Sequential Acquisition of Norfloxacin and Ofloxacin Resistance by Methicillin-Resistant and -Susceptible *Staphylococcus aureus*

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Received 19 April 1993/Returned for modification 1 June 1993/Accepted 16 August 1993

The acquisition of ofloxacin resistance by a susceptible clinical *Staphylococcus aureus* strain was found to be achieved in two sequential steps: the first step was accompanied by 4-fold increases in the ofloxacin MIC and 8- to 16-fold increases in the norfloxacin MIC. The second step was accompanied by further increases in both the ofloxacin and the norfloxacin MICs. A mutation of the *gyrA* gene resulting in an amino acid substitution was found in the second-step but not in the first-step resistant subclone. On the other hand, there was no difference in the accumulation of norfloxacin in the parent strain and the resistant subclones of each step. The rates of mutation to resistance in the steps were $(1.58$ to $6.81) \times 10^{-7}$ and $(0.71$ to $2.59) \times 10^{-9}$, respectively, and did not depend on whether the parent strain was resistant to methicillin. Some implications of these observations for clinical as well as mechanistic aspects of the prevalence of methicillin- and ofloxacin-resistant *S. aureus* are discussed.

Oflxacin-resistant isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) have become prevalent in the last few years (15, 16). In a recent survey of 13 medical facilities in Japan, ofloxacin-resistant isolates represented more than 64% of the MRSA isolates in 1990 (4). These isolates were characterized by high-level resistance to β-lactam antibiotics (methicillin MIC, ≥100; imipenem MIC, ≥25) as well as to tobramycin (11) and by their production of type II coagulase (22). In contrast, the majority of the population of methicillin-susceptible *S. aureus* (MSSA) still retained susceptibility to ofloxacin (MIC, ≤1.56). This prevalence of quinolone resistance in MRSA rather than in MSSA has been reported by other authors (1, 15, 16). In consideration of the low in vitro frequency rates (on the order of less than $10^{-12}$) that have been reported for ofloxacin resistance in *S. aureus* (3, 17), the recent clinical prevalence of ofloxacin-resistant MRSA is difficult to understand. In this study, clinical *S. aureus* strains isolated from 11 medical facilities throughout Japan in 1987 (the year in which ofloxacin-resistant MRSA began to be isolated) were analyzed for susceptibility to β-lactams as well as to the newer quinolone antibiotics. With these clinical strains, the rates of appearance of ofloxacin-resistant subclones and their mechanism of resistance were studied.

**MATERIALS AND METHODS**

**Bacterial strains.** A total of 362 clinical *S. aureus* strains, including 129 MRSA, were studied and tested for susceptibility to norfloxacin, ofloxacin, and methicillin. The 190 clinical strains collected from 10 Japanese medical centers in 1981 to 1983 included 33 MRSA strains. The remaining 172 strains were collected from 11 medical centers in 1987 and included 96 MRSA strains. All the clinical strains were isolated from different patients.

**MIC determinations.** MICs of β-lactams as well as of norfloxacin and ofloxacin were determined by the plate dilution method recommended by the Japanese Society of Chemotherapy as previously described (5). An inoculum of $1 \times 10^8$ to $5 \times 10^8$ cells was spotted on the antibiotic-containing Mueller-Hinton agar plates (Difco), and cell growth was evaluated after 24 h of incubation at 35°C. FDA209P was used as a control strain.

**Evaluation of mutation rates.** An inoculum of fewer than 100 cells was added to 1 ml of heart infusion (HI) broth (Difco) in 50 culture tubes and incubated at 37°C for 18 h. Portion of the resulting cultures were spread onto HI agar plates (Difco) containing 3.13 µg of norfloxacin per ml. Colonies appearing after 48 h of incubation at 37°C were enumerated, and clones were designated first-step mutants. First-step mutants were subjected to the second selection. In the same manner as that described above, portions of bacterial cultures of first-step mutants were spread on HI agar plates containing 3.13 µg of ofloxacin per ml. After incubation at 37°C for 48 h, colonies were enumerated and clones were designated second-step mutants. The mutation rates were calculated according to the formula of Luria and Delbrück (9).

**Measurement of accumulation of norfloxacin in bacterial cells.** The accumulation of norfloxacin in bacterial cells was measured by the method of Yoshida et al. (24), with slight modifications. The bacteria were grown to the late logarithmic phase ($A_{570} = 0.65$) in antibiotic medium 3 (Difco), and norfloxacin was added to the bacterial culture at a final concentration of 10 µg/ml. The culture was incubated at 37°C with intensive shaking. In some experiments, an energy inhibitor, carbonyl cyanide m-chlorophenylhydrazone (CCCP; Sigma), was added to the culture to a final concentration of 100 µM 11 min after the addition of norfloxacin. At 5, 10, 20, and 30 min after the addition of norfloxacin, 40-ml portions of the culture were taken, divided into four aliquots of 10 ml each, and immediately chilled on ice. The cells were collected by centrifugation and washed once with 5 ml of ice-cold saline. The cells were then resuspended in 1 ml of 5% acetic acid solution and boiled for 7 min to extract norfloxacin from the cells. After centrifugation at 15,000 × g
for 10 min, the concentration of norfloxacin in the supernatant was measured by high-performance liquid chromatography (24).

Nucleotide sequence analysis of the gyrA genes from quinolone-susceptible and -resistant strains. Polymerase chain reaction (PCR) amplification of part of the gyrA gene from quinolone-susceptible clinical strains and their corresponding resistant mutants was performed with Ampli-Taq (Perkin-Elmer Cetus) in a 100-µl reaction mixture (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl2, 0.001% [wt/vol] gelatin, 200 µM each deoxynucleoside triphosphate, 1.0 µM each of two primers, template DNA). Synthetic oligonucleotides used as primers were 5'-GAAACAACAAT GAACCTGA-3' and 5'-GTGTGATTITTAGCTACGCC-3', which corresponded to the nucleotide positions 2088 to 2107 and to the complementary nucleotides from positions 2660 to 2679, respectively (10). Nucleotide sequence analysis of the PCR products was performed with an automated sequencer (ABI; Applied Biosystems) by the dideoxynucleotide chain termination method of Sanger et al. (14).

RFLP analysis. For restriction fragment length polymorphism (RFLP) analysis, a 592-bp gyrA fragment was amplified from each subclone by PCR. The synthetic oligonucleotide primers used for PCR corresponded to nucleotide positions 2088 to 2107 and to complementary nucleotide positions 2660 to 2679 of the reported S. aureus gyrB and gyrA genes, respectively (10). The PCR products were digested with HinfI and subjected to electrophoresis in a 6% acrylamide gel. A mutation at nucleotide position 251 of the gyrA gene abrogates one of the two HinfI sites and results in an amino acid substitution at Ser-84 in the GyrA protein (19).

RESULTS

Norfloxacin and ofloxacin resistance of clinical S. aureus strains. Table 1 shows the distribution of the clinical S. aureus strains collected in 1987 according to ofloxacin and methicillin MICs. It was evident that there was a higher frequency of ofloxacin resistance (MIC, ≥6.25) in MRSA (methicillin MIC, ≥12.5) than in MSSA; this result was consistent with previous reports of other authors (1, 15, 16).

Figure 1 shows the pattern for norfloxacin and ofloxacin resistance of each MRSA strain plotted against ofloxacin and norfloxacin MICs. The norfloxacin-resistant (MIC, ≥25) MRSA population consisted of strains with various degrees of susceptibility to ofloxacin, from a borderline level (MIC, 1.56 to 3.13) to a moderate level (MIC, 6.25 to 12.5) or a high level (MIC, ≥25) of resistance. No population of strains was resistant to ofloxacin and susceptible to norfloxacin. This pattern of distribution of quinolone resistance indicated that

| Table 1. Methicillin and ofloxacin MICs for clinical S. aureus strains isolated in 1987 |
|-----------------------------------------------|
| Ofloxacin MIC (µg/ml) | No. of isolates for which the methicillin MIC (µg/ml) was: |
|------------------------|----------------------------------------------------------|
| 50                     | 0.39 0.78 1.56 3.13 6.25 12.5 25 50 100 ≥100           |
| 25                     | 2 1 2 1 2 1 4 4 4                                   |
| 12.5                   | 4 1 2 1 1 2 9 9 9                                   |
| 6.25                   | 1 2 1 1 1 1 1 1 1                                   |
| 3.13                   | 1 1 1 1 1 1 1 1 1                                   |
| 1.56                   | 1 1 1 1 1 1 1 1 1                                   |
| 0.78                   | 1 1 1 1 1 1 1 1 1                                   |
| 0.39                   | 1 1 1 1 1 1 1 1 1                                   |

FIG. 1. Correlation of ofloxacin and norfloxacin susceptibility in 172 clinical S. aureus strains isolated in 1987. A total of 172 strains were plotted against norfloxacin and ofloxacin MICs. The numeral at each coordinate is the number of strains for which the quinolone MIC was as indicated. Norfloxacin-resistant isolates had various degrees of resistance to ofloxacin, up to a borderline level (MIC, 1.56 to 3.13) to high level (MIC, ≥50). No norfloxacin-resistant isolate remained susceptible to norfloxacin.

ofloxacin resistance might have been derived from norfloxacin-resistant clinical S. aureus strains.

Sequential acquisition of ofloxacin and ofloxacin resistance in S. aureus. Five strains each were selected from the MRSA and MSSA populations of the clinical S. aureus strains described above on the basis of ofloxacin and ofloxacin MICs of 1.56 and 0.39 µg/ml, respectively. Fewer than 100 cells of each strain were inoculated into 3 ml of L broth and cultivated for 18 h at 37°C. A portion of the culture was spread on HI agar plates containing various concentrations of ofloxacin and ofloxacin. After 48 h of incubation at 37°C, spontaneous resistant mutants that grew on plates containing up to 6.25 µg of ofloxcin per ml appeared at frequencies of (0.24 to 211.25) × 10−8 (first-step mutants) (Table 2). In contrast, even when 5.26 × 1010 CFU of the parent strains was plated, no spontaneous mutant appeared on plates containing more than 1.56 µg of ofloxacin per ml (frequency, <1.9 × 10−11). The MICs of ofloxacin and ofloxacin for these first-step mutants were 12.5 to 25 µg/ml and 1.56 µg/ml, respectively. The first-step mutants derived from the clinical strains were designated by the name of the clinical strain followed by the suffix “-S” (for first-step mutant). One representative resistant mutant for each strain was picked and diluted, and fewer than 100 cells were inoculated into drug-free L broth and cultured for 18 h at 37°C. Then, a portion of the culture was spread on HI agar plates containing various concentrations of ofloxacin. Spontaneous mutants that grew on plates containing up to 3.13 µg of ofloxacin per ml appeared at frequencies of (0.2 to 7.11) × 10−8 (second-step mutants) (Table 3). The second-step mutants derived from the first-step mutants were designated by the name of the clinical strain followed by the suffix “-S” (for second-step mutant). The MICs of ofloxacin and ofloxacin for these second-step mutants were 25 to 100 µg/ml and 6.25 to 12.5 µg/ml, respectively. Therefore, a significant increase in ofloxacin resistance was obtained by sequentially exposing cells to norfloxacin and ofloxacin but not directly from exposure to ofloxacin. As this result could have been
TABLE 2. Appearance of norfloxacin-resistant mutants (first-step mutants)

| Strains | No. of bacteria/ml (10^9) | No. of mutants selected with a norfloxacin concn (µg/ml) of: | No. of mutants selected with an ofloxacin concn (µg/ml) of: | Rate of appearance of first-step mutants (10^{-8}) |
|---------|---------------------------|-------------------------------------------------|-------------------------------------------------|----------------------------------|
|         |                           | 3.13 | 6.25 | 12.5 | 25   | 0.39 | 0.78 | 1.56 |
| MS5935  | 8.8                       | 27   | 27   | 1    | 0    | >1,000 | 126 | 0 | 3.07 |
| MS5952  | 9.6                       | 74   | 4    | 4    | 0    | >1,000 | 332 | 0 | 0.42 |
| MS5977  | 11.0                      | 21   | 4    | 3    | 0    | >1,000 | 43  | 0 | 0.36 |
| MS5986  | 3.4                       | 7    | 2    | 0    | 0    | >1,000 | 54  | 0 | 0.59 |
| MS6001  | 4.2                       | 29   | 1    | 1    | 0    | >1,000 | 4   | 0 | 0.24 |
| MR5845  | 7.6                       | 40   | 4    | 1    | 0    | >1,000 | 152 | 0 | 0.53 |
| MR5867  | 2.4                       | 665  | 507  | 168  | 0    | >1,000 | 668 | 0 | 211.25 |
| MR5924  | 4.5                       | 24   | 24   | 13   | 0    | >1,000 | 17  | 0 | 5.33 |
| MR5982  | 6.2                       | 115  | 22   | 1    | 0    | >1,000 | 212 | 0 | 3.55 |
| MR6009  | 10.0                      | 109  | 25   | 1    | 0    | >1,000 | 196 | 0 | 2.50 |

a A total of 0.3 ml of a culture was spread on an HI agar plate containing each selective concentration of the new quinolone antibiotics.
b Calculated on the basis of the number of mutants selected with 6.25 µg of norfloxacin per ml.

due to a relatively stronger bactericidal activity of ofloxacin than of norfloxacin, we tried to select mutants by spreading 10^{10} CFU of the parent strains on HI agar plates containing a low concentration (1.56 µg/ml) of ofloxacin. Mutants were obtained at frequencies of (0.19 to 4.26) × 10^{-9} (Ofx' mutants). A total of 13 Ofx' mutants derived from two MRSA and two MSSA parent strains were tested for susceptibility to norfloxacin and ofloxacin. The MICs for the Ofx' mutants thus obtained were 12.5 to 25 µg/ml for norfloxacin and 1.56 µg/ml for ofloxacin and were the same as those for the first-step mutants. Therefore, we concluded that ofloxacin resistance cannot be achieved directly from S. aureus strains that are susceptible to both norfloxacin and ofloxacin. In our survey of 190 clinical S. aureus strains (including 33 MRSA strains) isolated from 10 Japanese medical centers from 1981 to 1983, norfloxacin-resistant clinical strains for which the norfloxacin and ofloxacin MICs were the same as the MICs for the first-step mutants were found to represent 8 and 21% of the MSSA and MRSA, respectively. One MSSA and eight MRSA strains were chosen from these norfloxacin-resistant populations and evaluated for frequencies for selection to ofloxacin resistance. Ofloxacin-resistant mutants that could grow on HI agar plates containing 3.13 µg of ofloxacin per ml were obtained at frequencies of (0.11 to 8.66) × 10^{-8} from five MRSA strains. This result strongly supports the view that the ofloxacin-resistant clinical S. aureus strains were derived from the norfloxacin-resistant S. aureus strains (equivalent to the first-step mutants in this study) at a relatively high frequency.

Estimation of mutation rates in the emergence of norfloxacin- and ofloxacin-resistant mutants from MRSA and MSSA strains. Fewer than 100 cells of each parent strain were inoculated into 1 ml of HI broth in 50 culture tubes and cultivated for 18 h at 37°C. A total volume of 0.3 ml from each of the cultures was spread on HI agar plates containing

TABLE 3. Appearance of ofloxacin-resistant mutants (second-step mutants)

| Strains | No. of bacteria/ml (10^9) | No. of mutants selected with a norfloxacin concn (µg/ml) of: | No. of mutants selected with an ofloxacin concn (µg/ml) of: | Rate of appearance of second-step mutants (10^{-8}) |
|---------|---------------------------|-------------------------------------------------|-------------------------------------------------|----------------------------------|
|         |                           | 12.5 | 25 | 50 | 100 | 1.56 | 3.13 | 6.25 |
| MS5935-F | 2.9                       | >1,000 | 6 | 0 | 0 | >1,000 | 5 | 7 | 1.72 |
| MS5935-F | 7.1                       | >1,000 | 171 | 0 | 0 | >1,000 | 4 | 0 | 0.78 |
| MS5952-F | 5.1                       | >1,000 | 9 | 0 | 0 | >1,000 | 9 | 2 | 2.25 |
| MS5952-F | 4.1                       | >1,000 | 16 | 0 | 0 | >1,000 | 0 | 0 | <0.20 |
| MS5977-F | 3.1                       | >1,000 | >1,000 | 0 | 0 | >1,000 | 7 | 0 | 2.56 |
| MS5977-F | 5.0                       | >1,000 | 19 | 1 | 0 | >1,000 | 9 | 5 | 1.45 |
| MS5977-F | 6.2                       | >1,000 | >1,000 | 2 | 1 | >1,000 | 37 | 4 | 6.98 |
| MS6001-F | 5.3                       | >1,000 | >1,000 | 2 | 1 | >1,000 | 0 | 0 | <0.40 |
| MR5845-F | 2.5                       | >1,000 | 0 | 0 | 0 | >1,000 | 5 | 1 | 1.39 |
| MR5867-F | 2.4                       | >1,000 | 1 | 0 | 0 | >1,000 | 1 | 1 | 0.42 |
| MR5924-F | 7.7                       | >1,000 | 19 | 160 | 0 | >1,000 | 8 | 4 | 0.91 |
| MR5924-F | 2.0                       | >1,000 | 14 | 1 | 0 | >1,000 | 26 | 0 | 2.60 |
| MR5982-F | 4.5                       | >1,000 | >1,000 | 83 | 0 | >1,000 | 32 | 14 | 7.11 |
| MR5982-F | 6.9                       | >1,000 | >1,000 | 83 | 0 | >1,000 | 0 | 0 | <0.14 |
| MR6009-F | 6.6                       | >1,000 | >1,000 | 83 | 0 | >1,000 | 0 | 0 | <0.15 |

a A total of 0.3 ml of a culture was spread on an HI agar plate containing each selective concentration of the new quinolone antibiotics.
b Calculated on the basis of the number of mutants selected with 3.13 µg of ofloxacin per ml.
TABLE 4. Mutation rates in the emergence of the first-step mutants

| Strain   | No. of cultures (C) | Mean no. of resistant mutants isolated/culture tube (r) | Variance (var)* | No. of bacteria/culture (10^9) (NN) | Mutation rate (10^-4) (a) |
|----------|---------------------|--------------------------------------------------------|----------------|-------------------------------------|--------------------------|
| MS5935   | 49                  | 68.37                                                  | 5,807.5        | 3.24                                | 3.36                     |
| MS5952   | 50                  | 101.87                                                 | 11,749.0       | 6.03                                | 2.54                     |
| MS5977   | 49                  | 130.75                                                 | 17,920.0       | 5.78                                | 3.31                     |
| MS5986   | 50                  | 13.33                                                  | 367.5          | 1.41                                | 1.92                     |
| MS6001   | 50                  | 58.13                                                  | 4,454.9        | 2.63                                | 3.59                     |
| MR5845   | 100                 | 27.73                                                  | 2,054.6        | 2.87                                | 1.58                     |
| MR5867   | 50                  | 34.07                                                  | 1,897.2        | 2.53                                | 2.54                     |
| MR5924   | 50                  | 38.80                                                  | 2,028.4        | 0.98                                | 6.82                     |
| MR5982   | 50                  | 96.47                                                  | 10,702.3       | 2.80                                | 5.23                     |
| MR6009   | 50                  | 69.60                                                  | 6,075.3        | 3.40                                | 3.24                     |

* Calculated according to the Luria-Delbrück formula (9): r = aNln(NtCa). The selective concentration of norfloxacin was 3.13 μg/ml.

# Calculated according to the formula var = rNtCa/NtCa.

3.13 μg of norfloxacin per ml. The mutation rates for this first-step acquisition of norfloxacin resistance were calculated to be (1.58 to 6.81) × 10^-9, and there was no appreciable difference between MRSA and MSSA (Table 4). In the same manner as for the first-step mutation, a portion of each of the 50 cultures of the first-step mutants was spread on HI agar plates containing 3.13 μg of ofloxacin per ml. The mutation rates for the second-step mutants were calculated to be (0.71 to 2.59) × 10^-9 (Table 5). The variance in each experiment was far greater than unity, confirming that the acquisition of resistance to norfloxacin as well as to ofloxacin was caused by spontaneous mutation and not by drug-induced adaptation (9).

**Evolution of cell-associated norfloxacin accumulation.** The NorA protein is considered a component of the presumptive efflux pump of hydrophilic quinolones (23). To study a possible role of this efflux pump in the two-step resistance acquisition, the cell-associated concentration of norfloxacin was measured with MRSA strain MR5982, MSSA strain MS5977, and their first- and second-step mutants. A standard laboratory *S. aureus* strain, FDA209P, was used as a control strain. The quantity of cell-associated norfloxacin incubated in antibiotic medium 3 in the absence of CCCP was approximately 0.2 μg/mg of bacterial cells with FDA 209P, whereas it was approximately 0.02 μg/mg of bacterial cells with MR5982, MS5977, and their first- and second-step mutants (Fig. 2). The difference in norfloxacin accumulation between FDA209P and the clinical strains was abolished by the addition of CCCP, which disturbs the proton motive force; the levels of norfloxacin associated with all the strains were the same (0.541 ± 0.041 and 0.332 ± 0.014 μg/ml in experiments A and B, respectively). The same pattern of norfloxacin accumulation was observed with the other two isogenic sets of parent and mutant strains as well (data not shown). Despite the 10-fold-higher concentration of norfloxacin associated with FDA209P than with the clinical strains, the MIC of norfloxacin for FDA209P was only 3-fold lower than those for the clinical strains. Also noteworthy was the fact that although the MICs of norfloxacin for the first-step quinolone-resistant mutants were more than 64-fold higher than those for their parent strains, there was no significant difference in the quantities of cell-associated norfloxacin.

**Nucleotide sequence analysis and RFLP analysis of the gyrA genes of the parent strains and mutants.** The partial nucleotide sequences corresponding to the nucleotides from positions 2283 to 2657 of the gyrA genes of the parent strains and their first- and second-step mutants were determined (10). A common point mutation, a transition from C to T at nucleotide 251 of the gyrA gene, which should result in an amino acid substitution from Ser to Leu at amino acid 84 of the GyrA protein, was found in the second-step mutants. This specific base substitution in gyrA was observed in the second- but not in the first-step mutants or in the Ofr' mutants. In other words, in the first-step mutants, regardless of selection by norfloxacin or ofloxacin, a mutation at nucleotide position 251 of the gyrA gene was not found. This point mutation in the second-step mutants was identical to that previously reported for ciprofloxacin-resistant clinical

| Strain   | No. of cultures (C) | Mean no. of resistant mutants isolated/culture tube (r) | Variance (var)* | No. of bacteria/culture (10^9) (NN) | Mutation rate (10^-4) (a) |
|----------|---------------------|--------------------------------------------------------|----------------|-------------------------------------|--------------------------|
| MS5935-F | 49                  | 21.70                                                  | 821.0          | 1.58                                | 2.59                     |
| MS5952-F | 50                  | 48.60                                                  | 3,276.9        | 4.35                                | 1.86                     |
| MS5977-F | 50                  | 20.07                                                  | 729.5          | 3.29                                | 1.16                     |
| MS5986-F | 50                  | 13.47                                                  | 733.5          | 2.66                                | 2.35                     |
| MS6001-F | 50                  | 9.00                                                   | 240.1          | 3.66                                | 0.71                     |
| MR5845-F | 50                  | 13.73                                                  | 386.9          | 1.74                                | 1.60                     |
| MR5867-F | 50                  | 30.07                                                  | 1,445.1        | 3.68                                | 1.46                     |
| MR5924-F | 50                  | 18.53                                                  | 639.6          | 1.69                                | 2.12                     |
| MR5982-F | 50                  | 42.67                                                  | 2,712.7        | 3.25                                | 2.32                     |
| MR6009-F | 50                  | 14.65                                                  | 431.1          | 2.67                                | 1.10                     |

* Calculated in the same manner as for the first-step mutants (see Table 4, footnote a). The selective concentration of ofloxacin was 3.13 μg/ml.

* See Table 4, footnote b.
S. aureus isolates by Sreedharan et al. (18). As they reported that the \( \text{Hinfl} \) restriction site located at nucleotide position 251 is abolished by the C-to-T mutation, a \( \text{Hinfl} \) RFLP analysis was performed with all of the first- and second-step mutants obtained in this study. Representative data are shown in Fig. 3. According to the RFLP analysis of the \( gyra \) genes of the first- and second-step mutants derived from 10 clinical strains (5 MRSA and 5 MSSA), the mutation causing the loss of the \( \text{Hinfl} \) site was observed only in the second-step mutants. A total of 13 \( \text{Opx}^+ \) mutants obtained from 2 strains each of MRSA and MSSA, were also tested by \( \text{Hinfl} \) RFLP analysis; no strain with this \( gyra \) mutation was found. To further establish the role of the \( gyra \) mutation in clinical strains with ofloxacin resistance, 68 of 172 clinical \( S. aureus \) strains collected in 1987, including methicillin-resistant and

-susceptible populations with various degrees of ofloxacin resistance, were tested by \( \text{Hinfl} \) RFLP analysis. The loss of the \( \text{Hinfl} \) site was observed in 18 strains for which ofloxacin MICs were all over 12.5 \( \mu \text{g/ml} \); on the other hand, there was no loss of the \( \text{Hinfl} \) site in strains for which ofloxacin MICs were less than 6.25 \( \mu \text{g/ml} \) (data not shown). These results strongly indicate that the DNA gyrase A subunit mutation at nucleotide position 251 is responsible for resistance to ofloxacin not only in the second-step mutants in the in vitro study but also in the ofloxacin-resistant clinical strains.

**DISCUSSION**

Some investigations have indicated that resistance in vitro to ofloxacin is less frequently obtained than that to some other, newer quinolone antibiotics (3, 17). In fact, the mutation rates for the acquisition of resistance for ofloxacin-susceptible strains in vitro were reported to be less than \( 10^{-12} \) in one study (17). However, in the clinical experience, ofloxacin-resistant \( S. aureus \) has emerged very rapidly, and the majority of the ofloxacin-resistant strains have consisted mainly of MRSA (16). There has been no explanation for this discrepancy between the in vitro and clinical observations. As resistance to newer quinolones has been found in association with a mutation in the \( gyra \) gene (18) or the \( norA \) gene (23), it appears that MRSA carries a kind of “mutator gene,” as previously reported for *Escherichia coli* (20). To test this possibility, rates of mutation to rifampin resistance were evaluated with three strains each of MRSA and MSSA; they were \( (3.30 \text{ to } 6.20) \times 10^{-8} \) for MRSA and \( (3.72 \text{ to } 8.44) \times 10^{-8} \) for MSSA (4). Thus, there was no apparent difference between MRSA and MSSA in rates of mutation to rifampin resistance. Therefore, the carriage of a mutator gene by MRSA was considered a remote possibility.

In this study, we found that the acquisition of ofloxacin resistance in \( S. aureus \) was achieved rather frequently through two steps of genetic events. At first, we tried to determine the rates of appearance of ofloxacin- and ofloxacin-resistant mutants in each step to compare them between

**FIG. 2.** Accumulation of cell-associated ofloxacin in \( S. aureus \) strains and their resistant subclones. Each datum point represents the mean of four measurements of cell-associated ofloxacin. Symbols: (A) ●, FDA209P (ofloxacin MIC, 0.2); ○, MR5982 (MIC, 1.56); □, MR5982-F (MIC, 25); (B) ●, FDA209P; ○, MS5977 (MIC, 1.56); □, MS5977-F (MIC, 12.5); ■, MS5977-S (MIC, 50). CCCP was added at the time indicated by the arrow.

**FIG. 3.** \( \text{Hinfl} \) RFLP analysis of the \( gyra \) genes in the parent strains and first- and second-step mutants. Lanes: 1, MS5977; 2, MS5977-F (a first-step mutant of MS5977); 3, MS5977-S (a second-step mutant of MS5977); 4, uncut PCR product of MS5977-S. Molecular size markers are \( \text{Hinfl} \) digests of fox174 (Nippon Gene, Tokyo, Japan) and are indicated in kilobases to the left of the gel.
MRSA and MSSA. However, those data were not appropriate for a comparative evaluation because of an unacceptable level of variation in the data (Tables 2 and 3). As the number of resistant mutants in a series of similar cultures are considered to be distributed in accordance with Poisson's law, we proceeded to evaluate the mutation rates for resistance acquisition on the basis of the Luria-Delbrück formula (9). The mutation rates in both steps were on the order of $10^{-9}$, and there was no significant difference between MRSA and MSSA in both steps of resistance acquisition. On the basis of these data, the prevalence of ofloxacin-resistant clinical S. aureus strains could be explained by their rather frequent derivation from low-level quinolone-resistant counterparts among clinical S. aureus strains. The higher prevalence of ofloxacin-resistant strains in MRSA than in MSSA may reflect the higher prevalence of low-level quinolone-resistant strains in MRSA, from which the ofloxacin-resistant strains emerged. In Japan, the presence of more low-level quinolone-resistant strains in MRSA populations than in MSSA populations may be the result of the much more frequent therapeutic use of norfloxacin to treat MRSA infections than to treat MSSA infections.

Mutation of the gyrA gene seems to have a definite role in the ofloxacin resistance observed in this study. All of the second-step mutants that we studied harbored the common gyrA mutation, a point mutation at nucleotide position 251 in the gyrA gene that seems to be necessary for ofloxacin resistance. The observation that the point mutation in the gyrA gene was also found in all 18 clinical strains for which the MICs of ofloxacin were over $12.5 \mu g/ml$ supports this hypothesis. However, as our attempts to isolate subclones with this gyrA mutation directly from ofloxacin-susceptible strains failed, the point mutation, as a single event, may not be sufficient for the expression of ofloxacin resistance. The possibility exists that the acquisition of a single mutation at nucleotide position 251 in the gyrA gene is lethal to the cell. The mutation may introduce into the GyrA protein a strong conformational instability, which is unacceptable for the physiological function of the protein in DNA replication. A previous increase in mutation rate of one or another domain of the GyrA protein, the GyrB protein, or another, unknown gene product by the first-step resistance acquisition may be necessary first to counteract such an instability caused by a mutation at nucleotide position 251. A complete nucleotide sequence analysis of the gyrA and gyrB genes of the parent strains and first-step mutants will be necessary to further clarify this question.

There have been many reports suggesting that the reduction of the intracellular accumulation of quinolones may cause resistance to newer quinolones in S. aureus (2, 6, 8, 13, 23, 24). Kaat and Seo (7) and Neyfakh (12) suggested that the overexpression of the active efflux pump conferred fluoroquinolone resistance to S. aureus and Bacillus subtilis. However, there has been no study in which resistance acquisition and quinolone accumulation are directly correlated in defined sets of isogenic bacterial strains. Because of this issue, it has been difficult to evaluate whether the reduction in accumulated quinolones really contributes to quinolone resistance. In this study, using norfloxacin- and ofloxacin-resistant isogenic mutants derived from susceptible clinical strains, we compared the changes in the cell-associated norfloxacin concentrations before and after resistance acquisition. The amounts of cell-associated norfloxacin were not appreciably different between the parent strains and their corresponding resistant mutants. This result suggested that the mechanism of resistance to norfloxacin in the first-step mutants was not caused by a reduction in accumulated quinolone. It seems that the decreased level of accumulated norfloxacin is responsible for only a slight decrease in S. aureus norfloxacin susceptibility; the norfloxacin MICs were 0.2 for FDA209P and 1.56 for MS5977 and MR5982.

In view of the above-described results, the existence of a novel mechanism is suggested to explain quinolone resistance in the first-step mutants. Neither the gyrA gene mutation at nucleotide 251 nor the acceleration of quinolone efflux seems to be responsible. In this context, although an as-yet-undefined mutation of the gyrA or gyrB gene is a candidate, a newly identified locus, which determines fluoroquinolone resistance and is designated cfxB or cfxC, is also an attractive candidate. The cfxB or cfxC mutant reported by Trucksis et al. exhibits a level of ciprofloxacin resistance (MIC, 2) similar to that of the first-step quinolone-resistant mutants (MIC, 3.13) described in this study (21).

ACKNOWLEDGMENTS

We thank K. Asada for valuable technical assistance in DNA sequencing.

This work was supported by grant 03670222 from the Japanese Ministry of Education.

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