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Quinolone Susceptibility of *norA*-Disrupted *Staphylococcus aureus*

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The MIC of norfloxacin for the *norA*-disrupted mutant termed RDN1, obtained from quinolone-susceptible *Staphylococcus aureus* RN4220, was eightfold lower than that for RN4220. The increase in susceptibility was related to an increase of drug accumulation by RDN1. These results indicate that NorA plays an important role in the susceptibility of quinolone-susceptible *S. aureus* to selected quinolones.

Quinolones demonstrate a potent antimicrobial effect against gram-positive and gram-negative bacteria (5). These drugs have been widely used as effective antimicrobial agents, but quinolone-resistant strains have emerged in many pathogens including methicillin-resistant *Staphylococcus aureus* (1, 11).

NorA is considered to be one factor relating to quinolone resistance in *S. aureus*, besides mutational alterations in *gyrA* (8) and *grlA* (4) which produce the target proteins for quinolones. NorA has been reported to act as an active efflux pump for some quinolones and is classified as a member of the efflux protein superfamily along with Bmr and Tet (3, 9, 14). Several studies suggest that overproduction of this efflux protein results in quinolone resistance (7, 12). However, because studies on the efflux of quinolones mediated by *norA* were mostly performed by using a *norA* recombinant plasmid, the role of native *norA* in *S. aureus* is less well comprehended. Here, we report that we made a *norA*-disrupted mutant from quinolone-susceptible *S. aureus* and evaluated the effects of *norA* disruption on the susceptibilities of seven quinolones.

In order to make the *norA* knockout strain, we cloned a 5.5-kbp *Hin*dIII fragment containing *norA* from *S. aureus* SA113 into pUC19 in which multiple cloning sites were deleted but not the *Hin*dIII site. The *aadD* gene encoding tobramycin resistance obtained from *Hin*dIII-*Bam*HI-digested chromosomal DNA of TK784 (13) was cloned into pUC19. Then, the 2.0-kbp *Sph*I-*Kpn*I fragment containing the *norA* gene was replaced by a 2.2-kbp *Hin*dIII-*Bam*HI fragment containing the *aadD* gene, so that the *norA* coding region was completely removed. Each cohesive end had been blunt ended by mung bean nuclease before ligation. This *norA*-deleted plasmid was introduced into RN4220. Five milliliters of a cell culture shaken at 37°C for 18 h in 10 ml of brain heart infusion broth (Difco) was inoculated into 100 ml of the same broth and incubated with shaking at 37°C for 3 h. Then, cells were collected, washed three times with 1.1 M sucrose below 4°C, and suspended in 1 ml of the same solution. The plasmid was mixed with 100 μl of the cell suspension and introduced by electroporation (BTX, Inc.; Electro Cell Manipulator 600; charge voltage: 1.5 kV; maximum voltage range: 2.5 kV; timing mode: resistance; resistance timing: 129 Ω). A *norA* knockout strain, termed RDN1, was grown on a Luria-Bertani agar plate containing tobramycin (20 μg per ml). The *norA* gene was not detected in RDN1 by Southern hybridization using a PCR product which involved a part of the *norA* coding region (forward primer: 5′-ATGTTTGCAGTTGGCCACAATTTTTC; reverse primer: 5′-ATGACCAAGCTATAAATGTTAAC) as a probe (Fig. 1). Although NorA is considered to function as a native membrane protein in *S. aureus*, its disrupted mutant demonstrated a colony morphology and a cell growth rate that were similar to those of RN4220. Therefore, NorA is suggested to be dispensable for the growth of RN4220 in the normal in vitro environment.

To investigate the influence of *norA* disruption, we determined the MICs of norfloxacin, pazufloxacin, ciprofloxacin, enoxacin, ofloxacin, tosufloxacin, and sparfloxacin for RN4220 and RDN1 by the agar dilution method (6). We also determined the drug accumulations of pazufloxacin and norfloxacin in both strains according to the method reported by Chapman et al. (2). The MIC of norfloxacin for RDN1 was eightfold lower than that for RN4220. On the other hand, the MICs of pazufloxacin were the same for both strains (Table 1). The accumulation of norfloxacin in RDN1 was threefold more than that in RN4220 (114 versus 35 ng/mg of dry cells, respectively) and reached almost the same level as that in pazufloxacin, for which drug accumulation was similar in both strains (120 and 139 ng/mg of dry cells in RN4220 and RDN1, respectively).

Moreover, we measured the drug concentration in a 50 mM phosphate buffer (pH 7.0) layer after mixing with an equal volume of chloroform and incubating at 37°C for 16 h in order to measure the hydrophobicities of quinolones. All the quinolones for which susceptibilities were influenced by *norA* disruption demonstrated low hydrophobicities (Table 1). Yoshida et al. (14) considered that the relative hydrophobicities of the quinolones affected their efflux mediated by NorA. However, the relative hydrophobicity of pazufloxacin is similar to that of enoxacin, whose MIC for RDN1 was fourfold lower than that

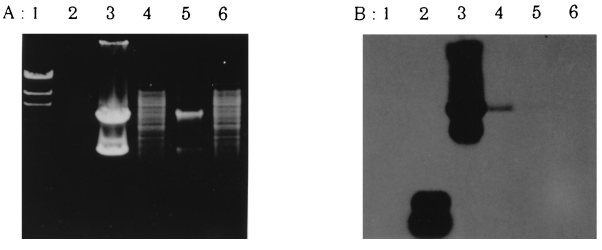

FIG. 1. Southern hybridization analysis of RN4220 and RDN1. The 5.5-kbp *Hin*dIII fragment of the *norA* gene was not detected in RDN1 chromosomal DNA. (A) Agarose gel electrophoresis (stained by ethidium bromide). (B) Southern hybridization. Lanes (both panels): 1, molecular weight marker (λ-DNA–*Hin*dIII digestion); 2, PCR product of the *norA* fragment (used as a probe); 3, *Hin*dIII-digested, *norA*-cloned pUC19; 4, *Hin*dIII-digested chromosomal DNA of RN4220; 5, *Hin*dIII-digested, *norA*-disrupted plasmid; 6, *Hin*dIII-digested chromosomal DNA of RDN1.

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TABLE 1. Relationship between the MICs for RN4220 and RDN1 and the relative hydrophobicities of the quinolones

Compound	MIC (μg/ml) for:		Hydrophobicity
	RN4220	RDN1	
Norfloxacin	1.56	0.2	0.33
Ciprofloxacin	0.39	0.1	0.79
Enoxacin	1.56	0.39	1.16
Pazufloxacin	0.2	0.2	1.28
Ofloxacin	0.39	0.39	4.00
Tosufloxacin	0.05	0.05	7.23
Sparfloxacin	0.1	0.1	17.35

for RN4220. Takenouchi et al. (10) reported that the properties of the C-7 and the C-8 substituents of quinolones were important for efflux-mediated resistance. Actually, the quinolones whose susceptibilities were influenced by *norA* disruption have piperazinyl residues at the C-7 position. However, we have not clarified whether this residue makes the compound hydrophilic or relates to recognition by NorA.

These results indicate that NorA plays an important role in the susceptibility to selected quinolones of quinolone-susceptible *S. aureus*. Therefore it may be useful to clarify the substrate specificity of NorA and what mutations result in the resistance, as well as to determine other mechanisms of resistance, in order to develop novel quinolones effective against quinolone-resistant strains.

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