

Enrichment of Fluoroquinolone-Resistant *Staphylococcus aureus*: Oscillating Ciprofloxacin Concentrations Simulated at the Upper and Lower Portions of the Mutant Selection Window[∇]

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The time inside the mutant selection window (MSW), T_{MSW} , appears to be less predictive of the selection of fluoroquinolone-resistant *Staphylococcus aureus* than is the ratio of the area under the concentration-time curve (AUC) to the MIC. This observation might be attributed to the fact that T_{MSW} does not consider the actual position of simulated antibiotic concentrations inside the MSW, which also might influence the amplification of resistant mutants. To test this hypothesis, the enrichment of ciprofloxacin-resistant *S. aureus* was studied at ciprofloxacin (CIP) concentrations that oscillate near the mutant prevention concentration (MPC), i.e., closer to the top of the MSW (“upper case”), and closer to the MIC, i.e., at the lower limit of the MSW (“lower case”) at the same T_{MSW} . Two methicillin-resistant strains of *S. aureus*, ATCC 6538 and ATCC 43300 (MICs of 0.25 and 0.5 mg/liter, respectively, and MPCs of 4 and 2 mg/liter, respectively), were exposed to twice-daily CIP treatments for three consecutive days. With *S. aureus* ATCC 6538, the simulated ratios of the AUC at 24 h (AUC_{24}) to the MIC were 50 and 260 h (T_{MSW} 75% of the dosing interval). With *S. aureus* ATCC 43300, the simulated AUC_{24}/MIC s were 30 and 100 h (T_{MSW} 56%). With each organism, mutants resistant to CIP were enriched in an AUC_{24}/MIC -dependent manner: the higher the AUC_{24}/MIC ratio, the lower the growth on CIP-containing plates. For example, the area under the time-kill curve of mutants resistant to $4\times$ MIC of CIP in the upper case was three times smaller than that in the lower case for both *S. aureus* strains. Similar differences were seen at the higher ($8\times$ MIC) and lower ($2\times$ MIC) CIP concentrations. These data highlight differences in the selection of resistant *S. aureus*, depending on the position of simulated concentrations inside the MSW at a given T_{MSW} . This explains why T_{MSW} -based predictions of resistance are less accurate than those based on AUC/MIC and AUC/MPC .

Emerging bacterial resistance has stimulated a thorough evaluation of novel antibiotics, with a special emphasis on the abilities of these antibiotics to prevent the enrichment of resistant mutants. Over the last 5 years, this phenomenon has been studied intensively in vitro by using dynamic models; the results of these studies have recently been reviewed and discussed in detail elsewhere (9). Previously reported complex relationships between the enrichment of resistant mutants and in vitro-simulated pharmacokinetics of fluoroquinolones (5–7, 16) and glycopeptides (8) support the hypothesis of the mutant selection window (MSW) (14). Based on these studies, both mutant selection and the concomitant loss in susceptibility depend on the simulated ratio of area under the curve (AUC) to the MIC in a bell-shaped manner. Together with AUC/MIC , the time inside MSW (T_{MSW}) has been proposed as a predictor of resistance (5), although it appeared to be less predictive of the selection of fluoroquinolone-resistant and, especially, glycopeptide-resistant *Staphylococcus aureus* than was the $AUC/$

MIC ratio (1, 6–8, 12). This observation might be attributed to the fact that T_{MSW} does not consider the position of simulated antibiotic concentrations within the MSW, which also might influence the amplification of resistant mutants (15).

To test this hypothesis, the enrichment of ciprofloxacin-resistant *S. aureus* was studied at ciprofloxacin concentrations oscillating around the mutant prevention concentration (MPC), i.e., closer to the top of the MSW (“upper case”) and closer to the MIC, i.e., at the lower boundary of the MSW (“lower case”) over the same T_{MSW} . To make this comparison more comprehensive, *S. aureus* strains with distinctly different MPC/MIC ratios were chosen.

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MATERIALS AND METHODS

Antimicrobial agents, bacterial strains, and susceptibility testing. Ciprofloxacin powder was kindly provided by Bayer Corporation (West Haven, CT). Two methicillin-resistant strains of *S. aureus* were selected for the study, *S. aureus* ATCC 6538 and *S. aureus* ATCC 43300. The MICs were determined prior to, during, and after a 3-day-simulated treatment with ciprofloxacin. Susceptibility testing was performed in triplicate by broth microdilution techniques at 24 h postexposure, with the organism grown in Ca^{2+} - and Mg^{2+} -supplemented Mueller-Hinton broth (MHB) at an inoculum size of 5×10^5 CFU/ml. The cipro-

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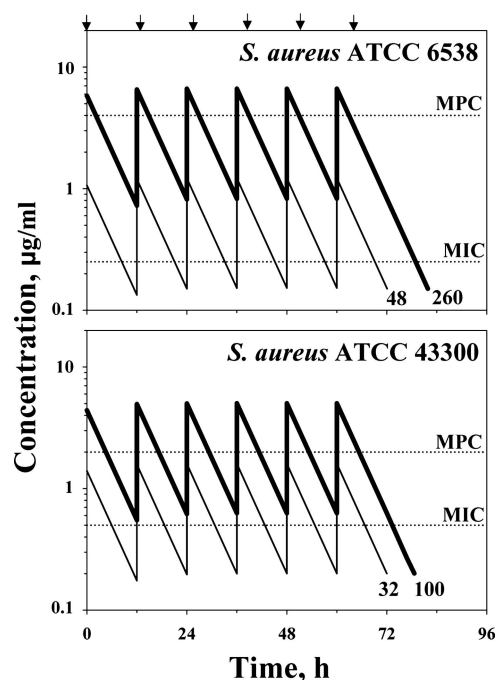


FIG. 1. Pharmacokinetic profiles of ciprofloxacin simulated in studies with two strains of *S. aureus*. Lower case CIP concentrations are indicated by thin lines and upper case CIP concentrations are indicated by bold lines. Arrows reflect ciprofloxacin administration. The respective AUC_{24}/MIC ratio (h) is indicated by number at the end of each curve.

floxacin MICs for *S. aureus* ATCC 6538 and 43300 were 0.25 and 0.5 $\mu\text{g}/\text{ml}$, respectively.

The MPCs were determined as described elsewhere (14). Briefly, the tested microorganisms were cultured in MHB and incubated for 24 h. Then, the suspension was centrifuged ($4,000 \times g$ for 10 min) and resuspended in MHB to yield a concentration of 10^{10} CFU/ml. A series of agar plates containing known ciprofloxacin concentrations was then inoculated with $\sim 10^{10}$ CFU of *S. aureus*. The inoculated plates were incubated for 48 h at 37°C and screened visually for growth. To estimate the MPC, logarithms of bacterial numbers were plotted against ciprofloxacin concentrations. The MPC was taken as the point where the plot intersected the x axis, i.e., the lowest ciprofloxacin concentration that completely inhibited growth. The MPCs for *S. aureus* ATCC 6538 and ATCC 43300 were estimated at 4 and 2 $\mu\text{g}/\text{ml}$, respectively.

Thus, in terms of the MPC/MIC ratio, the two organisms differed over a fourfold range, from 4 (for *S. aureus* ATCC 43300) to 16 (for *S. aureus* ATCC 6538).

Simulated pharmacokinetic profiles. A series of monoexponential profiles that mimic twice-daily dosing of ciprofloxacin was simulated with a half-life ($T_{1/2}$) of 4 h. The simulated $T_{1/2}$ s represented weighted means of the values reported for humans: 3.2 to 5.0 h (13).

To ensure the selection of resistant mutants that can occur at T_{MSW} s of more than 20 to 50% of the dosing interval (1, 5), the simulated T_{MSW} s were designed to be more than 50%. To provide the same T_{MSW} s for the lower and upper cases, simulated ratios of 24-h AUC (AUC_{24}) to MIC were designed at 50 h (lower case) and 260 h (upper case) with *S. aureus* ATCC 6538 (T_{MSW} 75% of the dosing interval) and at 30 h (lower case) and 100 h (upper case) with *S. aureus* ATCC 43300 (T_{MSW} 56%). Simulated concentration-time profiles are shown in Fig. 1.

In vitro dynamic model. A previously described dynamic model (3) was used in the study. Briefly, the model consisted of two connected flasks (one containing fresh MHB and the other with a magnetic stirrer, the central unit), with the same broth containing either a bacterial culture alone (control growth experiments) or a bacterial culture plus antibiotic (killing/regrowth experiments). Peristaltic pumps circulated fresh nutrient medium to the flasks and from the central 60-ml unit at a flow rate of 10.4 ml/h. The clearance provided by this flow rate, plus the

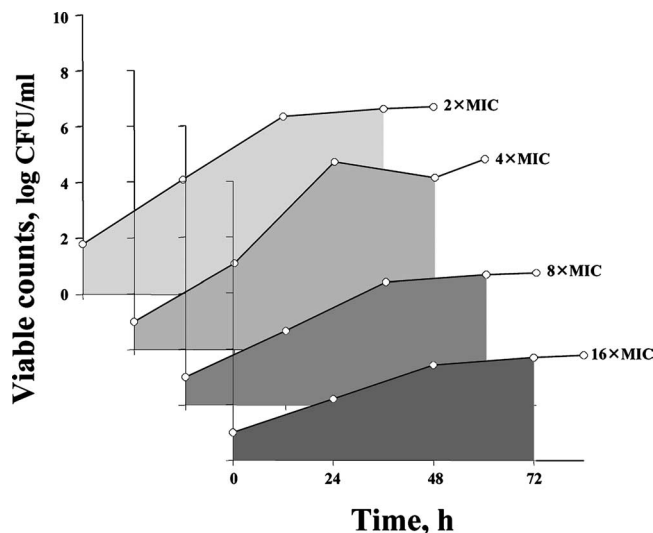


FIG. 2. $AUBC_M$ analysis of the time-kill curves with mutants grown on plates containing ciprofloxacin (*S. aureus* ATCC 43300 exposed to the higher ciprofloxacin concentrations [upper case]). Shaded areas reflect $AUBC_{MS}$.

volume of the central unit, ensured monoexponential elimination of ciprofloxacin bacteria from the system at an elimination rate constant of 0.17 h^{-1} .

The system was filled with sterile MHB and placed in an incubator at 37°C . The central unit was inoculated with an 18-h culture of *S. aureus*. After a 0.5-h incubation of the bacteria, the resulting exponentially growing cultures reached approximately 10^8 CFU/ml (6×10^9 CFU per 60-ml central compartment) and ciprofloxacin was injected into the central unit. The duration of the experiment was defined in each case as the time after the last dose when ciprofloxacin-exposed bacteria reached the maximum numbers observed in the absence of antibiotic (10^8 CFU/ml). All experiments were performed at least in duplicate within a 2-week interval. The reliability of ciprofloxacin pharmacokinetic simulations and the high reproducibility of the time-kill curves provided by the model have previously been reported (4).

Quantitation of the antimicrobial effect and susceptibility changes. In each experiment, multiple sampling of bacteria-containing medium from the central compartment was performed throughout the observation period. One-hundred-microliter samples were serially diluted as appropriate and plated onto Mueller-Hinton agar plates containing 0x, 2x, 4x, 8x, and 16x MIC of ciprofloxacin. The lower limit of accurate detection was 200 CFU/ml.

The cumulative effect of each simulated treatment on the susceptible *S. aureus* subpopulations was expressed by area under the time-kill curve ($AUBC$ [3]) measured from time zero to 72 h. A similar analysis of the time courses of mutants grown on ciprofloxacin-containing plates allowed calculation of the $AUBC$ ($AUBC_M$) over the same time interval. An example of the $AUBC_M$ determination is shown in Fig. 2.

To reveal changes in susceptibility, ciprofloxacin MICs of bacterial cultures sampled from the model were determined 24, 48, and 72 h after beginning treatment and at the end of the observation period if it was longer than 72 h. The final MIC (MIC_{final}) was then related to the initial value (MIC_{initial}).

RESULTS

Time courses of ciprofloxacin-exposed *S. aureus* ATCC 6538 and 43300 grown on antibiotic-free and antibiotic-containing medium are shown in Fig. 3. With each organism, both the extent of killing of the susceptible subpopulation and the time to regrowth (Fig. 3) increased with an increase of the simulated AUC_{24}/MIC ratio. A 5-fold increase in the simulated AUC_{24}/MIC (from 50 to 260 h) led to a 1.7-fold decrease in the $AUBC$ and, by that, to the same increase in the cumulative effect of ciprofloxacin on *S. aureus* ATCC 6538. With *S. aureus* ATCC

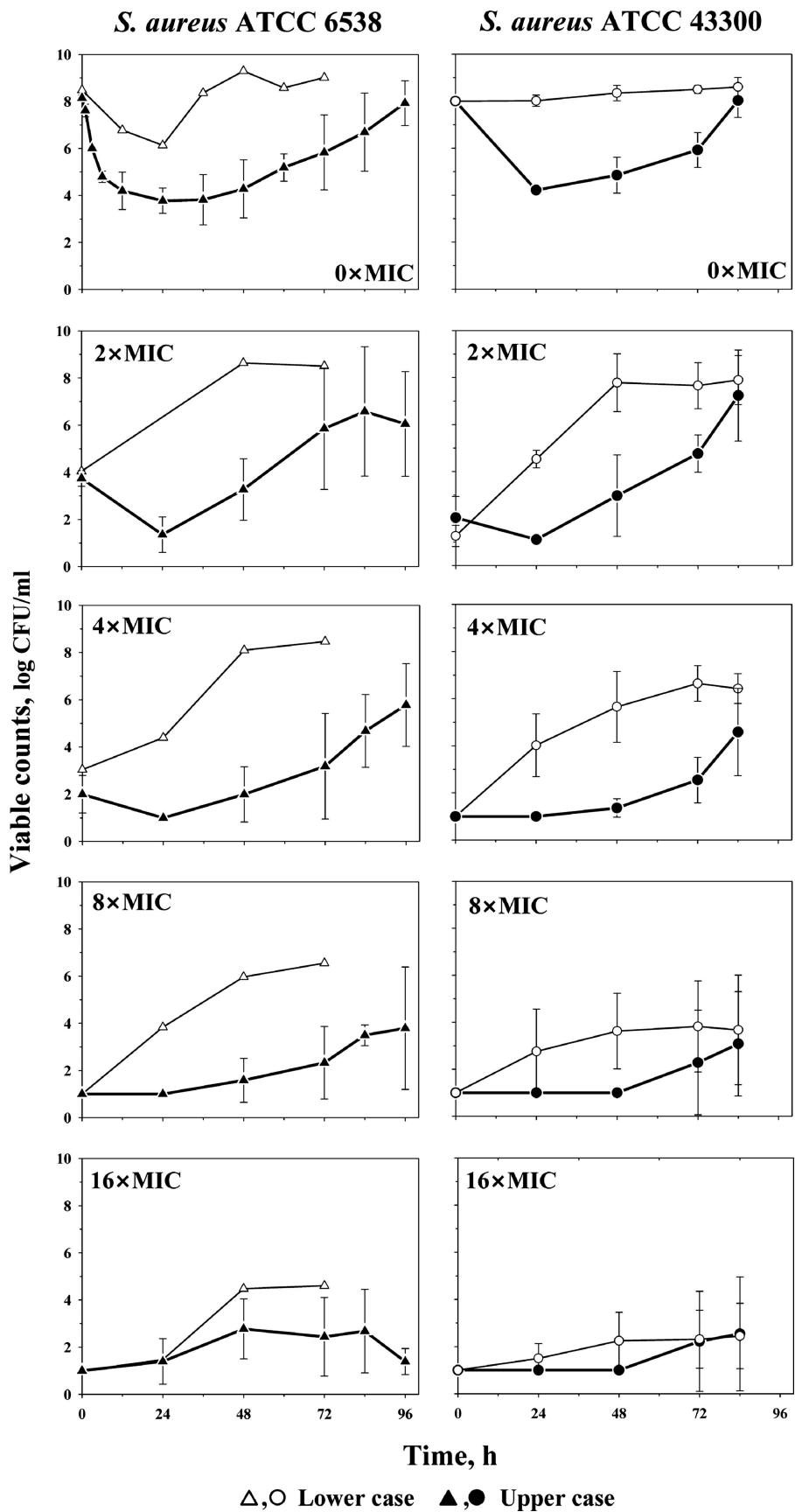


FIG. 3. Time courses of ciprofloxacin-exposed *S. aureus* ATCC 6538 and 43300 indicated by open symbols with thin lines (lower case) and filled symbols with bold lines (upper case). Total bacterial counts (0x MIC) and subpopulations resistant to ciprofloxacin (from 2x MIC to 16x MIC) in samples collected from the in vitro model. Results are expressed as means \pm standard deviations (error bars).

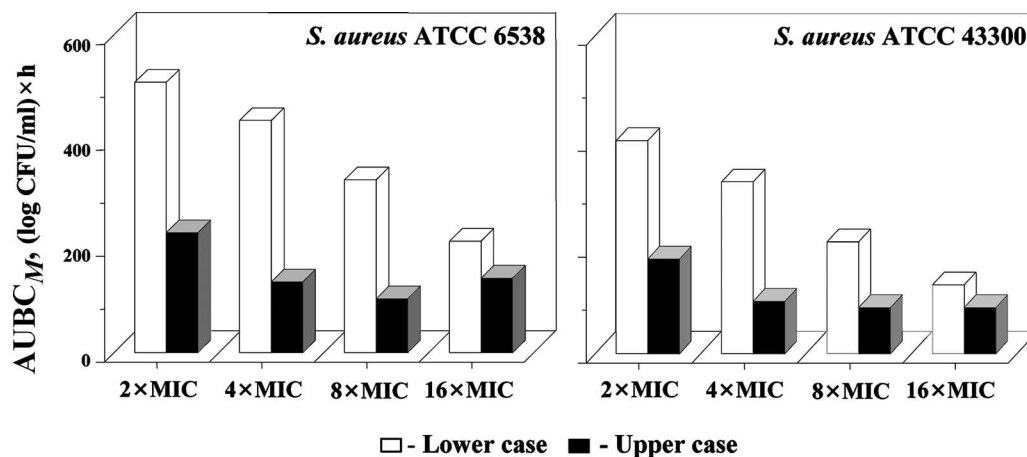


FIG. 4. Enrichment of *S. aureus* resistant to 2×, 4×, 8×, and 16× MIC of ciprofloxacin expressed by AUBC.

43300, a 3-fold increase in the simulated AUC_{24}/MIC (from 30 to 100 h) was accompanied by a 1.5-fold increase in the cumulative antimicrobial effect.

As seen on the lower four plots shown in Fig. 3, mutants resistant to 2×, 4×, and 8× MIC of ciprofloxacin were enriched also in an AUC_{24}/MIC -dependent manner. Bacterial growth on antibiotic-containing plates started earlier and was more pronounced at the smaller AUC_{24}/MIC s (lower case) than at the higher AUC_{24}/MIC s (upper case). At the highest ciprofloxacin concentrations on plates (16× MIC), the AUC_{24}/MIC -associated differences in the growth of mutants were minimal with *S. aureus* ATCC 6538 and almost negligible with *S. aureus* ATCC 43300. In simulations of the lower case, the susceptible subpopulation of *S. aureus* ATCC 6538 in samples withdrawn from the model after a 48-h exposure was almost completely replaced by mutants resistant to 2× and 4× MIC of ciprofloxacin. By the same time, susceptible cells of *S. aureus* ATCC 43300 were replaced by mutants resistant to 2× MIC and, by 72 h, these cells were replaced by mutants resistant to 4× MIC of ciprofloxacin. In simulations of the upper case with both organisms, similar replacement occurred only by mutants resistant to 2× MIC.

The described differences between the lower and upper case simulations were confirmed by the $AUBC_M$ analysis of the time courses of ciprofloxacin-resistant mutants of *S. aureus* ATCC 6538 and 43300 (Fig. 4). The cumulative production of the mutants resistant to 2×, 4×, and 8× MIC of ciprofloxacin appeared more than twofold greater in the lower case than in the upper case. These differences are consistent with a slightly more pronounced loss in susceptibility of staphylococci in the lower case than in the upper case, although this trend was not statistically significant (data not shown).

DISCUSSION

This study exposed two strains of *S. aureus* to ciprofloxacin concentrations that fell into the MSW for more than half of the dosing interval, with T_{MSW} s of 56% with *S. aureus* ATCC 43300 and 75% with *S. aureus* ATCC 6538. As expected from the first study of this type (5), resistant mutants were enriched at these conditions, with a concomitant loss in susceptibility.

Although the same T_{MSW} was designed with each organism, this enrichment was more pronounced at ciprofloxacin concentrations that were closer to the lower limit of the MSW than to the upper limit. Due to the fact that the numbers of resistant mutants were determined during treatment as well as after treatment, the regimen-associated differences in mutant selection could be presented objectively, by using the areas under the time courses of mutants grown on antibiotic-supplemented plates. For both *S. aureus* ATCC 6538 and 43300 mutants resistant to 2×, 4×, and 8× MIC of ciprofloxacin, the upper case was associated with $AUBC_M$ s more than twofold smaller than those of the lower case. Similar position-associated differences in the selection of mutants of these two strains have recently been reported in a dynamic model study that simulates constant ciprofloxacin concentrations (T_{MSW} 100%) (2). Also, these findings are consistent with in vitro data reported at static conditions, in which many more mutants were observed at antibiotic concentrations in the lower than in the upper portions of the MSW (2, 15).

Overall, the present study with two strains of *S. aureus* with different MPC/MIC ratios supports the MSW hypothesis and provides an explanation for the limited ability of T_{MSW} to predict the selection of fluoroquinolone-resistant staphylococci. Indeed, the enrichment of ciprofloxacin-resistant mutants depends not only on the T_{MSW} but also on the position of antibiotic concentrations within the MSW, making T_{MSW} -based predictions of resistance less accurate than those based on AUC/MIC or AUC/MPC . Also, this study suggests that together with the population analysis profile method (10, 11), the $AUBC_M$ analysis is useful for objective presentation of resistance data.

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Each author certifies that he or she has no commercial association that might pose a conflict of interest in connection with this article.

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