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First *Streptococcus agalactiae* Isolates Highly Resistant to Quinolones, with Point Mutations in *gyrA* and *parC*

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Three isolates of *Streptococcus agalactiae* highly resistant to multiple fluoroquinolones were isolated in Japan. Compared with susceptible strains of *S. agalactiae*, these quinolone-resistant strains had double point mutations within the quinolone resistance-determining regions of *gyrA* and *parC*; Ser-81 was changed to Leu (TCA → TTA) in the amino acid sequence deduced from *gyrA*, and Ser-79 was changed to Phe (TCC → TTC) in the amino acid sequence deduced from *parC*. Comparative sequence analysis revealed the possibility of gene transfer between *S. agalactiae* and another beta-hemolytic streptococcus, *Streptococcus difficile*.

Streptococcus agalactiae, a beta-hemolytic group B streptococcus, is an important pathogen causing serious neonatal infections characterized by sepsis and meningitis and also maternal infections. *S. agalactiae* is also associated with bacteremia, endocarditis, skin and tissue infections, and osteomyelitis in nonpregnant persons (13).

Quinolones were introduced into clinical use in the mid-1980s for the treatment of infections. This class of antibiotics had been very effective for infections caused by streptococci. However, from the mid-1990s, there were many reports describing *Streptococcus pneumoniae* isolates that were resistant to quinolones (1, 3–6, 10, 15, 17). A highly quinolone-resistant strain of *S. pyogenes* was also reported (16). Fortunately, almost all *S. agalactiae* isolates remain susceptible to a number of antibiotics (9, 11). *S. agalactiae* isolates highly resistant to quinolones have not been reported, though one report stated that for some strains of *S. agalactiae*, ofloxacin and grepafloxacin MICs were slightly increased (8).

From February to December 2002, we isolated quinolone-resistant *S. agalactiae* strains in Tottori prefecture in Japan. The majority of quinolone resistance has been thought to be due to point mutations in *gyrA* and *parC*, in the internal regions called the quinolone resistance-determining region (QRDR). Therefore, we determined the nucleotide sequences and deduced amino acid sequences of *gyrA* and *parC* genes including QRDRs in resistant isolates. These sequences were compared with those of susceptible strains of *S. agalactiae* and other beta-hemolytic streptococci, including *Streptococcus dysgalactiae* subsp. *equisimilis*, which causes streptococcal toxic shock syndrome (14).

Case history. In this study, we used three isolates from three epidemiologically unrelated patients. The first patient was a newborn male of low birth weight who became infected 20 h after birth. *S. agalactiae* was the predominant organism in nasal discharge, throat swab, and eye discharge. This strain was given reference number 02Z95 (=GTC1966).

The second patient was a 77-year-old male hospitalized due to an acute aortic dissection. In the hospital, he had been receiving intravenous hyperalimentation. After 1 month in the hospital, he was prescribed antibiotics (vancomycin and arbekacin) to cure a methicillin-resistant *Staphylococcus aureus* infection around the intravenous catheter. *S. agalactiae* was obtained from a throat swab after 2 months in the hospital. The number of this isolate was 02Z106 (=GTC1967).

The third patient was a 75-year-old female, admitted to the hospital for insertion of tension-free vaginal tape. On admission, *S. agalactiae* was detected in her urine. The strain was designated 02Z119 (=GTC2001).

We surveyed all medical records for these three patients to determine whether they had received any quinolones; however, we could not find any information.

The susceptibility testing of these isolates were carried out at the clinical laboratory section of the hospital. The MicroScan Walk Away system (Dade Behring Co., Tokyo, Japan) was used to determine the MICs of 33 antimicrobial agents, including 3 penicillins, 12 cephems, 3 aminoglycosides, 3 macrolides, 3 tetracyclines, 1 quinolone (levofloxacin [LVX]), and 1 glycopeptide (vancomycin). The three *S. agalactiae* isolates were susceptible to all antimicrobial agents used in this study except LVX (MIC > 8 μg/ml). To confirm the resistance to the quinolones, an E-test (AB Biodisk Sweden) was carried out for six classes of fluoroquinolones: LVX, ciprofloxacin, norfloxacin, ofloxacin, fleroxacin, and sparfloxacin. The MICs for the type strain (GTC1234^T [=NCTC 8181^T]) and three reference strains of *S. agalactiae* (GIFU10482, GIFU10483, and GIFU10484) were determined (Table 1). However, three clinical isolates (GTC1966, GTC967, and GTC2001) were not inhibited in these tests, confirming that these isolates were multiply highly quinolone-resistant *S. agalactiae* strains.

To determine the QRDRs of *gyrA* and *parC*, we amplified

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TABLE 1. MICs (E-test) of quinolones for *S. agalactiae* and mutations in *gyrA* and *parC*

Strain	Status	MIC (μg/ml)[a]						Codon (amino acid) at:	
		LVX	NOR	FLE	SPX	OFX	CIP	Position 81 in *gyrA*	Position 79 in *parC*
GTC1234	Type strain	0.5	3	3	0.38	1	0.38	TCA (Ser)	TCC (Ser)
GIFU10482	Reference strain	0.38	3	3	0.38	1	0.5	TCA (Ser)	TCC (Ser)
GIFU10483	Reference strain	0.5	4	4	0.5	1.5	0.5	TCA (Ser)	TCC (Ser)
GIFU10484	Reference strain	0.75	6	4	0.75	1.5	0.75	TCA (Ser)	TCC (Ser)
GTC1966	Clinical isolate	>32	>256	>256	>32	>32	>32	TTA (Leu)	TTC (Phe)
GTC1967	Clinical isolate	>32	>256	>256	>32	>32	>32	TTA (Leu)	TTC (Phe)
GTC2001	Clinical isolate	>32	>256	>256	>32	>32	>32	TTA (Leu)	TTC (Phe)

[a] LVX, levofloxacin; NOR, norfloxacin; FLE, fleroxacin; SPX, sparfloxacin; OFX, ofloxacin; CIP, ciprofloxacin.

DNA fragments from chromosomal DNA by PCR using previously reported PCR primers for *gyrA* and *parC* (16). However, the primers amplified the target fragments from *S. pyogenes* but not from *S. agalactiae* strains (GTC1234, GIFU10482, GIFU10483, and GIFU10484). Therefore, we designed new PCR primers from the region common to *S. pyogenes* and *S. agalactiae*: GyrA-forward, 5′ GACAAGTGAAA TGAAAACGAG (positions 33 to 53); GyrA-reverse, 5′ CGC TCCATTGACTAATAAATTAGG (positions 484 to 507); ParC-forward, 5′ CAAAACATGTCCCTTGAGGA (positions13 to 32); and ParC-reverse, 5′ CTAGCTTTGGGATGA TCAATCAT (positions 577 to 599). After confirmation of a single 474- or 586-bp amplification product of *gyrA* or *parC*, respectively, on 1% agarose gels, sequences were determined with an automatic sequencer (model 3100; Applied Biosystems) with a dye terminator reaction kit (Applied Biosystems). DNA and protein sequence comparisons were done with DNASIS software (Hitachi Software Co., Yokohama, Japan).

First, we estimated the specificity of our PCR primers using the type strains of several beta-hemolytic streptococci. Subsequently, our primers for both *gyrA* and *parC* could amplify the target fragments from the following species: *S. agalactiae* (GTC1234^T [=NCTC 8181^T]), *Streptococcus pyogenes* (GTC262^T [=ATCC 12344^T]), *Streptococcus equi* subsp. *equi* (GTC269^T [=NCTC 9682^T]), *Streptococcus equi* subsp. *zooepidemicus* (GTC542^T [=ATCC 43079^T]), *Streptococcus iniae* (GTC244^T [=ATCC 29178^T]), *Streptococcus canis* (GTC423^T [=ATCC 43496^T]), *Streptococcus dysgalactiae* subsp. *dysgalactiae* (GTC431^T [=NCFB 2023^T]), *S. dysgalactiae* subsp. *equisimilis* (GTC842^T [=NCFB 1356^T]), *Streptococcus porcinus* (GTC543^T [=ATCC 43138^T]), and *Streptococcus difficile* (GTC730^T [=ATCC 51487^T]). All of these species belong to the pyogenic group of the genus *Streptococcus* (7). The species of the anginosus group are also beta-hemolytic, although our primers did not amplify fragments from the type strain of each species in this group (*S. anginosus* GTC268^T [=NCTC10713^T]; *Streptococcus intermedius* GTC216^T [=ATCC27335^T]; and *Streptococcus constellatus* subsp. *constellatus* GTC221^T [=ATCC27823^T]). Because the anginosus group is phylogenetically distant from the pyogenic group (7), the sequences of *gyrA* and *parC* may be slightly different from those in *S. agalactiae* and *S. pyogenes*.

Comparative amino acid sequences deduced from *gyrA* and *parC* (including the QRDR) from representative strains of both quinolone-susceptible and -resistant *S. agalactiae* strains and some other beta-hemolytic streptococci are shown in Fig. 1 and 2, respectively.

All four quinolone-susceptible *S. agalactiae* strains (GTC1234, GIFU10482, GIFU10483, and GIFU10484) shared the same deduced amino acid sequences for the QRDRs of both *gyrA* and *parC*. Similarly, three isolates of highly quinolone-resistant strains (GTC1966, GTC1967, and GTC2001) had identical amino acid sequences. However, compared with susceptible strains, these quinolone-resistant *S. agalactiae* strains carried double point mutations of DNA with the following inferred amino acid substitutions involving the QRDRs of *gyrA* and *parC*; Ser-81 to Leu (TCA → TTA) for *gyrA* and Ser-79 to Phe (codon TCC → TTC) for *parC*. The mutations at these positions were previously described as contributing to quinolone resistance (4–6, 10, 15–17).

Three major mutation sites have been previously reported for quinolone-resistant streptococci, namely, position 81 in *gyrA* and positions 79 and 83 in *parC*. Yokota et al. found that some quinolone-resistant *S. pneumoniae* strains had other mutations, including Ser-114 to Gly in *gyrA* and Ser-52 to Gly, Asn-91 to Asp, and Glu-135 to Asp in *parC* (17). We concluded that these mutations were not related to quinolone resistance, because, as shown in Fig. 1 and 2, many quinolone-susceptible streptococci had the same amino acid sequences.

Surprisingly, *S. agalactiae* exhibited sequences different from those of other beta-hemolytic streptococci: *S. agalactiae* strains had Met in position 132 of the *gyrA* product, whereas all other beta-hemolytic streptococci in this study had Leu (Fig. 1). All beta-hemolytic streptococci and even *S. pneumoniae* strains have same amino acid at positions 69, 97, 99, and 113 of the *parC* product (Ile, Ile, Val, and Pro, respectively); however, *S. agalactiae* strains (seven strains including the type strain) and the type strain of *S. difficile* have different amino acids (Val, Thr, Ile, and Ala, respectively, at these positions) (Fig. 2). We cannot easily explain why only *S. agalactiae* and *S. difficile* have different amino acids at these positions. According to a FASTA homology search (12) on the DDBJ website (http://www.ddbj.nig.ac.jp), there was no more closely related organism than beta-hemolytic streptococci (data not shown). At this time, we do not expect that the genes were transferred from other organisms.

We were also surprised that *S. agalactiae* and *S. difficile* shared identical amino acid sequences of QRDRs in both *gyrA* and *parC* (Fig. 1 and 2). There were numerous silent nucleotide base substitutions, especially in *parC*: for example, only

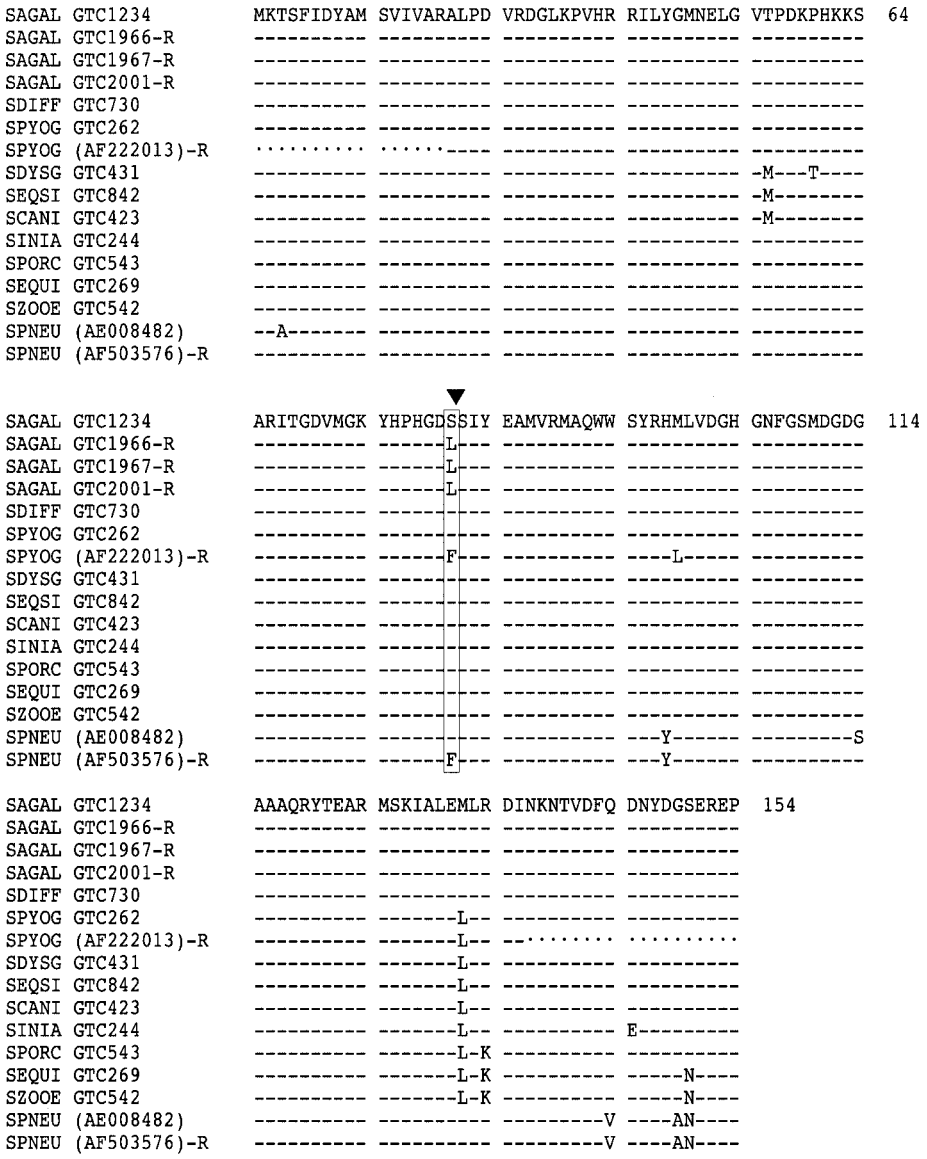

FIG. 1. Comparison of the deduced amino acid sequences of a region of *gyrA* containing a QRDR from quinolone-susceptible and -resistant strains of *S. agalactiae* and other beta-hemolytic streptococci. SAGAL, *S. agalactiae*; SDIFF, *S. difficile*; SPYOG, *S. pyogenes*; SDYSG, *S. dysgalactiae* subsp. *dysgalactiae*; SEQSI, *S. dysgalactiae* subsp. *equisimilis*; SCANI, *S. canis*; SINIA, *S. iniae*; SPORC, *S. porcinus*; SEQUI, *S. equi* subsp. *equi*; SZOOE, *S. equi* subsp. *zooepidemicus*; SPNEU, *S. pneumoniae*. "R" following the strain number indicates quinolone resistance. The sequence data for the resistant strain of *S. pyogenes* (AF222013) and *S. pneumoniae* (AF503576) were taken from DDBJ. Only amino acids different from those in *S. agalactiae* GTC1234^T are given; dashes indicate amino acid identity, and a dot indicates no sequence information. The amino acids involved in quinolone resistance (▼) are boxed. Quinolone-susceptible strains of *S. agalactiae* (GIFU10482, GIFU10483, and GIFU10484) shared identical sequences with *S. agalactiae* GTC1234^T.

two amino acid differences were found between *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus*, although there were 21 silent base substitutions (data not shown). In the case of *S. agalactiae* and *S. difficile*, only one base difference was found. Strains of *S. agalactiae* have been isolated from homeothermic animals, including humans, whereas *S. difficile* has been isolated from poikilothermic animals, such as fish (2). Horizontal gene transfer is one possible explanation for the presence of the same sequences in these two species.

Munoz and Campa had reported that *parC* was the primary target of quinolones in *S. pneumoniae* (10). In this study we isolated three resistant strains of *S. agalactiae*, with the same point mutation in both *gyrA* and *parC*. We cannot tell which gene is the primary target of the quinolones in *S. agalactiae* on the basis of our isolates; further study is needed.

Nucleotide sequence accession numbers. The nucleotide sequences of *gyrA* and *parC* for each strain were deposited in DDBJ under the following respective accession numbers: AB101448 and AB101464 for *S. agalactiae* GTC1234^T; AB101449 and AB101465 for *S. agalactiae* GTC1966; AB101450 and AB101466 for *S. agalactiae* GTC1967; AB101451 and AB101467 for *S. agalactiae* GTC2001;

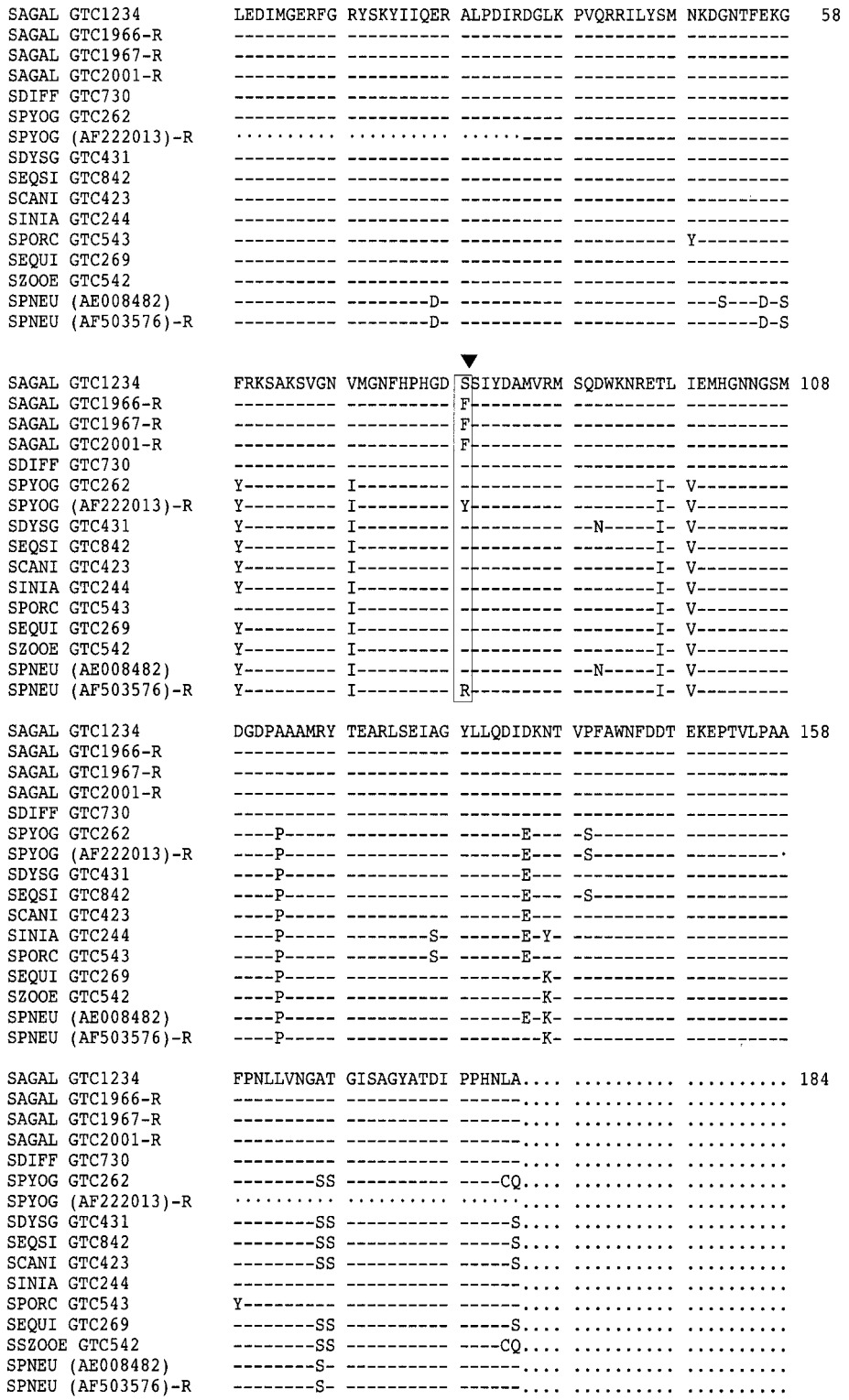

FIG. 2. Amino acid sequences deduced from a region of *parC* containing a QRDR from quinolone-susceptible and -resistant strains of *S. agalactiae* and other beta-hemolytic streptococci. Symbols and abbreviations are as in Fig. 1.

AB101452 and AB101468 for *S. agalactiae* GIFU10482; AB101453 and AB101469 for *S. agalactiae* GIFU10483; AB101454 and AB101470 for *S. agalactiae* GIFU10484; AB101455 and AB101471 for *S. pyogenes* GTC262^T; AB101456 and AB101472 for *S. dysgalactiae* subsp. *dysgalactiae* GTC431^T; AB101457 and AB101473 for S. *dysgalactiae* subsp. *equisimilis* GTC842^T; AB101458 and AB101474 for *S. canis* GTC423^T; AB101459 and AB101475 for *S. iniae* GTC244^T; AB101460

and AB101476 for *S. porcinus* GTC543^T; AB101461 and AB101477 for *S. equi* subsp. *equi* GTC269^T; AB101462 and AB101478 for *S. equi* subsp. *zooepidemicus* GTC542^T; and AB101463 and AB101479 for *S. difficilis* GTC730^T.

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