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NOTES

Efflux Pump-Mediated Quinolone Resistance in *Staphylococcus aureus* Strains Wild Type for *gyrA*, *gyrB*, *grlA*, and *norA*

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Fluoroquinolone efflux was studied in 47 *Staphylococcus aureus* clinical strains with MICs of ciprofloxacin (CFX) of ≤2 μg/ml. Forty-three strains were wild type for *gyrA*, *gyrB*, and *grlA* quinolone resistance-determining regions and for *norA* and its promoter region. Forty of these strains (MICs of CFX, 0.1 to 0.2 μg/ml) did not show efflux of fluoroquinolones. Three strains (MICs of CFX, 1 to 2 μg/ml) showed efflux. These results suggest that efflux can appear in *S. aureus* clinical strains in the absence of mutations in *norA* and its promoter.

Fluorinated quinolones (FQ) appeared in the 1980s as synthetic broad-spectrum antibiotics that were very active mainly against gram-negative bacteria. Mutations in *grlA*, the gene encoding topoisomerase IV subunit A, seem to be the main factor in *Staphylococcus aureus* resistance to older FQ, such as ciprofloxacin and ofloxacin (1, 2, 10). Mutations in DNA gyrase (*gyrA* mainly) usually appear in the presence of previous mutations in *grlA*, leading to additional increases in MICs (10). Some newer FQ have a different behavior. The main target for some FQ, in gram-positive bacteria such as *Streptococcus pneumoniae* is DNA gyrase, instead of topoisomerase IV (11). Nevertheless, this characteristic has not been shown for other gram-positive bacteria.

Another mechanism involved in quinolone resistance in *S. aureus* is overexpression of *norA*. This gene encodes a multidrug efflux protein (NorA) capable of transporting FQ outside the bacteria (4–6, 16, 17). Overexpression of *norA* has been related to mutations 89 bp upstream from the putative ATG start codon (4, 9). NorA-mediated resistance has been described in the apparent absence of mutations in topoisomerase genes (6). Moreover, NorA-mediated resistance can appear both in the presence (4, 9) and in the absence of promoter mutations (5). *S. aureus* strains derived from a single strain (SA-1199) that can overexpress *norA* in a constitutive or inducible manner have been reported. Inducible strains lack promoter mutations (6).

NorA has been shown to play a role even in quinolone-susceptible strains, since *norA* disruption leads to MICs eightfold lower than for the parent strain (16). We have studied the presence of mutations in the genes encoding DNA gyrase and topoisomerase IV and in the *norA* gene and its promoter in FQ-sensitive and borderline strains. When strains with the same genotype had different FQ susceptibilities, efflux activity was studied.

Bacterial strains. Forty-seven *S. aureus* clinical strains with ciprofloxacin MICs of ≤2 μg/ml were studied.

Antimicrobial susceptibility. MICs of ofloxacin, ciprofloxacin, sparfloxacin, and trovafloxacin were determined by the agar dilution method, according to National Committee for Clinical Laboratory Standards guidelines (8). Drugs were obtained from their respective manufacturers as standard powders. MIC determinations were also performed in the presence of 20 μg of reserpine per ml.

PCR procedures. *gyrA* (13), *grlA* (1), and *gyrB* (3) quinolone resistance-determining regions (QRDRs) and *norA* (4) and its promoter region were amplified and sequenced (12) according to previously described methods.

TABLE 1. In vitro activities of four quinolones, alone and combined with reserpine, against the three groups of *S. aureus* strains studied

Group	No. of strains	MIC range (μg/ml) of quinolone alone/quinolone plus reserpine (20 μg/ml)			
		Ciprofloxacin	Ofloxacin	Sparfloxacin	Trovafloxacin
A1	40	0.1–0.2/0.1–0.2	0.1–0.2/0.1–0.2	0.01–0.03/0.01–0.03	≤0.008/≤0.008
A2	3	1–2/0.1–0.2	1–2/0.1–0.2	0.06/0.01–0.03	0.03/≤0.008
B	4	1–2/1–2	1–2/1–2	0.1/0.1	0.03/0.03

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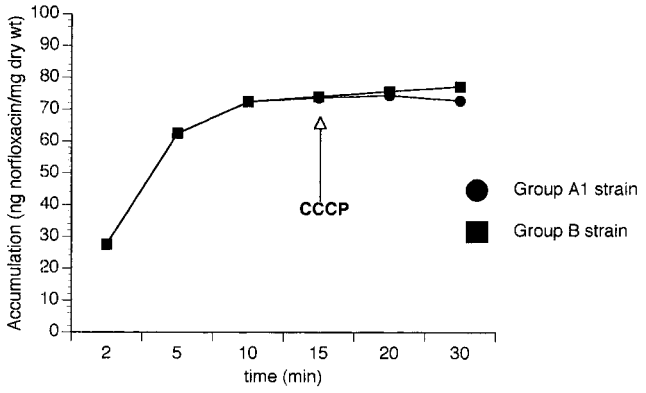

FIG. 1. Example of norfloxacin accumulation in strains of groups A1 and B.

Uptake of quinolones. Uptake and accumulation of fluoroquinolones was determined by the method described by Takenouchi et al. Fluorescence was used as a means of quantifying FQ concentrations (15).

The results of sequencing of the four genes appear in Table 1. Among the 47 strains with ciprofloxacin MICs of ≤2 μg/ml, four strains showed a mutation in *grlA* leading to a Ser80-to-Ile substitution (group B), and 43 strains were wild type for *gyrA*, *gyrB*, *grlA*, and *norA*. The four strains with mutations in *grlA* showed MICs of ciprofloxacin of 1 to 2 μg/ml. Among the 43 wild-type strains, we found two groups of strains. Forty strains showed ciprofloxacin MICs of around 0.1 to 0.2 μg/ml (group A1). Three strains showed MICs similar to *grlA* mutant strains (1 to 2 μg/ml) (group A2).

The study of FQ uptake by the wild-type strains in group A1 and the three *grlA* mutant strains (group B) led to results similar to the uptake curve appearing in Fig. 1 (which corresponds to one of the strains). Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) did not modify their behavior. When FQ uptake was studied in the three wild-type strains with higher ciprofloxacin MICs (group A2), we observed the behavior shown in Fig. 2. FQ uptake increased in the presence of CCCP.

NorA-mediated quinolone efflux has been extensively studied in *S. aureus*. Previous studies have shown that *norA* overexpression can lead to FQ resistance, both in the presence and in the absence of topoisomerase alterations (4, 5). Quinolone resistance due to *norA* overexpression was first related to mutations in the *norA* promoter region (4, 9). Previous studies suggested that the thymine-to-adenine mutation in the *norA* promoter region might be responsible for increased *norA* transcription (4, 9), but *norA* overexpression happens independently of this mutation (5), suggesting that *norA* regulation can be located elsewhere in the chromosome (6). Recent studies suggest that this mutation might be necessary for constitutively increased *norA* expression, but it is not necessary when overproduction is inducible (6). Efflux-pump mediated quinolone-resistance has not been described in *gyrA* or *grlA* wild-type clinical strains. Our results show that efflux-mediated resistance might be not so infrequent in this kind of strain. The three *S. aureus* strains in group A2 were wild type for *norA* and its promoter region. Results obtained in this study suggest that resistance in these strains is directly related to efflux. We did not find any mutation in *gyrA*, *gyrB*, and *grlA* QRDRs and *norA* and its promoter region. *grlB* mutations have been described in *S. aureus* only following *gyrA* and *grlA* mutations (14). Moreover, MICs of ciprofloxacin decreased by eightfold in the three strains when ciprofloxacin was combined with reserpine (Table 1). One possible explanation is that mutations in areas other than the *norA* promoter region might be involved in constitutive *norA* overexpression. Nevertheless, since *norA* expression has not been determined, we cannot know certainly the origin of the efflux system, and the possibility of efflux pumps other than NorA cannot be disregarded. In other species, such as *Pseudomonas aeruginosa*, the presence of several efflux systems able to efflux quinolones with different levels of efficacy has been described (7).

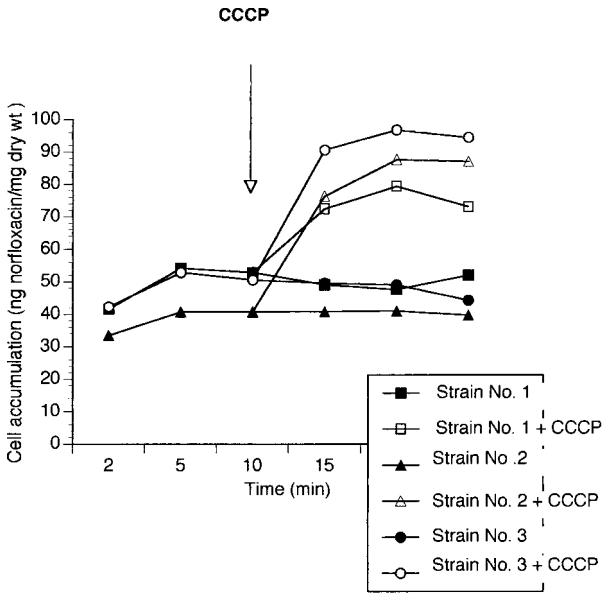

FIG. 2. Norfloxacin accumulation in strains of group A2.

In the three strains in group A2, efflux was associated with MICs similar to those caused by *grlA* mutations in the strains in group B. Changes were not the same for all of the quinolones tested (Table 1). Ciprofloxacin and ofloxacin are hydrophilic quinolones, and MICs of these compounds ranged from 0.1 to 0.2 μg/ml in wild-type strains without efflux activity. MICs of these FQ against strains with efflux activity ranged between 1 and 2 μg/ml and reverted to 0.1 to 0.2 μg/ml when tested in presence of reserpine. MICs of sparfloxacin, a more hydrophobic compound, were less affected. MICs ranged between 0.01 and 0.03 μg/ml for strains without efflux activity, and MICs for the three strains with efflux activity were 0.06 μg/ml. MICs for trovafloxacin remained very low for all strains. MICs for strains with efflux activity were 0.03 μg/ml, while MICs for strains without efflux activity were ≤0.008 μg/ml (four- to eightfold lower).

These results show that efflux can appear in clinical strains, even in the absence of mutations in the genes usually involved in quinolone resistance, particularly in strains with to MICs at or above the breakpoint. The mechanisms controlling this efflux, and the possibility that these strains might even increase their efflux activity in an inducible way, are now being studied.

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