Relationship between mutations in the DNA gyrase and topoisomerase IV genes and nadifloxacin resistance in clinically isolated quinolone-resistant Staphylococcus aureus

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Abstract We analyzed the relationship between resistance to nadifloxacin, a fluoroquinolone antimicrobial agent, and mutations in the quinolone resistance-determining regions (QRDRs) of the A subunit of DNA gyrase and topoisomerase IV, in 24 clinical isolates of Staphylococcus aureus. Seven known mutations were found in the QRDRs. The minimum inhibitory concentration (MIC) of nadifloxacin increased in a manner that was dependent on mutations in the A subunit of DNA gyrase, and did not appear to be related to mutations in the A subunit of topoisomerase IV. The type 9 mutant, which included four mutations, was highly resistant to ofloxacin, norfloxacin and sparfloxacin, but only moderately resistant to nadifloxacin (MIC, 12.5 µg/ml). One of the norfloxacin-resistant strains that expressed high levels of norA was not resistant to nadifloxacin. To the best of our knowledge, this is the first report describing a fluoroquinolone antimicrobial agent whose primary target is suggested to be DNA gyrase in S. aureus.

Key words Topoisomerase IV · DNA gyrase · Staphylococcus aureus · Nadifloxacin · Fluoroquinolone resistance · norA
**S. aureus** FDA-209P was used as the standard strain. We used clinical isolates of *S. aureus*, obtained from the Department of Dermatology, Kansai Medical University and Nagoya University Hospital, before the introduction of nafcillin to the market.

Nafcillin was synthesized by Otsuka Pharmaceutical (Tokushima, Japan). Norfloxacin, ofloxacin, levofloxacin, enoxacin, ciprofloxacin, lomefloxacin, tosufloxacin, and sparfloxacin were extracted and purified from commercially available products.

MICs were determined at the inoculum size of 10^6 CFU/ml by the twofold agar dilution method, using Mueller-Hinton agar (Difco Laboratories, Detroit, MI, USA), according to the standard methods of the Japan Society of Chemotherapy.

The genomic DNA of *S. aureus* was prepared using TRIzol (GIBCO BRL, Gaithersburg, MD, USA). The gyrA, grlA, gyrB, and grlB gene regions were amplified by polymerase chain reaction (PCR) in a DNA thermal cycler (model RJ1000; Perkin-Elmer Cetus, Norwalk, CT, USA) from bp 2370 to 2653, from bp 2090 to 2409, from bp 1252 to 1467, and from bp 1252 to 1467, respectively, to allow cloning of the PCR fragments into pCR2.1 plasmids (Invitrogen, San Diego, CA, USA). Nucleotide sequences are numbered according to Margerrison et al. for gyrA and gyrB, and according to Ferrero et al. for grlA and grlB. The DNA sequence was determined with an ALF DNA sequencer (Pharmacia Biotech, Upsala, Sweden).

DNA probes used for the dot blotting of *norA* mRNA were prepared by performing PCR on the genomic DNA isolated from *S. aureus* FDA-209P, using synthesized primers. The nucleotide sequences of *norA* are numbered according to the GenBank STANORA11 database. Positions 929 to 1107 of the *norA* gene were amplified by PCR. The PCR product was labeled with [α-35S]dCTP (DuPont-NEN, Boston, MA, USA; specific activity, 46.3 TBq/mmol), using a random primer DNA labeling kit (Ver. 2.0; Takara Shuzo, Otsu, Japan), and was then used as a DNA probe.

*S. aureus* was cultured at 37°C while being shaken in 5 ml of antibiotic medium 3 (Difco Laboratories), either norfloxacin-free or containing either 1/8 of the MIC or 1/4 of the MIC of norfloxacin, until the culture reached an optical density at 610 nm (OD 610) of approximately 1. Total RNA was then extracted using TRIzol (GIBCO BRL). Ten μg of total RNA was then applied to a Hybond-N+ filter (Amersham, Little Chalfont, UK), hybridized with the 35S-labeled probe, and autoradiographed. The autoradiograms were then scanned with a Dual-Wave-length Flying-Spot Scanner (model CS-9000; Shimadzu, Kyoto, Japan).

The MICs of the various fluoroquinolones for one standard (FDA-209P) and the 24 clinically isolated *S. aureus* strains that were genetically analyzed are shown in Table 1. The MIC of nafcillin for the FDA-209P strain was 0.006 μg/ml or less. Type 9 was highly resistant to ofloxacin, norfloxacin, and sparfloxacin (all MICs were 50 μg/ml or greater), while it was moderately resistant to nafcillin and tosufloxacin (MIC, 12.5 μg/ml).

Mutant strains with amino acid alterations are shown in Table 1. In DNA gyrase A subunits, mutations of Ser-84 to Leu, Ser-85 to Pro, and Glu-88 to Gly or Lys were found. In topoisomerase IV A subunits, mutations of Ser-80 to Tyr or Phe and Glu-84 to Lys were found. Thus, there were seven different mutations at five different sites, all of which are known mutations associated with fluoroquinolone resistance. The mutations of Asp-437 or Arg-458 in the gyrase A gene, the MICs of nafcillin for type 4 and type 6 increased dependant on mutations in the *gyrA* gene. The target of nafcillin, at least for these three types, was DNA gyrase. The MIC for type 3 was 0.006 μg/ml or less. These results suggested that the primary target of nafcillin was DNA gyrase. This is the first report to describe a fluoroquinolone antibacterial agent whose primary target in *S. aureus* is suggested to be DNA gyrase. Mutants that included only gyrA mutations were not found in this study. One of the reasons for this finding is thought to be the use of clinical isolates of *S. aureus* obtained prior to the introduction of nafcillin to the market.

Type 9, which included two mutations in the *gyrA* gene, was only moderately resistant to nafcillin (MIC, 12.5 μg/ml).

It has been demonstrated that NorA protein is responsible for norfloxacin resistance in *S. aureus*, and that this resistance is due to increased *norA* expression. We, therefore, decided to analyze the relationship between the level of *norA* expression and nafcillin resistance in clinically isolated quinolone-resistant *S. aureus* strains. Although both strain SA011 and strain SA014 possessed the same mutations in the *grlA* gene, the MICs of norfloxacin for strain SA011 and strain SA014 were 0.39 μg/ml and 50 μg/ml, respectively. Similar to findings in previous reports of *S. aureus* expressing high levels of *norA*, the ciprofloxacin, enoxacin, and norfloxacin MICs for strain SA014 were increased, compared with strain SA011 but the sparfloxacin and tosufloxacin MICs were not (Table 1). As the norfloxacin resistance found in strain SA014 could have been caused by the overexpression of *norA*, we examined the levels of *norA* expression in these two strains. The levels of *norA* expression were determined in the presence of various concentrations of norfloxacin, because the *norA* gene is drug-inducible or consistent.

The expression of *norA* in the SA011 strain (MIC of norfloxacin, 0.39 μg/ml) was 100%, 78%, and 167% for norfloxacin-free, 1/8, and 1/4 MIC treatments, respectively, and the expression of *norA* in the SA014 strain (MIC...