					**Asp**				–	8	2
L21	R	N	N	N	Rough				**Thr**									Asn	–	16	2
L26	R	N	N	N	Rough	Phe												Asn	–	16	16
Atypical pneumococci																					
a																					
L3	R	N	P	P	Rough													Asn	–	4	1
L8	R	N	P	P	Rough					**Gly**	His	Gly			**Asp**			Asn	–	8	2
L9	R	N	P	P	Rough		**Gly**		**Thr**									Asn	–	4	2
L10	R	N	P	P	Rough	Phe												Asn	–	4	2
L4	R	P	P	P	Rough	Phe													–	4	2
L1	R	I	P	P	Rough	Phe							Phe			Asp		Asn	–	>32	16
b																					
L25	S	I	N	P	Rough													Asn	–	4	2
L27	S	I	P	P	Rough					**Gly**					**Asp**	Asp			–	4	1
L11	S	I	P	P	Rough					**Gly**					**Asp**	Asp			–	8	2

[a] Within the atypical pneumococcal group, all isolates possess the *lytA* and *ply* genes, but group "a" strains have reduced susceptibilities to optochin, while group "b" strains are optochin susceptible. Isolates may be bile soluble (P), insoluble (N), or incompletely soluble (I). OPT, optochin test; Bile, bile solubility test; *lytA*, autolysin gene; *ply*, pneumolysin gene.

[b] Position of amino acid substitution compared to the corresponding amino acid position of *S. pneumoniae* R6. Bold characters indicate QRDR and amino acid substitutions unique compared to those of R6 and more compatible with those of viridans streptococci. –, not done.

83-Tyr or Asn, and Lys-137-Asn in ParC; Ser-494-Thr in GyrB; and Ile-460-Val in ParE. These included the predominant amino acid substitutions described previously associated with fluoroquinolone resistance in pneumococci from Hong Kong (17).

The viridans streptococcal and "atypical" strains showed a number of unique amino acid substitutions within the QRDRs of the GyrA and -B and ParC and -E proteins compared to the corresponding regions of the R6 strain in particular, with amino acid substitutions at Ser-114-Gly in GyrA, Ser-494-Thr in GyrB, and Ser-52-Gly and Asn-91-Asp in Par C.

The gene sequences of the QRDR of each of the four genes were aligned (see the supplemental material), and minimum evolution trees were constructed by the neighbor-joining method using MEGA 3.1 (23) (Fig. 2a to d). With 354 bp of *gyrA* sequence used for alignment (Fig. 2a), the major group of isolates that descend from a single node included all of the typical *S. pneumoniae* isolates, R6, and the ATCC 49619 strain. In addition, all the "atypical pneumococcal" isolates except

one, L9, clustered within this group, suggesting a common origin in this *gyrA* region, possibly through horizontal transfer. All the LEV-resistant isolates, including those of atypical strains and viridans streptococci, were present within this cluster. Similarly, with 429 bp of *gyrB*, 231 bp of *parC*, and 267 bp of *parE* used for the analyses (Fig. 2b to d), the major group included all the typical *S. pneumoniae* isolates, R6, and ATCC 49619, with the exception of one strain (L24). L24 has a unique *gyrB* amino acid substitution, Ser-494-Thr, which was not present in other *S. pneumoniae* isolates but was seen in the viridans streptococcal group. This suggests a possible transfer of this *gyrB* fragment from viridans streptococci into pneumococcus. With *parC*, six of the nine strains of "atypical pneumococci" also clustered into the major "typical *S. pneumoniae*" group, suggesting the origin of this gene. Interestingly, with *parE*, only one (L8) atypical strain was clustered with the "typical *S. pneumoniae*" group. In all cases, the majority of the viridans streptococci clustered away from the major group, suggesting unique sequences that differ from those of *S. pneumoniae*.
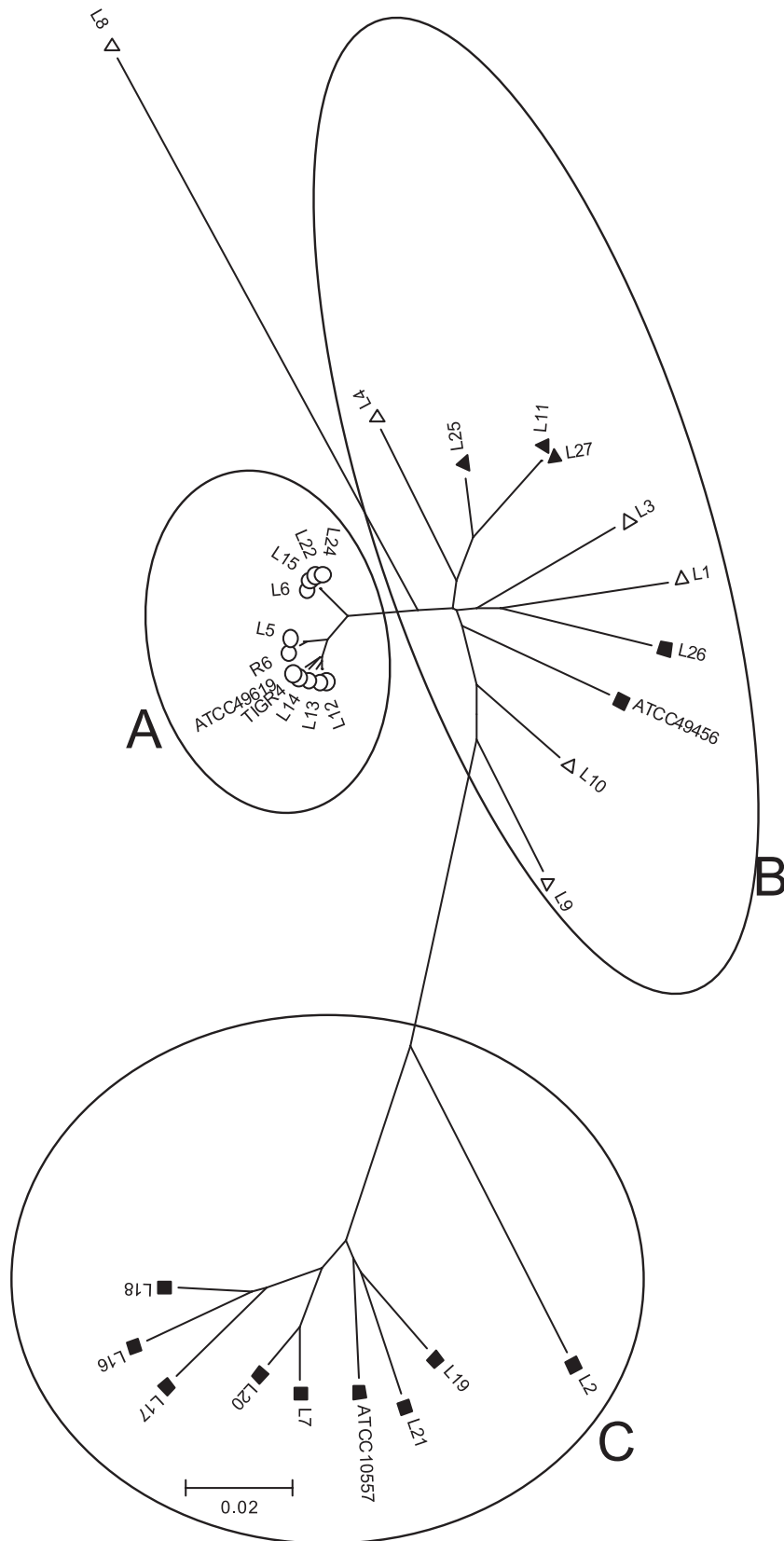
FIG. 1. Minimum evolution tree showing relationship based on concatenated sequences of six housekeeping genes. The tree was constructed by the neighbor-joining method using MEGA 3.1. Isolates included in group A are those of the typical *S. pneumoniae* group and ATCC 49619 (Table 1) and are indicated by ○. ■ represents the viridans streptococcal group, and △ or ▲ represents the "atypical pneumococcal" group. Isolates in group B included *S. mitis* ATCC 49456 and mainly "atypical pneumococcal" strains. Isolates in group C included the viridans streptococcal group and *S. oralis* ATCC 10557.
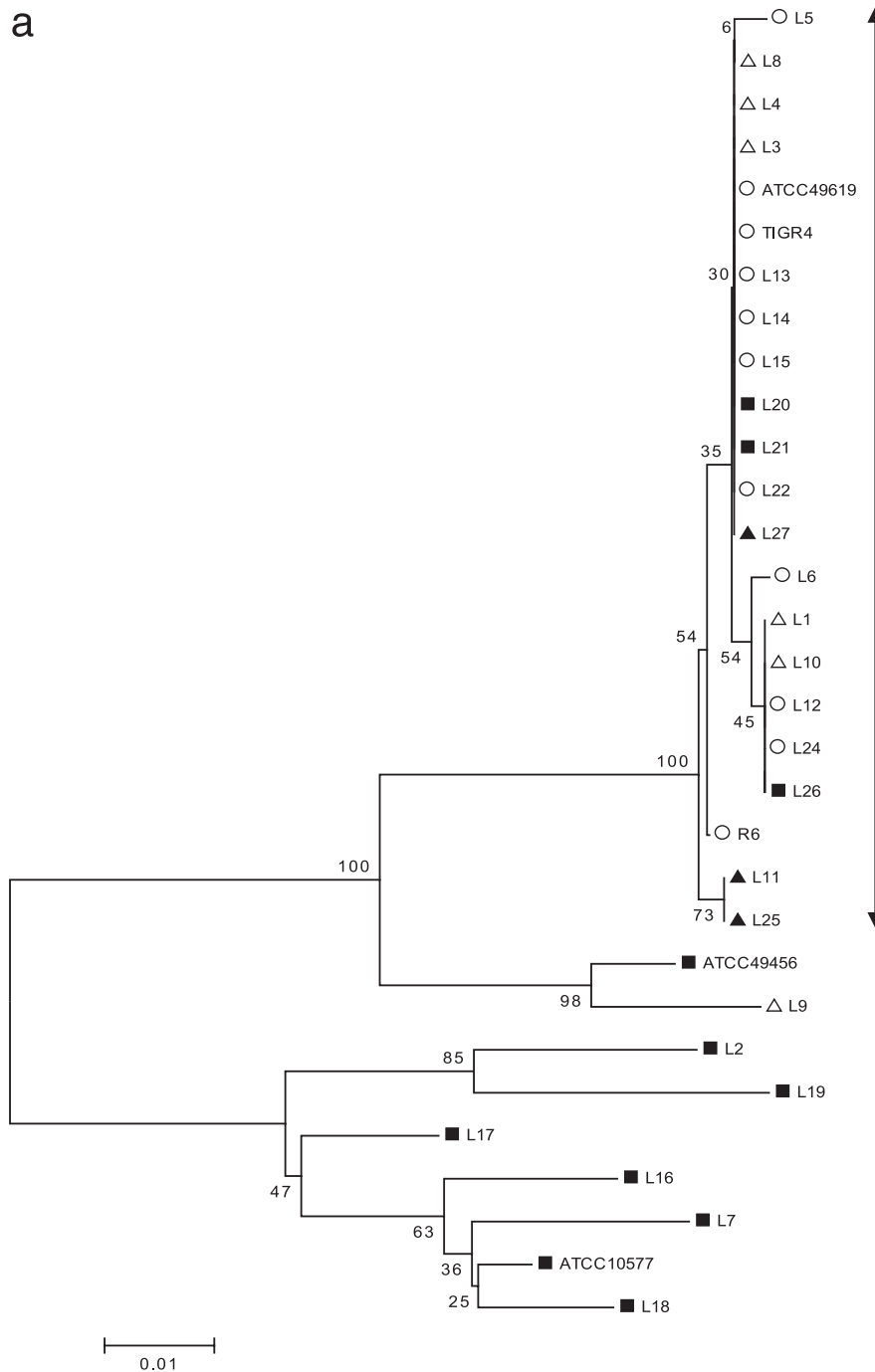
a

FIG. 2. (a) Minimum evolution tree constructed from 354 bp of *gyrA* gene sequence data using the neighbor-joining method. The scale represents the number of nucleotide substitutions per site. ○ indicates the typical *S. pneumoniae* group and ATCC 49619; ■ represents the viridans streptococcal group, *S. mitis* ATCC 49456, and *S. oralis* ATCC 10557; and △ and ▲ represent "atypical pneumococcal" strains "a" and "b," respectively (see Table 1 for corresponding isolates). (b) Minimum evolution tree constructed from 429 bp of *gyrB* gene sequence data using the neighbor-joining method. The scale represents the number of nucleotide substitutions per site. ○ indicates the typical *S. pneumoniae* group and ATCC 49619; ■ represents viridans streptococcal group, *S. mitis* ATCC 49456, and *S. oralis* ATCC 10557; and △ and ▲ represent "atypical pneumococcal" strains "a" and "b," respectively (see Table 1 for corresponding isolates). (c) Minimum evolution tree constructed from 231 bp of *parC* gene sequence data using the neighbor-joining method. The scale represents the number of nucleotide substitutions per site. ○ indicates the typical *S. pneumoniae* group and ATCC 49619; ■ represents the viridans streptococcal group, *S. mitis* ATCC 49456, and *S. oralis* ATCC 10557; and △ and ▲ represent "atypical pneumococcal" strains "a" and "b," respectively (see Table 1 for corresponding isolates). (d) Minimum evolution tree constructed from 267 bp of *parE* gene sequence data using the neighbor-joining method. The scale represents the number of nucleotide substitutions per site. ○ indicates the typical *S. pneumoniae* group and ATCC 49619; ■ represents the viridans streptococcal group, *S. mitis* ATCC 49456, and *S. oralis* ATCC 10557. △ and ▲ represent "atypical pneumococcal" strains "a" and "b," respectively (see Table 1 for corresponding isolates).
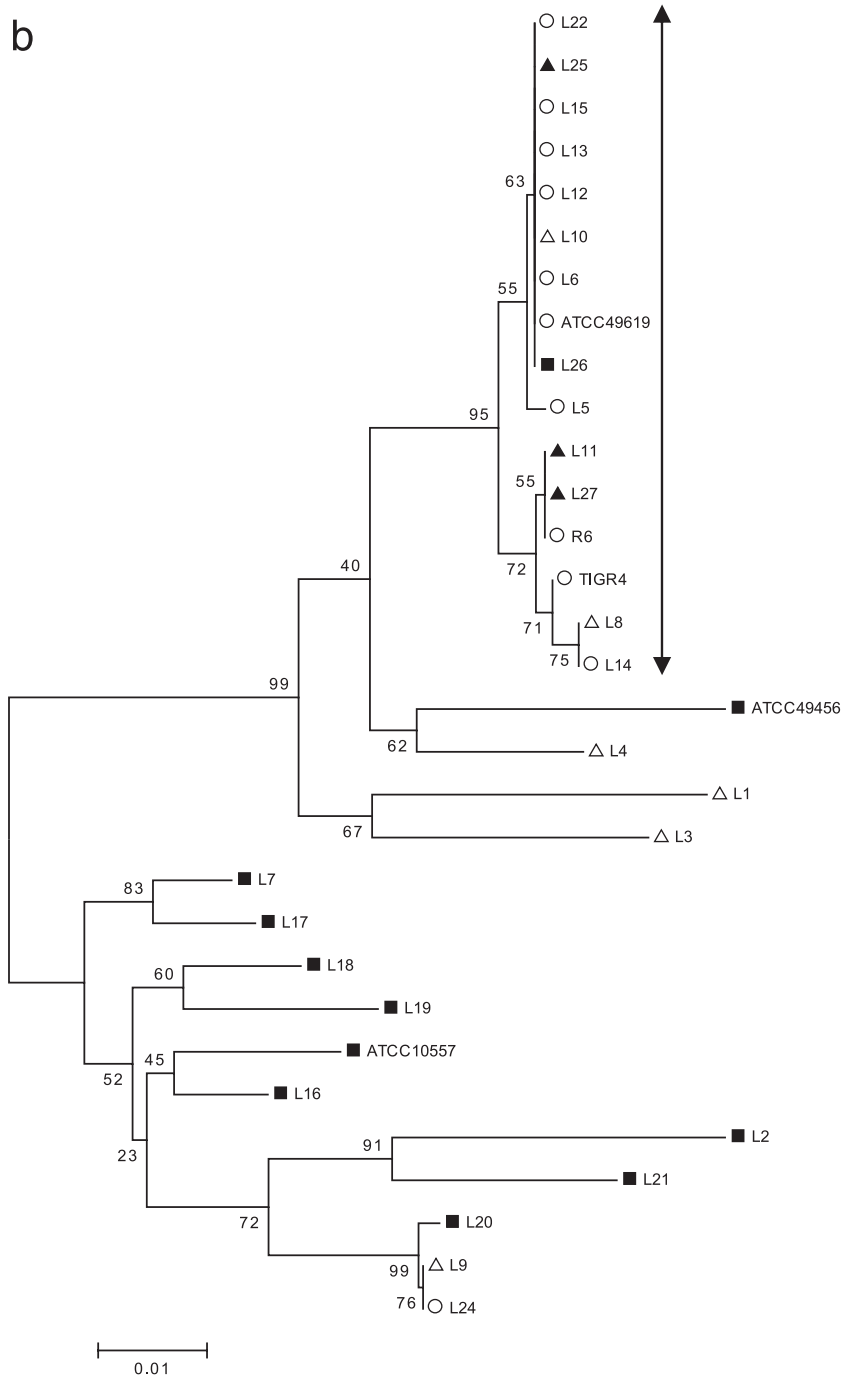
FIG. 2—*Continued.*

Fluoroquinolone susceptibility in *S. oralis* and *S. mitis*. The MICs of CIP and LEV were determined for 32 isolates of *S. oralis* and *S. mitis* and are listed in Table 2. Fifty-six percent (18/32) of the strains have CIP MICs of ≥4.0 μg/ml, while 9.4% (3/32) have LEV MICs of ≥4.0 μg/ml. The efflux phenotype was detected (using inhibition by reserpine) in all but one strain (HK467, *S. mitis*) with CIP MICs of ≥2.0 μg/ml and negative in those strains with CIP MICs of ≥32 μg/ml.

Mutations at the QRDRs of gyrase and topoisomerase genes in *S. oralis* and *S. mitis*. The QRDR sequences of the *parC* and *gyrA* genes were determined for all of the clinical strains, while the QRDR sequences of the *parE* and *gyrB* genes were determined for only four strains, for which the CIP MICs were ≥4 μg/ml. The amino acid sequences of QRDRs were analyzed by comparison to susceptible strain R6, and the amino acid substitutions are summarized in Table 2.
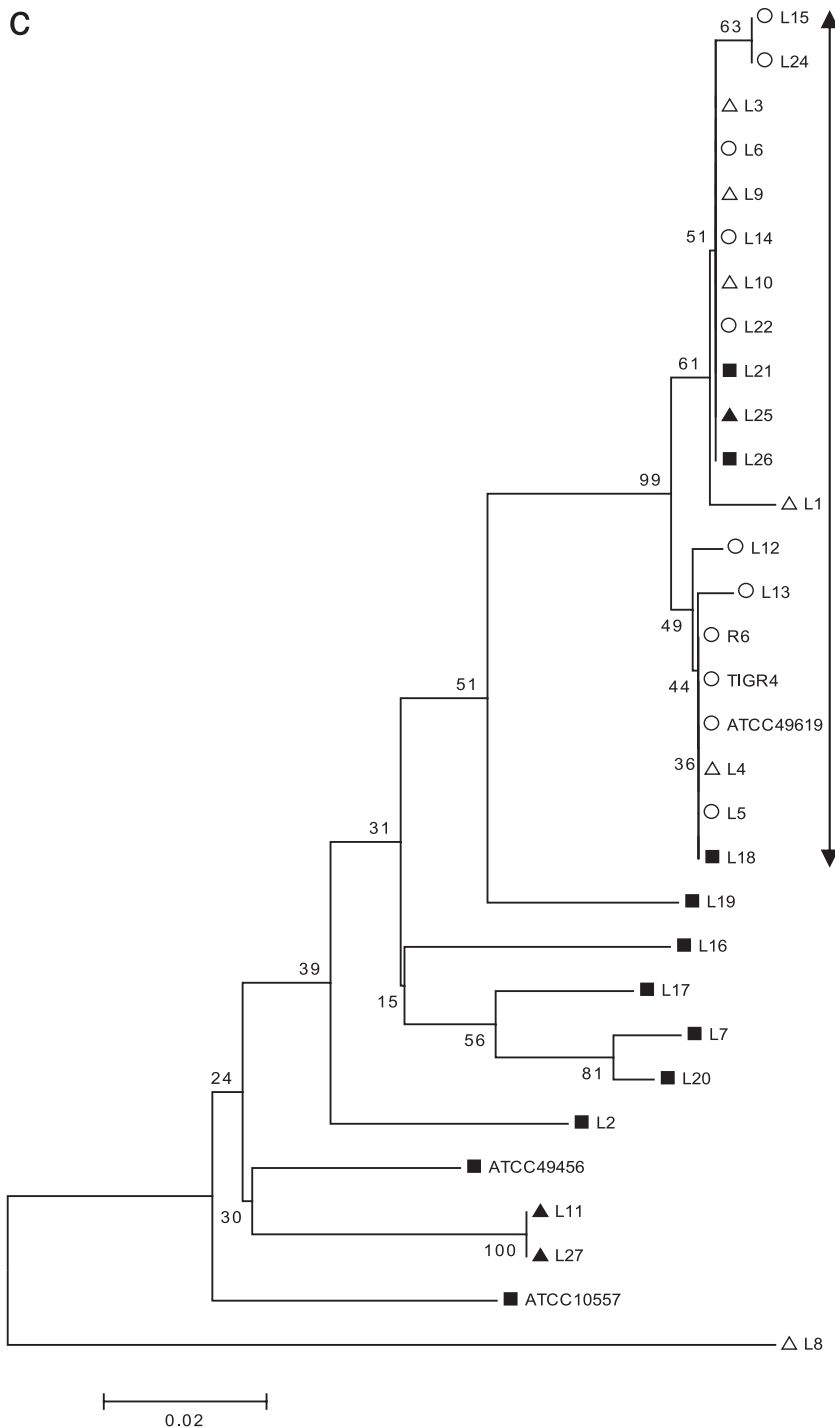
C

FIG. 2—*Continued.*

The *parC* and *gyrA* nucleotide sequences showed high diversity among these strains, with 25.5% and 27.4% variations, respectively (data not shown). Comparisons of amino acid sequences with those of R6 revealed two common substitutions in ParC (Ser-52-Gly and Asn-91-Asp) and one in GyrA (Ser-114-Gly) in all *S. oralis* and *S. mitis* isolates. These do not contribute to fluoroquinolone resistance. In the three CIP-resistant strains (MICs $\geq$ 32 μg/ml; HK816, HK754, and HK677), amino acid substitutions at Ser-79-Tyr and Asp-83-Tyr in ParC and Ser-81-Leu in GyrA were noted. Other substitutions observed (Glu-135-Asp, Pro-140-Ser, and Ala-142-Ser in ParC and Leu-155-Val in GyrA) were present only in strains with CIP MICs of $\leq$2 μg/ml. For the limited sequence analyses for *parE* and *gyrB*, the following amino acid substitutions were noted: Arg-447-Ser in ParE and Ile-385-Val, Met-483-Thr, and Ser-494-Thr in GyrB.
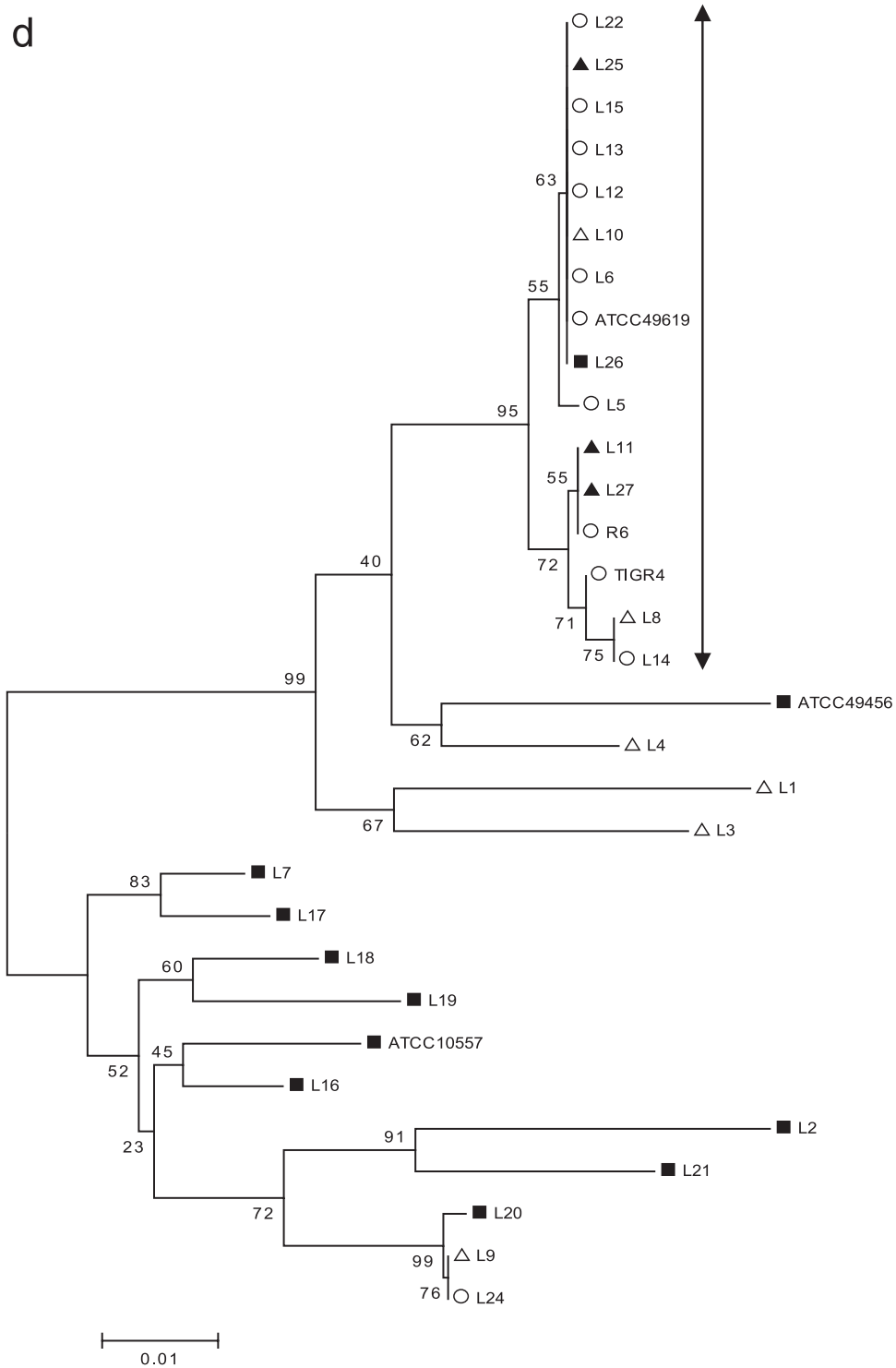
d

FIG. 2—*Continued.*

DISCUSSION

The emergence of nontypeable *S. pneumoniae* and other atypical pneumococci (10, 21, 30) brings a challenge in the identification of pneumococci. As the prevalent capsular types of *S. pneumoniae* are being eradicated by the use of the seven-valent conjugate vaccine, noncapsulate types become more commonly encountered in a diagnostic laboratory. Both optochin-resistant pneumococci (2, 37) and optochin-susceptible viridans streptococci (5, 26) have been described previously. Based on the analysis of the concatenated sequences of the six MLST loci (13, 14), the "atypical pneumococci" described in this study clustered closely with *S. mitis*. As with

TABLE 2. Fluoroquinolone susceptibilities of *S. oralis* and *S. mitis* and their respective amino acid substitutions at the QRDRs of the gyrase and topoisomerase genes[a]

| Isolate no. | MIC (µg/ml) for: | | Result for efflux phenotype detection | Amino acid substitution at indicated QRDR[b] | | | | | | | | | | | | | |
| | CIP | LEV | | *gyrA* | | | *gyrB* | | | *parC* | | | | | | | *parE* |
				Ser 81	Ser 114	Leu 155	Ile 385	Met 483	Ser 494	Ser 52	Ser 79	Asp 83	Asn 91	Glu 135	Pro 140	Ala 142	Arg 447
S. pneumoniae R6	0.5	0.5	–														–
S. oralis																	
ATCC 10557	2	1	N		Gly				–				Asp				–
HK575	1	1	–		Gly	Val			–	Gly			Asp		Ser		–
HK323	2	1	P		Gly				–	Gly			Asp				–
HK417	2	1	P		Gly				–	Gly			Asp				–
HK496	2	1	P		Gly				–	Gly			Asp				–
HK553	2	1	P		Gly				–	Gly			Asp				–
HK62	4	1	P		Gly				–	Gly			Asp				–
HK122	4	1	P		Gly				–	Gly			Asp				–
HK123	4	1	P		Gly				–	Gly			Asp				–
HK271	4	1	P		Gly				–	Gly			Asp				–
HK300	4	1	P		Gly				–	Gly			Asp				–
HK312	4	1	P		Gly				–	Gly			Asp				–
HK51	4	2	P		Gly				–	Gly			Asp				–
HK299	4	2	P		Gly				–	Gly			Asp				–
HK579	4	2	P		Gly				–	Gly			Asp				–
HK587	4	2	P		Gly				–	Gly			Asp				–
HK386	4	2	P		Gly				–	Gly			Asp				–
HK619	4	2	P		Gly				–	Gly			Asp				–
HK574	8	2	P		Gly		Val	Thr	Thr	Gly			Asp				
HK816	32	32	N	Leu	Gly				–	Gly	Tyr		Asp				
HK754	128	≥32	N	Leu	Gly				Thr	Gly		Tyr	Asp				Ser
S. mitis																	
ATCC 49456	1	1	–		Gly					Gly			Asp	Asp		Ser	
HK260	<0.5	<0.25	–		Gly				–	Gly			Asp				–
HK141	1	0.5	–		Gly				–	Gly			Asp				–
HK188	1	0.5	–		Gly				–	Gly			Asp	Asp			–
HK651	1	0.5	–		Gly				–	Gly			Asp				–
HK137453	2	1	P		Gly				–	Gly			Asp				–
HK680	2	1	P		Gly				–	Gly			Asp				–
HK125807	2	1	P		Gly				–	Gly			Asp				–
HK467	2	1	N		Gly	Val			–	Gly			Asp				–
HK666	2	2	P		Gly				–	Gly			Asp	Asp			–
HK135786	4	1	P		Gly				–	Gly			Asp				–
HK29	4	2	P		Gly				–	Gly			Asp				–
HK677	128	≥32	N	Leu	Gly				Thr	Gly	Tyr		Asp				–

[a] P, positive; N, negative; –, not done.
[b] Position of amino acid substitution compared to the corresponding amino acid position of *S. pneumoniae* R6.

previous reports, DNA-based methods for the detection of the *ply* and *lytA* genes did not reliably confirm the identity of *S. pneumoniae* (13, 20, 32, 39). The 16s rRNA genes of *S. mitis*, *S. oralis*, and *S. pneumoniae* were homologous and do not allow distinction between these species (20, 29) based on sequence analyses. Any possibility for the "atypical" pneumococci to be *Streptococcus pseudopneumoniae* (2) was also excluded using optochin susceptibility testing in an ambient atmosphere.

However, when the sequences of the QRDRs of the *gyrA*, *gyrB*, *parC*, and *parE* genes of these "atypical pneumococci" were analyzed, they were found to cluster with sequences of the "typical *S. pneumoniae*" group, especially for the *gyrA* and *parC* genes. All "atypical" strains and five of nine strains from the viridans streptococcal group possessed one to three *gyrA*, *gyrB*, *parC*, and *parE* genes that were clustered within the *S. pneumoniae* group. The fact that the atypical strains clustered closely with *S. mitis* by MLST suggests the occurrence of horizontal transfer from recombination and supports the postulation that horizontal transfer of these genes from pneumococ-

cus into viridans streptococci is probably a more frequent event than vice versa (36).

Substitutions at position 81 of GyrA and positions 79 and 83 of ParC were all important in the development of LEV resistance in *S. pneumoniae* and viridans streptococci. Interestingly, among the *S. mitis* and *S. oralis* isolates isolated in blood/body fluids, position 81 of GyrA was replaced by Leu and position 79 of ParC by Tyr instead of Phe in the pneumococcal or "atypical" isolates. The other amino acid substitutions (Ser-114-Gly at GyrA; Ser-52-Gly, Asn-91-Asp, and Glu-135-Asp at ParC; and Ser-494-Thr at GyrB) have been previously shown not likely to confer CIP resistance (3, 4, 11, 19, 27).

In Hong Kong, the high fluoroquinolone resistance in *S. pneumoniae* was reported to be due to the dissemination of a variant of the Spain [23F]-1 clone (13). In this study, we identified evidence that suggests horizontal transfers of the QRDR of the gyrase and topoisomerase genes from pneumococci into viridans streptococci. These included amino acid substitutions that conferred LEV resistance in viridans streptococcus L26 and the atypical strain L1. The LEV-resistant isolates of *S. oralis*

and *S. mitis* have a unique amino acid substitution at position 81 by Leu in GyrA, differing from those of fluoroquinolone-resistant pneumococcal and "atypical" isolates. This study reflects the pool of fluoroquinolone resistance determinants that are presently seen among clinical isolates of viridans streptococci.

Previously, it was demonstrated in vitro that recombinational events occurred more frequently from *S. mitis* or *S. oralis* to *S. pneumoniae* (19). Increasingly, members of the pneumococcal clone Spain [23F]-1 have been shown to act as frequent donors of fluoroquinolone-nonsusceptible loci (36). Both recombinational events and selective pressure from the use of fluoroquinolones with de novo mutations play an important role in the development of fluoroquinolone resistance.

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