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NOTES

In Vivo Selection during Pefloxacin Therapy of a Mutant of *Staphylococcus aureus* with Two Mechanisms of Fluoroquinolone Resistance

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Staphylococcus aureus **BM4626 (ciprofloxacin MIC, 0.5 μg/ml) and BM4627 (ciprofloxacin MIC, 32 μg/ml) were isolated from the same patient before and during pefloxcin therapy for septic tibial nonunion, respectively. The two strains had similar serotypes and indistinguishable phage types and *SmaI*-generated restriction fragment length polymorphisms. Portions of the *gyrA* (codons 60 to 120) and the *gyrB* (codons 420 to 480) genes of each clinical isolate were amplified by PCR and sequenced. Strain BM4627 had a serine-to-leucine substitution resulting from a cytosine-to-thymidine mutation at codon 84 of *gyrA* relative to the sequence of the *gyrA* gene of BM4626. Norfloxacin accumulation, measured in a whole-cell uptake assay, was significantly lower in BM4627 than BM4626. These data indicate that double mutants can be selected in vivo under fluoroquinolone therapy.**

Fluoroquinolones are synthetic broad-spectrum antibiotics that were introduced in clinical practice in the early 1980s. They were initially active against methicillin-susceptible as well as methicillin-resistant strains of *Staphylococcus aureus*, a major community-acquired and nosocomially acquired pathogen. Unfortunately, fluoroquinolone resistance emerged and spread rapidly in this species, especially among methicillin-resistant strains (15).

Three mechanisms of resistance to fluoroquinolones have been described in *S. aureus*. The first one consists of a mutational alteration of DNA gyrase, a topoisomerase which catalyzes ATP-dependent negative supercoiling of DNA and is thought to be the primary target for quinolone action (21). The enzyme is a tetramer of two A and two B subunits, coded for by the *gyrA* and *gyrB* genes, respectively. In *S. aureus* (6, 16, 17), like in *Escherichia coli* (4, 7, 23), clustered point mutations in *gyrA* have been associated with fluoroquinolone resistance. Resistance in *E. coli* can also result from a mutation in *gyrB* (22) that has not been found in *S. aureus*. Active efflux of quinolones from the cell has been proposed as a second mechanism of resistance in *S. aureus* (9, 24). The gene involved, *norA*, is part of the *S. aureus* chromosome and encodes a polypeptide that acts like a quinolone efflux pump. A third resistance locus, termed *flqA*, has been shown to be distinct from the structural genes for the DNA gyrase and from the *norA* gene, but the mechanism of resistance remains unknown (20). The *norA* and *flqA* loci are implicated in low-level resistance to fluoroquinolones, whereas mutations in *gyrA* have been found only in strains with high levels of resistance. However, the precise contribution of *gyrA* mutations to resistance remains to be established.

We studied three pairs of fluoroquinolone-susceptible and -resistant matched clinical strains of methicillin-susceptible *S. aureus* isolated in three geographically distant hospitals. Each pair of strains was isolated from the same patient before and during pefloxacin therapy. *S. aureus* BM4626 and BM4627 were isolated from the pus of a patient with a septic tibial nonunion, BM4628 and BM4629 were isolated from bronchial aspirations of a polytraumatized patient, and BM4630 and BM4631 were isolated from the pus of a foot osteitis of a diabetic patient. The MICs of pefloxacin, norfloxacin, ciprofloxacin, and sparfloxacin for these strains were determined by dilution in agar with Mueller-Hinton medium and an inoculum of 10^4 CFU per spot (Table 1).

The strains were compared by serotyping (13), phage typing (2), and pulsed-field gel electrophoresis analysis after *SmaI* digestion of total DNA. In the last technique, isolation, deproteinization, and restriction of DNA were performed as described previously (18). Restriction fragments were separated in 1% agarose gels run at 200 V in 0.5× Tris-borate-EDTA buffer by using a CHEF-DR II system (Bio-Rad Laboratories, Nazareth, Belgium). Optimal separation was achieved by using a 5- to 35-s-pulse-time linear gradient for 23 h. The two members of each pair had similar (BM4626 and BM4627; BM4630 and BM4631) or identical (BM4628 and BM4629) serotypes and phage types (Table 1) and indistinguishable *SmaI* macrorestriction patterns (data not shown) and were thus clonally related. The differences in serotypes and phage types between members of two of the pairs confirm that identification methods based on the genotype provide data that are usually more stable than those based on phenotypic characters.

We tested the possibility that the resistant strains carry mutations in the genes for the DNA gyrase by amplification

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TABLE 1. Serotypes, phage types, and susceptibilities to selected fluoroquinolones of *S. aureus* strains

Strain	Serotype	Phage type	MIC (μg/ml)				
			Norfloxacin	Norfloxacin + reserpine (20 μg/ml)	Pefloxacin	Ciprofloxacin	Sparfloxacin
BM4626	a5/h2/p	3A/	1	0.5	0.5	0.5	0.125
BM4627	h2	3A/	128	16	64	32	8
BM4628	a5/h2/o/p	NT[a]	1	1	0.5	1	0.125
BM4629	a5/h2/o/p	NT	16	8	8	4	0.25
BM4630	a5/(e)/h2/k1/m/o/p	29/81/	2	1	0.5	0.5	0.125
BM4631	a5/h2/k1/m/(o)	29/80/81/	32	16	8	8	0.25

[a] NT, nontypeable.

and then sequence determination of critical regions of *gyrA* (codons 60 to 120) and *gyrB* (codons 400 to 460). All the mutations associated with quinolone resistance described in *S. aureus* (codons 84, 85, and 88 of *gyrA*) and *E. coli* (codons 67, 81, 83, 84, 87, and 106 of *gyrA*; codons 427 and 446 of *gyrB*) reside in these two portions, which may be part of the quinolone-binding site (4, 6, 7, 16, 17, 23). The *S. aureus gyrB* and *gyrA* genes are contiguous (10), and we amplified by PCR a 1,232-bp fragment that includes the 737-bp 3′ portion of *gyrB*, the 36-bp small intergenic region, and the 459-bp 5′ portion of *gyrA*. The heptadecanucleotides used as primers consisted of 5′-GTGAAGTAACACGTCGT-3′ (*gyrB* positions 1186 to 1202) and 5′-TTACCATCATAGTTATC-3′ (complementary to *gyrA* positions 443 to 459). One microgram of chromosomal DNA isolated from each strain by phenol-chloroform extraction (5) was added to a PCR mixture of 50 μl (1 U of *Taq* DNA polymerase [Amersham Corp., Arlington Heights, Ill.] in its buffer [1×] with 10 pmol of each primer and 5 nmol of each of the 2′-deoxynucleoside 5′-triphosphates). PCR conditions were as follows: 92°C for 30 s, 50°C for 1 min, and 72°C for 1 min for 35 cycles. The amplification products were purified with the QUIAEX DNA purification kit (Diagen GmbH, Hilden, Germany) and were partially sequenced on both strands by the dideoxy-chain termination method (14) by using the primers for PCR and primers 5′-ACCAAATGCTGTG ATCAT-3′ (complementary to *gyrB* positions 1430 to 1446) and 5′-TTAGATTATGCGATGAG-3′ (*gyrA* positions 68 to 84).

The sequences of the *gyrA* portions from the fluoroquinolone-susceptible strains BM4626, BM4628, and BM4630 and from the fluoroquinolone-resistant isolates BM4629 and BM4631 were identical to that reported previously for the susceptible strain *S. aureus* 81231 (10). The *gyrA* portion from the resistant isolate BM4627 had a C-to-T transition at nucleotide position 251, resulting in a serine-to-leucine substitution at codon 84 (Ser-84 to Leu substitution). The partial *gyrB* sequences obtained were identical to (BM4630 and BM4631) or differed only by three silent mutations (BM4626, BM4627, BM4628, and BM4629) from that reported previously for *S. aureus* 601055 (3).

An amino acid substitution similar to that found in GyrA of strain BM4627 has already been detected in methicillin-resistant strains of *S. aureus* (6, 16, 17). Other substitutions in GyrA have also been associated with quinolone resistance in *S. aureus*: Ser-84 to Ala; Ser-85 to Pro, which can be associated with a Ser-84 to Leu substitution; and Glu-88 to Lys. These four substitutions are associated with high-level quinolone resistance (ciprofloxacin MICs, ≥16 μg/ml), as is the case in strain BM4627.

We also studied the kinetics of accumulation of norfloxacin by the six clinical isolates. The isogenic strains SA-1199 (quinolone susceptible; ciprofloxacin MIC, 0.25 μg/ml) and SA-1199B (quinolone-resistant; ciprofloxacin MIC, 5.96 μg/ml) were used as controls (9). Norfloxacin accumulation in SA-1199B is significantly reduced compared with that in SA-1199 because of active efflux of the drug (9). The modified fluorescence method described by Mortimer and Piddock (11) was used, with the final norfloxacin concentration being 20 μg/ml. The fluorescences of the cells were measured with a spectrofluorometer at excitation and emission wavelengths of 277 and 440 nm, respectively, and the data were expressed as nanograms of norfloxacin per milligram of protein (Fig. 1). As in the control strains, norfloxacin accumulation in the resistant strain BM4627 was significantly reduced compared with that in the susceptible strain BM4626. Resistant strains BM4629 and BM4631 accumulated norfloxacin similarly to their respective susceptible counterparts BM4628 and BM4630 (data not shown). Resistance in SA-1199B is associated with enhanced activity of a chromosomally encoded protein, NorA, which actively exports fluoroquinolones from the cells, in particular, hydrophilic molecules (9). Protein NorA and the closely related *Bacillus subtilis* protein Bmr are multidrug efflux transporters, and their activities are inhibited by the plant alkaloid reserpine (12). At a concentration which does not affect bacterial growth (20 μg/ml), reserpine reduces markedly the MIC of norfloxacin for SA-1199B (eightfold), whereas that for

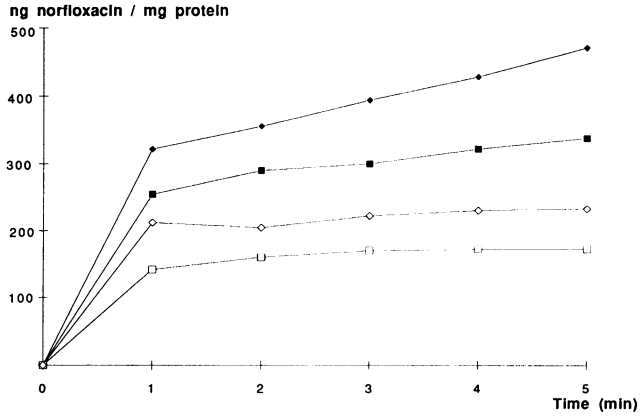

FIG. 1. Uptake of norfloxacin (20 μg/ml) by whole cells of *S. aureus* strains. Results are means of three separate experiments. ■, SA-1199; □, SA-1199B; ◆, BM4626; ◇, BM4627.

SA1199 is reduced only twofold (9). Similar results were obtained for strains BM4627 and BM4626 (Table 1). This suggests that a NorA-related efflux mechanism is involved in the reduced norfloxacin uptake of strain BM4627.

In conclusion, a minimum of two mechanisms account for high-level (64-fold increase in the ciprofloxacin MIC) fluoroquinolone resistance in strain BM4627: a mutation of the *gyrA* gene at position 251 resulting in a Ser-to-Leu substitution at position 84 of the GyrA protein and reduced accumulation of the drugs, probably involving NorA. So far, mutational alterations of GyrA have been found only in high-level fluoroquinolone-resistant strains of *S. aureus*. However, spontaneous high-level-resistant mutants of *S. aureus* cannot be selected in a single step (1, 8, 19). Recently, a *gyrA* mutation similar to that present in BM4627 was found in second-step but not in first-step mutants of *S. aureus* obtained in vitro (8). It was proposed that this *gyrA* mutation may occur only in strains of *S. aureus* that have already undergone another mutation. Our results indicate that the sequential acquisition of fluoroquinolone resistance in *S. aureus* also occurs in vivo.

The mechanism responsible for the low-level quinolone resistance of strains BM4629 and BM4631 remains unexplained. The reduction in the novobiocin MIC for strain BM4631 was slight (twofold) but reproducible compared with that for the parent strain. This observation suggests the presence of a *flqA* mutation, since it is known that mutations in this locus render the cells more susceptible to novobiocin (20). Mutations in the nonsequenced regions of the genes for the DNA gyrase or drug impermeation not detected by the norfloxacin uptake experiment are also possible.

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