Evolution of Ciprofloxacin-Resistant *Staphylococcus aureus* in In Vitro Pharmacokinetic Environments

Jeffrey J. Campion,¹ Patrick J. McNamara,² and Martin E. Evans^{1*}

Division of Infectious Diseases, Department of Internal Medicine, College of Medicine,¹ and Division of Pharmaceutical Sciences, College of Pharmacy,² University of Kentucky, Lexington, Kentucky

Received 8 April 2004/Returned for modification 26 July 2004/Accepted 19 August 2004

The development of novel antibacterial agents is decreasing despite increasing resistance to presently available agents among common pathogens. Insights into relationships between pharmacodynamics and resistance may provide ways to optimize the use of existing agents. The evolution of resistance was examined in two ciprofloxacin-susceptible *Staphylococcus aureus* strains exposed to in vitro-simulated clinical and experimental ciprofloxacin pharmacokinetic profiles for 96 h. As the average steady-state concentration ($C_{avg\ ss}$) increased, the rate of killing approached a maximum, and the rate of regrowth decreased. The enrichment of subpopulations with mutations in *grlA* and low-level ciprofloxacin resistance also varied depending on the pharmacokinetic environment. A regimen producing values for $C_{avg\ ss}$ slightly above the MIC selected resistant variants with *grlA* mutations that did not evolve to higher levels of resistance. Clinical regimens which provided values for $C_{avg\ ss}$ intermediate to the MIC and mutant prevention concentration (MPC) resulted in the emergence of subpopulations with *gyrA* mutations and higher levels of resistance. A regimen producing values for $C_{avg\ ss}$ close to the MPC selected *grlA* mutants, but the appearance of subpopulations with higher levels of resistance was diminished. A regimen designed to maintain ciprofloxacin concentrations entirely above the MPC appeared to eradicate low-level resistant variants in the inoculum and prevent the emergence of higher levels of resistance. There was no relationship between the time that ciprofloxacin concentrations remained between the MIC and the MPC and the degree of resistance or the presence or type of ciprofloxacin-resistance mutations that appeared in *grlA* or *gyrA*. Regimens designed to eradicate low-level resistant variants in *S. aureus* populations may prevent the emergence of higher levels of fluoroquinolone resistance.

The rapid ascent of fluoroquinolone resistance in *Staphylococcus aureus* is one of the best contemporary examples of biological evolution. *S. aureus* isolates resistant to ciprofloxacin were described shortly after introduction of the agent into clinical practice, and presently up to 89% of isolates are resistant to the antimicrobial in some areas of the world (11). Two principal mechanisms of fluoroquinolone resistance have been reported. The first involves point mutations in the *grlA/grlB* and *gyrA/gyrB* genes, which encode the subunits of topoisomerase IV and DNA gyrase, respectively (16, 19, 27, 42, 50, 54, 55, 59). In *S. aureus*, the primary target of ciprofloxacin is topoisomerase IV, and DNA gyrase is a secondary target (16, 42, 59). The second mechanism involves efflux of fluoroquinolones by the membrane-associated protein NorA. This efflux pump actively transports fluoroquinolones and several other structurally unrelated compounds out of the bacterial cell (29, 60).

Since few novel antimicrobials are being developed for the treatment of bacterial infections, it would be of value to optimize the use of agents that are already available (53). One step in this direction is to gain a better understanding of the relationship between antimicrobial concentrations and the evolution of resistance. Previous investigators sought to establish relationships between pharmacokinetic parameters, such as the peak concentration (C_{max}) or the 24-h area under the

concentration-time curve (AUC_{24}), and pharmacodynamic parameters, such as the MIC, to optimize clinical and microbiological outcomes. Data from a variety of in vitro and clinical studies suggest that C_{max}/MIC ratios ranging from ~6 to >12 and AUC_{24}/MIC ratios ranging from ~35 to ≥ 125 are optimal for ensuring acceptable clinical and microbiological outcomes with fluoroquinolones and *S. aureus* (6, 18, 35, 45). However, considerable uncertainty about these values exists (57), and the emphasis of most of these studies was on bacterial eradication or clinical cure rather than prevention of the emergence of resistance.

Others have examined the relationship between antimicrobial concentrations and the evolution of resistance. Baquero and Negri proposed that environments providing small differences in antimicrobial concentrations might have a selective effect on bacterial cultures comprised of subpopulations with heterogeneous resistance phenotypes. These selective compartments were delimited by the concentration that inhibits susceptible bacteria and the concentration that inhibits organisms with low-level resistance (2). Drlica and colleagues refined this concept in the context of fluoroquinolones and *S. aureus* by defining a mutant selection window (MSW) as the concentration range between the MIC and the mutant prevention concentration (MPC; the concentration which inhibits growth of first-step mutants). They provided experimental data and theoretical analyses suggesting that regimens providing fluoroquinolone concentrations within the MSW select resistant variants, whereas regimens providing levels above the MPC prevent the emergence of resistant strains (7, 12, 61). Firsov and coworkers examined changes in the susceptibility of

* Corresponding author. Mailing address: Division of Infectious Diseases, Department of Internal Medicine, Room MN672, University of Kentucky Medical Center, 800 Rose St., Lexington, KY 40536-0298. Phone: (859) 323-8178. Fax: (859) 323-8926. E-mail: Martin.Evans@uky.edu.

S. aureus exposed to four fluoroquinolones in an in vitro system and reported that the greatest increases in MIC occurred with AUC₂₄/MIC ratios that corresponded to antimicrobial concentrations that fell within the MSW over more than 20% of the dosing interval (17).

In the work reported here, we examine the interplay of pharmacokinetics, bacterial subpopulation dynamics, and the mechanisms underlying resistance in *S. aureus* exposed to ciprofloxacin in an in vitro system to gain insights into the effects of pharmacokinetic environments on the evolution of resistance.

MATERIALS AND METHODS

Bacterial strains. Two methicillin-resistant *S. aureus* clinical isolates (MRSA 8043 and MRSA 8282) were used as parent strains for all in vitro system experiments. These bacteria were ciprofloxacin susceptible and had unrelated genomic DNA restriction patterns as assessed by pulsed-field gel electrophoresis (14). SA1199 (a wild-type *S. aureus* strain for which the ciprofloxacin MIC was 0.25 to 0.5 µg/ml) and the genetically related SA1199B (a strain with constitutively up-regulated efflux for which the ciprofloxacin MIC was 4 to 8 µg/ml) served as controls in efflux screening experiments (28, 30).

Antimicrobial agent. Analytical-grade ciprofloxacin powder (Bayer Diagnostics, Kankakee, Ill.) was used. Stock solutions (1,000 µg/ml) were prepared in sterile water, frozen (−80°C) in aliquots, and used within 30 days.

Susceptibility tests. MICs and minimal bacterial concentrations were determined by the broth microdilution method (40, 41) in triplicate for each organism before exposure to ciprofloxacin and for organisms recovered at 0, 24, 48, and 96 h during in vitro system experiments. MICs were also determined in the presence of 20 µg of reserpine (Sigma, St. Louis, Mo.)/ml, a competitive inhibitor of NorA, to screen for possible ciprofloxacin efflux (29).

MPC determinations. The MPCs for MRSA 8043 and MRSA 8282 were determined using a modification of a previously described method (12). Single bacterial colonies from overnight growth on Mueller-Hinton agar (MHA; Difco, Detroit, Mich.) were grown for 24 h at 37°C in Trypticase soy broth (TSB; Difco), concentrated by centrifugation, and resuspended in TSB to final concentrations of $\sim 2 \times 10^{10}$ to 5×10^{10} CFU/ml. Samples (200 µl) were applied to MHA containing ciprofloxacin (0, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 3.5, and 4 µg/ml) and incubated at 37°C for 48 h. The MPC was defined as the lowest concentration at which no bacteria were detected. Nucleotide sequence analysis was performed on DNA extracted from colonies recovered on ciprofloxacin-containing agar (see below).

In vitro system experiments. A two-compartment hollow-fiber in vitro system that permits exposure of bacteria to fluctuating concentrations of antimicrobial agents was used (5). The system consists of a sterile central reservoir connected to a series of hollow-fiber bioreactors (Cell Pharm Mini-Bioreactor System BR110; BioVest International, Inc., Minneapolis, Minn.). During experiments, the central compartment (central reservoir, connecting tubing, and lumina of hollow-fiber bundles), representing the systemic circulation, and the peripheral compartment (extracapillary chambers of each bioreactor and connecting tubing), representing extravascular infection sites, were filled with Mueller-Hinton broth (Difco) supplemented with calcium (25 µg/ml) and magnesium (12.5 µg/ml) (CAMHB) to final volumes of 120 and 20 ml, respectively. Contents of the central and peripheral compartments were circulated by a peristaltic pump (Masterflex; Cole Parmer, Chicago, Ill.) at a rate of 3 ml/min. The entire system, except the infusion pumps and drug and waste reservoirs, was housed in an incubator maintained at 37°C.

A standardized inoculum for all in vitro system experiments was prepared from a single colony suspended in TSB, incubated overnight, centrifuged, resuspended in TSB-glycerol (90:10), and frozen in aliquots containing $\sim 2 \times 10^6$ CFU/ml for subsequent use. On the day of an experiment, aliquots of the frozen inoculum were thawed and incubated for 1 h at 37°C. The bacterial suspensions (1.0 ml) were then injected into the peripheral compartment of the in vitro system and allowed to grow for 3 h to achieve a starting density of $\sim 1 \times 10^7$ CFU/ml. Frozen ciprofloxacin stock solutions were thawed and diluted with CAMHB in the drug reservoir to final concentrations appropriate for each in vitro system experiment. The ciprofloxacin solutions were delivered via a computerized infusion pump (Gemini PC-2TX; IMED, San Diego, Calif.) into the central reservoir as intermittent 1-h infusions at 8- or 12-h intervals or as a continuous infusion, depending on the dosage regimen being simulated (see below).

The exponentially growing cultures were exposed to a series of intermittent and continuous-infusion intravenous ciprofloxacin pharmacokinetic profiles for 96 h. Two simulations reproduced the pharmacokinetics of clinical dosage regimens (400 mg administered intravenously every 8 and 12 h). These simulations were designed to achieve total drug concentrations in the central and peripheral compartments that mimicked profiles observed in the serum and skin blister fluid of healthy adults over time (10, 32, 52). Three experimental dosage simulations were also examined. These included a regimen of 750 mg every 12 h used to provide $C_{\max}$ /MIC ratios of ≥ 8 and AUC₂₄/MIC ratios of ≥ 125 h, which have been purported to provide optimal antimicrobial efficacy (6, 49); a regimen of 2,800 mg every 12 h used to produce concentrations that exceeded the MPC for the study strains for the entire duration of the experiment, and a 12.5-mg/h continuous-infusion regimen used to provide constant concentrations that were just above the MIC for the parent strains. The latter allowed an examination of the emergence of resistance under conditions in which the rate of bacterial killing by ciprofloxacin was less than maximal. During continuous infusion experiments, desired ciprofloxacin concentrations were immediately achieved by administration of a bolus dose into the central compartment prior to beginning the infusion. Pharmacokinetic profiles for all experimental regimens were based on the assumption of linear pharmacokinetics.

Drug, diluent, and elimination pumps of the in vitro system were set at appropriate rates to simulate a ciprofloxacin elimination half-life of 4 h based on pharmacokinetics in adults with normal renal function (10, 51, 52). Growth control experiments for each strain were conducted over 36 h at the same pump rates used for ciprofloxacin dosage regimen simulations. Every in vitro system simulation was performed in duplicate. The pH of CAMHB in the central and peripheral compartments was measured at 24-h intervals for the duration of each experiment.

Pharmacokinetic analysis. Samples (250 µl) were collected from the central and peripheral compartments of the in vitro system during the course of each ciprofloxacin experiment to confirm simulation of the desired pharmacokinetic profiles. Ciprofloxacin concentrations in the collected samples were determined using a previously described ion-pair high-performance liquid chromatography method (58), with modifications. The modified method did not require protein precipitation of the sample or addition of an internal standard. Central and peripheral (bacterial suspension) compartment samples were simply passed through a 0.45-µm-pore-size syringe filter (Acrodisc; Pall Corp., East Hills, N.Y.) and then directly injected onto a C18 analytical column (Adsorbosphere HS; Alltech Associates, Inc., Deerfield, Ill.) equipped with a guard column (All-Guard cartridge system; Alltech). The mobile phase employed was identical to the previously published method except that sodium dodecyl sulfate (2.8 mM) and triethylamine (4.7 mM) concentrations were altered, and the detection wavelength was set at 290 nm. Calibration curves were linear ($R^2 > 0.999$) over the concentration range tested (0.1 to 100 µg/ml). The within-run relative standard deviations (RSDs; $n = 5$) for ciprofloxacin quality control concentrations of 25, 2.5, and 0.25 µg/ml were 1.68, 0.99, and 1.36%. The between-run RSDs for the same quality control concentrations ($n = 16$) were 2.02, 2.25, and 1.41%. The reliable lower limit of quantification (defined as percent RSD and percent deviation from the nominal concentration of $<15\%$) was 0.06 µg/ml.

The central and peripheral compartment concentration-time data for ciprofloxacin were analyzed by standard noncompartmental methods (20). The extent of ciprofloxacin penetration into the peripheral compartment was estimated by comparing the AUC₂₄ ratios of the peripheral and central compartments. For intermittent-infusion dosage regimen simulations, the mean steady-state concentration ($C_{\text{avg,ss}}$) was calculated by dividing the AUC_{24,ss} by the dosing interval (τ).

A one-compartment pharmacokinetic model, with first-order elimination from the central compartment, was fit to ciprofloxacin concentrations in the central compartment using a nonlinear regression program (WinNonlin; Pharsight Corporation, Mountain View, Calif.). All pharmacokinetic data were weighted by the inverse of squared predicted concentrations based on the precision of the high-performance liquid chromatography assay. The goodness of fit between observed and model-predicted central compartment concentrations was evaluated by residual analysis and Pearson's correlation coefficient (r).

Pharmacodynamic analysis. Samples (150 µl) were removed from the peripheral compartment of the in vitro system at predetermined times during each experiment to determine the number of viable bacteria using the drop count method (36). Mean viable count data for replicate experiments were plotted as the number of log₁₀ CFU/ml versus time. Experiments were performed to assess the effect of ciprofloxacin carryover in bacterial samples collected from the in vitro system. Ciprofloxacin concentrations of 16 to 64 and 1 to 8 µg/ml resulted in significantly lower ($P < 0.05$; paired t test) viable counts for suspensions containing $\leq 4 \times 10^5$ CFU/ml and $\leq 3 \times 10^4$ CFU/ml, respectively. Ciprofloxacin at a concentration of 0.5 µg/ml did not significantly lower the colony counts

compared to those of the drug-free controls with suspensions containing $<3 \times 10^4$ CFU/ml. Thus, dilutions of at least 1,000- and 100-fold were necessary to prevent a significant lowering of colony counts in plated samples containing ciprofloxacin concentrations of 16 to 64 and 1 to 8 $\mu\text{g/ml}$, respectively.

When bacterial counts were expected to be unreliable because of antimicrobial carryover, a membrane filter count method was employed. Samples with potential drug carryover were diluted in cold 0.9% NaCl and then passed through a 0.45- μm -pore-size cellulose nitrate filter (Sartorius, Gottingen, Germany). Filters were aseptically placed on MHA and incubated for 48 h at 37°C, and the colonies were counted. Additional experiments using low-inoculum suspensions ($<10^3$ CFU/ml) were performed to determine the reliable limit of detection for membrane filter counts. The reliable lower limit of detection, defined as the most dilute suspension for which the coefficient of variation of viable counts was less than 20%, was 3.0×10^2 CFU/ml for 100- μl and 1.0×10^2 CFU/ml for 250- μl samples.

Resistant subpopulation analysis. Resistant bacterial subpopulations were quantified in samples (150 μl) drawn from the peripheral compartment of the in vitro system at 0, 24, and 36 h (growth control experiments) and at 0, 24, 48, and 96 h (ciprofloxacin experiments). Samples were serially diluted 10-fold with 0.9% NaCl and plated in triplicate onto MHA containing increasing ciprofloxacin concentrations in twofold dilutions. To detect resistant subpopulations in samples expected to contain small numbers of bacteria, the membrane filter count method was employed using filtered volumes of 100, 250, or 500 μl . Plates were incubated at 37°C for 48 h, after which colonies were counted. For resistant bacteria detected in the starting populations, the frequency of resistance at each ciprofloxacin concentration was calculated by dividing the number of colonies that appeared by the inoculum applied to each plate.

For the purposes of this paper, we defined ciprofloxacin-susceptible bacteria as those recovered only on drug-free agar. Bacteria with low-level ciprofloxacin resistance were defined as those recovered only on agar containing a ciprofloxacin concentration of 0.5, 1, or 2 $\mu\text{g/ml}$. Bacteria with high-level resistance were defined as those recovered on agar containing a ciprofloxacin concentration of 4, 8, or 16 $\mu\text{g/ml}$.

Nucleotide sequence analysis of *grrA/B* and *gyrA/B*. Colonies recovered from ciprofloxacin-containing agar in the MPC studies and at the start of the in vitro system experiments were analyzed for nucleotide changes within the quinolone resistance-determining regions (QRDRs) of *grrA/B* and *gyrA/B*. Colonies recovered on drug-free agar at the conclusion of the in vitro system experiments were replica plated onto agar containing increasing ciprofloxacin concentrations in twofold dilutions, and colonies that appeared on plates with the highest ciprofloxacin concentrations were chosen for sequence analysis. All colonies were examined if three or fewer colonies were present on a plate. Three colonies were randomly chosen for sequence analysis if more than three colonies were present. Genomic DNA was obtained by lysis of cells with lysostaphin (Sigma) followed by phenol-chloroform extraction (34). The nucleotide primers used for PCR of the QRDRs of *grrA/B* and *gyrA/B*, based on published gene sequences (8, 59), included the following: *grrA*, 5'-TGC CAG ATG TTC GTG ATG-3' (5' nucleotide at position 2467) and 5'-CCT TGA ATA CCA CCA GTT G-3' (5' nucleotide at position 3040); *grrB*, 5'-TGT TGT GTC TGT TCG TAT TCC-3' (5' nucleotide at position 1353) and 5'-GCA CCA TCA GTA TCA GCA TC-3' (5' nucleotide at position 1910); *gyrA*, 5'-GAG TGT TAT CGT TGC TCG TG-3' (5' nucleotide at position 2333) and 5'-GAC GGC TCT CTT TCA TTA CC-3' (5' nucleotide at position 2725); and *gyrB*, 5'-CCA CAA GTC GCA CGT ACA G-3' (5' nucleotide at position 1407) and 5'-ATC CAC ATC GGC ATC AGT C-3' (5' nucleotide at position 1817). Colonies with high-level resistance to ciprofloxacin and no mutations in the QRDRs of *grrA/B* and *gyrA/B* were further analyzed for nucleotide changes outside the QRDR in *grrA/B*. The PCR primers used for these analyses were as follows: *grrA*, 5'-GAC GAT GAA CCC AGA AAC AC-3' (5' nucleotide at position 2157) and 5'-TAA ACC ATC ACG AAC ATC TG-3' (5' nucleotide at position 2489); and *grrB*, 5'-GTT TGC AGG AGG CGA AAT C-3' (5' nucleotide at position 359) and 5'-GCA TAC CAC GTC CAT TAT CTT C-3' (5' nucleotide at position 622). PCR was performed using the GeneAmp reagent kit with AmpliTaq DNA polymerase (Perkin Elmer Applied Biosystems, Foster City, Calif.). Reaction conditions included an initial 2-min denaturation step at 95°C followed by 30 cycles of denaturation (30 s at 94°C), annealing (30 s at 56°C), and polymerization (45 s at 72°C). This was followed by a final incubation at 72°C for 5 min. PCR products were purified using QIAquick spin columns (QIAGEN, Valencia, Calif.) and directly sequenced by the dideoxy chain termination method (46). Sequences were considered correct only if there were no discrepancies between the forward and reverse sequences.

RESULTS

Susceptibilities of and MPCs for MRSA 8043 and MRSA 8282 parent cultures. The ciprofloxacin MIC for MRSA 8043 and MRSA 8282 was 0.5 $\mu\text{g/ml}$, and the minimal bactericidal concentrations were 1 and 2 $\mu\text{g/ml}$, respectively. Reserpine lowered the ciprofloxacin MICs for the starting cultures of MRSA 8043 ($n = 12$) and MRSA 8282 ($n = 12$) two- to fourfold (Fig. 1 and 2, boxes). The reserpine-mediated reductions in the ciprofloxacin MICs for MRSA 8043 and MRSA 8282 were the same as those for the wild-type *S. aureus* control strain, SA1199. In contrast, reserpine lowered the ciprofloxacin MIC for SA1199B, the strain with constitutively up-regulated efflux, four- to eightfold.

The ciprofloxacin MPCs for MRSA 8043 ranged from 3 to 3.5 $\mu\text{g/ml}$ (geometric mean, 3.30 $\mu\text{g/ml}$; $n = 3$) and for MRSA 8282 from 2.5 to 3 $\mu\text{g/ml}$ (geometric mean, 2.82 $\mu\text{g/ml}$; $n = 3$). The most resistant variants of MRSA 8043 and MRSA 8282, recovered on agar containing 2 to 3 $\mu\text{g/ml}$ of ciprofloxacin, had mutations in *grrA* (corresponding to S80Y or S80F).

Resistant-variant subpopulations in the starting cultures during in vitro system experiments. The majority ($>99\%$) of cells in the starting populations were susceptible to ciprofloxacin concentrations of $<0.5 \mu\text{g/ml}$ (Fig. 1 and 2, bars). Resistant subpopulations were detected in both strains at frequencies ranging from 9.8×10^{-5} to 9.0×10^{-4} , $<3.0 \times 10^{-7}$ to 3.0×10^{-6} , and $<1.1 \times 10^{-7}$ to 4.2×10^{-7} when the bacteria were subcultured on agar containing ciprofloxacin concentrations of 0.5, 1, and 2 $\mu\text{g/ml}$, respectively (Fig. 1 and 2). No bacteria were recovered on agar containing more than 2 μg of ciprofloxacin/ml. Variants resistant to ciprofloxacin concentrations of 1 and 2 $\mu\text{g/ml}$ were detected in the starting populations in 12 of 12 and 3 of 12 experiments with MRSA 8043 and in 10 of 12 and 0 of 12 experiments with MRSA 8282, respectively.

Direct sequencing of PCR-amplified DNA from 72 randomly chosen colonies of MRSA 8043 and MRSA 8282 recovered on drug-free agar revealed silent mutations or wild-type *grrA/B* and *gyrA/B* QRDR nucleotide sequences in all instances (Fig. 1 and 2). Nucleotide sequence analysis of 29 MRSA 8043 and 34 MRSA 8282 colonies recovered on agar containing 1 μg of ciprofloxacin/ml indicated that no mutations, silent mutations, or point mutations (corresponding to S80Y and S80F in MRSA 8043 and S80Y and A116P in MRSA 8282) were present within *grrA* (Fig. 1 and 2). Mutations in the *grrA* QRDR, however, were found in only 43% of the MRSA 8043 and 26% of the MRSA 8282 sequenced variants that were recovered on agar containing 1 μg of ciprofloxacin/ml. All low-level resistant variants of MRSA 8043 recovered on agar containing 2 μg of ciprofloxacin/ml were *grrA* mutants (with a mutation corresponding to S80Y). Variants with *grrA* mutations were detected in the starting populations in 7 of 12 experiments with MRSA 8043 and 5 of 12 experiments with MRSA 8282, respectively. No point mutations within the *grrB* or *gyrA/B* QRDRs were found among low-level ciprofloxacin-resistant subpopulations in the starting populations of either strain during any of the experiments.

Ciprofloxacin pharmacokinetics in the in vitro system. Desired ciprofloxacin pharmacokinetic profiles for the five regimens were accurately and reproducibly simulated in the in vitro system (Fig. 3). Peak ciprofloxacin concentrations in the

peripheral compartment, attained 15 to 30 min after the end of the 1-h intermittent infusions, were 85 to 99% of the corresponding central compartment concentrations. One hour after the end of the simulated intermittent infusions, the measured peripheral and central concentration profiles crossed, and the peripheral compartment concentrations remained 1 to 13% higher than central compartment values for the remainder of the dosing interval. The extent of ciprofloxacin penetration into the peripheral compartment, estimated by the ratio of the AUC in peripheral and central compartments, was between 97 and 105% for all experiments. The mean ($\pm$ SD) ciprofloxacin elimination half-lives in the central and peripheral compartments were 4.17 ± 0.12 h and 4.54 ± 0.08 h for all experiments.

A one-compartment pharmacokinetic model accurately described observed central compartment ciprofloxacin concentration-time profiles from individual experiments. The correlation (r) between measured and model-predicted central compartment concentrations ranged from 0.957 to 0.999 for all dosage regimen simulations. No systematic deviation in the residuals between observed and model-predicted concentrations was noted. Central compartment pharmacokinetic parameters obtained by nonlinear curve-fitting analysis were similar to those obtained by noncompartmental methods (data not shown). The model-predicted central compartment pharmacokinetic profiles mimicked pharmacokinetics in the peripheral compartment except that the model over- and underpredicted the measured peak and trough concentrations in the peripheral compartment by 1.6 to 10.3 and 1.1 to 15.5%, respectively (Fig. 3).

Bacterial population dynamics during in vitro system experiments. Viable count versus time profiles for MRSA 8043 and MRSA 8282 populations during in vitro system experiments are shown in Fig. 4. Bacteria were in log-phase growth at the start of each experiment. In the absence of ciprofloxacin, MRSA 8043 and MRSA 8282 populations continued to grow at an exponential rate until the carrying capacity of the in vitro system was reached ($\sim 10^9$ CFU/ml for MRSA 8043 and $\sim 10^{10}$ CFU/ml for MRSA 8282), and remained near this plateau for the remainder of each growth control experiment.

During ciprofloxacin dosage regimen simulations, the densities of the starting MRSA 8043 and MRSA 8282 cultures ranged from 5.0×10^6 to 1.9×10^7 CFU/ml (corresponding to a total population of $\sim 1.3 \times 10^8$ to 3.8×10^8 CFU in the 20-ml peripheral compartment). When the bacteria were exposed to the continuous-infusion regimen or that of 400 mg every 8 or 12 h, the numbers initially fell but then rebounded toward the carrying capacity of the in vitro system. A similar pattern of killing and regrowth was seen when MRSA 8043 bacteria were exposed to the simulation of 750 mg every 12 h. When MRSA 8282 populations were exposed to this regimen, bacterial counts in one of the replicate experiments increased after an

initial period of killing, but bacterial counts in the other experiment persisted near the lower limit of detection (Fig. 4, bottom). The inoculum used in the second experiment (6.7×10^6 CFU/ml) was smaller than that used in the first experiment (1.8×10^7 CFU/ml). When MRSA 8043 and MRSA 8282 were exposed to the simulation of 2,800 mg every 12 h, viable counts declined to below the reliable limit of detection. However, despite the rapid rate of killing achieved with this regimen, small numbers of bacteria (<100 CFU/ml) were recovered from the peripheral compartment of the in vitro system for the duration of the experiments. Overall, the initial rate of bacterial killing increased to a maximum as $C_{\text{avg ss}}$ increased (values are shown in Table 1). In those experiments in which regrowth of bacteria occurred, the apparent rate of regrowth decreased as $C_{\text{avg ss}}$ increased.

The slopes of the killing and regrowth portions of the viable count curves were similar for replicate dosage regimen simulations with a given strain (Fig. 4). However, bacterial counts were often shifted up or down along the ordinate in replicate experiments. The shifts in the curves were usually more pronounced for the regrowth phases than the initial killing phases. Variations in inoculum and resistant subpopulation size between experiments may have accounted for the shifts in the killing and regrowth curves, respectively.

Ciprofloxacin activity decreases at pH values below 6.8 (22). The pH (mean $\pm$ SD) of CAMHB in the central and peripheral compartments was 7.20 ± 0.04 at the beginning of the experiments. After 48 h, the pH decreased in those simulations in which regrowth occurred (6.97 ± 0.14 at 72 h and 6.91 ± 0.10 at 96 h) but remained between 7.17 and 7.21 in those experiments where viable counts declined to the lower limit of detection. The 24-h pH in the growth control experiments ranged from 6.79 to 6.85.

Changes in susceptibilities of bacterial populations during in vitro system experiments. No change in the ciprofloxacin MICs (boxes, Fig. 1 and 2) was observed during the growth control experiments. When the bacteria were exposed to the simulated continuous-infusion regimen, the ciprofloxacin MIC increased fourfold for MRSA 8043 and two- to fourfold for MRSA 8282. Greater increases in ciprofloxacin MICs for MRSA 8043 (16- to 32-fold) and MRSA 8282 (8- to 16-fold) were noted when these strains were exposed to the simulated clinical ciprofloxacin regimens. The simulated regimen of 750 mg every 12 h produced different susceptibility changes in the two strains. When MRSA 8043 was exposed to this regimen, the MIC increased 16-fold in replicate experiments. In contrast, the MIC for MRSA 8282 increased eightfold in the first experiment with the larger inoculum but was unchanged in the second experiment with the smaller inoculum. The MIC for MRSA 8043 and MRSA 8282 cultures exposed to the simu-

FIG. 1. Total MRSA 8043 bacterial counts (0 μ g of ciprofloxacin/ml) and subpopulations resistant to ciprofloxacin at 0.5 to 16 μ g/ml in samples collected from the in vitro system at the indicated times during growth control and simulated ciprofloxacin dosage regimen experiments. Bacteria isolated in numbers below the reliable limit of detection (100 CFU/ml) are shown as $2 \log_{10}$. Broth microdilution MICs for the total bacterial population in the absence of reserpine are indicated in boxes within the population profiles, and those in the presence of reserpine are shown in parentheses. Results are expressed as geometric means of results from two separate experiments. The *gtrA* and *gyrA* QRDR mutation(s) and the number of clones sequenced at specific ciprofloxacin concentrations are also shown. No mutations in the QRDRs of *gtrB* or *gyrB* were detected.

FIG. 2. Total MRSA 8282 bacterial counts (0 μ g of ciprofloxacin/ml) and subpopulations resistant to ciprofloxacin at 0.5 to 16 μ g/ml in samples collected from the in vitro system at the indicated times during growth control and simulated ciprofloxacin dosage regimen experiments. See legend to Fig. 1 for additional details.

FIG. 3. Measured concentrations in the central (filled symbols) and peripheral (open symbols) compartments for simulated ciprofloxacin dosage regimens. The fitted curves from the one-compartment pharmacokinetic model are also shown. The MICs (dashed-dotted line) and MPCs for MRSA 8043 (solid line) and MRSA 8282 (dotted line) are shown.

lated ciprofloxacin regimen of 2,800 mg every 12 h did not change during the course of the 96-h experiments.

Reserpine decreased the ciprofloxacin MIC by two- to four-fold in all experiments in which resistance developed, with one exception. The exception occurred with the first of the replicate simulations of 750 mg every 12 h with MRSA 8282. In this experiment, reserpine decreased the 96-h ciprofloxacin MIC eightfold (4 to 0.5 µg/ml), which is a reduction similar to that observed with SA1199B.

Changes in *grrA/B* and *gyrA/B* genotypes during in vitro system experiments. During growth control experiments, the number of low-level resistant variants with a mutation corresponding to S80Y or no mutation in *grrA* increased, although their relative frequency, as a percentage of the total popula-

tion, decreased (Fig. 1 and 2). When MRSA 8043 and MRSA 8282 were exposed to the continuous-infusion regimens, subpopulations with low-level resistance and *grrA* mutations (corresponding to S80Y and A116P) appeared. When the bacteria were exposed to the clinical ciprofloxacin regimens (400 mg every 8 and 12 h), high-level resistant subpopulations and mutations in *grrA* (corresponding to S80Y, S80F, or A116P), and sometimes *gyrA* (corresponding to S84L), appeared. Resistant subpopulation changes differed in the two bacteria when they were exposed to the ciprofloxacin regimen of 750 mg every 12 h. For MRSA 8043, high-level resistant subpopulations with mutations inside (S80Y) and outside (A176G) the QRDR of *grrA* were detected after 96 h (Fig. 1). In contrast, with MRSA 8282, resistant subpopulations were not detected in one exper-

FIG. 4. Viable count versus time profiles for MRSA 8043 (top) and MRSA 8282 (bottom) during exposure to various simulated ciprofloxacin (CIP) dosage regimens in the in vitro system. Control growth curves are also depicted. Viable counts are plotted as the means and range of results from two separate experiments. The lower limit of detection was 100 CFU/ml. The legend for both panels appears in a box inside the top panel.

iment but were recovered on agar containing up to 4 μ g of ciprofloxacin/ml in the other experiment, and no mutations were seen in the QRDR of *grlA* at 96 h (Fig. 2). Resistant subpopulations did not emerge when the bacteria were exposed to the simulated ciprofloxacin regimen of 2,800 mg every 12 h. Overall, different *grlA/B* and *gyrA/B* genotypes with the same susceptibility phenotype were often seen. No mutations in the QRDRs of *grlB* or *gyrB* were found in any resistant variants during the ciprofloxacin dosage regimen simulations.

Overall, different patterns of nucleotide sequence changes in *grlA/B* and *gyrA/B* were observed during exposures of the bacteria to ciprofloxacin. In some cases, no mutants were detected in the starting cultures, and resistant bacteria were recovered at 96 h with either no mutations or mutations in *grlA*. In others, *grlA* mutants were present in the starting populations, and

resistant bacteria were recovered at 96 h with either no additional mutations or mutations in *gyrA*. The latter pattern was observed only in those experiments that simulated clinical ciprofloxacin dosage regimens. In experiments where *grlA/gyrA* double mutants were found following ciprofloxacin exposure, there was always a *grlA* mutant detected in the starting cultures. The pattern of nucleotide sequence changes often varied with different dosage regimen simulations and between replicate experiments of the same simulation. For example, when MRSA 8043 was exposed to a dosage regimen of 400 mg every 8 h, *grlA/gyrA* double mutants were detected in one experiment, but only *grlA* mutants were found in the other.

Pharmacokinetic-pharmacodynamic parameters and the emergence of resistance. Ciprofloxacin concentrations were above the MIC 99.3 to 100% of the time during the initial 24 h

TABLE 1. Ciprofloxacin pharmacokinetic parameters^a relative to MICs and MPCs for MRSA 8043 and MRSA 8282 during in vitro system experiments

Simulated regimen ($C_{avg\ ss}$) ^b	Strain	Parameters during first 24 h					Steady-state parameters				
		C_{max}/MIC	AUC_{0-24}/MIC (h)	C_{max}/MPC	AUC_{0-24}/MPC (h)	$T_{0-24\ MSW}^c$ (%)	$C_{max\ ss}/MIC$	$AUC_{24\ ss}/MIC$ (h)	$C_{max\ ss}/MPC$	$AUC_{24\ ss}/MPC$ (h)	$T_{24ss\ MSW}^c$ (%)
12.5 mg/h (0.65 μ g/ml)	8043	1.16	28.8	0.17	4.34	100	1.28	30.7	0.19	4.63	100
	8282	1.12	28.6	0.20	5.06	100	1.30	31.2	0.23	5.53	100
400 mg every 12 h (1.77 μ g/ml)	8043	5.54	73.7	0.83	11.1	99.8	6.96	84.5	1.05	12.7	96.7
	8282	5.40	73.2	0.96	13.0	96.9	6.74	85.4	1.20	15.1	87.7
400 mg every 8 h (2.61 μ g/ml)	8043	5.84	113	0.88	17.1	91.5	8.74	125	1.32	18.8	75.7
	8282	5.38	109	0.95	19.4	81.1	7.74	125	1.37	22.2	63.2
750 mg every 12 h (3.30 μ g/ml)	8043	10.3	149	1.55	22.4	67.6	13.4	159	2.02	24.0	57.1
	8282	9.60	141	1.70	25.1	62.8	12.7	157	2.25	27.9	50.4
2,800 mg every 12 h (12.1 μ g/ml)	8043	39.0	565	5.88	85.1	0.63	47.2	578	7.11	87.1	0.00
	8282	38.8	557	6.88	98.8	0.54	46.5	584	8.24	103	0.00

^a Values are expressed as the means of results from two separate experiments. The subscript ss indicates steady-state conditions.

^b The average steady-state concentration for the simulated dosage regimens expressed as the mean of results from two separate experiments.

^c Expressed as the percentage of time in the MSW during a 24-h period.

and 100% of the time at steady state in all experiments. The fluctuating pharmacokinetic profiles of the simulated clinical regimens of 400 mg every 8 or 12 h, which produced ciprofloxacin C_{max}/MIC ratios which ranged from 5.38 to 8.74 and AUC_{24}/MIC ratios from 73.7 to 125 h (Table 1), were associated with the greatest (8- to 32-fold) increases in the MICs for both strains and the appearance of *grrA/gyrA* double mutants in many cases (Fig. 1 and 2). The T_{MSW} (the time ciprofloxacin concentrations were in the MSW) for a 24-h period ranged from 63.2 to 99.8% during these simulations. The fluctuating concentrations of the experimental regimen of 750 mg every 12 h, which produced C_{max}/MIC ratios ranging from 9.60 to 13.4 and AUC_{24}/MIC ratios from 142 to 152 h, was associated with a less pronounced eightfold increase in the ciprofloxacin MIC for MRSA 8043 and either no change or an eightfold increase in the ciprofloxacin MIC for MRSA 8282. The T_{MSW} for a 24-h period was between 50.4 and 67.6% in these simulations. Selective enrichment of low-level resistant variants with subsequent evolution to higher levels of resistance occurred in three of the four experiments with administration of 750 mg every 12 h. The constant concentrations of the continuous-infusion simulation, in which ciprofloxacin C_{max}/MIC ratios ranged from 1.12 to 1.30 and AUC_{24}/MIC ratios ranged from 28.6 to 31.2 h, was associated with the smallest increases in ciprofloxacin MIC (two- to fourfold). In these simulations, ciprofloxacin concentrations were between the MIC and the MPC for the entire 24-h period, and enrichment of low-level resistant subpopulations occurred. The fluctuating pharmacokinetic profile of the simulated experimental regimen of 2,800 mg every 12 h, which produced the highest C_{max}/MIC ratios (38.8 to 47.2) and AUC_{24}/MIC ratios (565 to 584 h) and the lowest T_{MSW} ($\leq 0.63\%$), did not result in the emergence of ciprofloxacin resistance. This regimen provided first-dose C_{max}/MPC and AUC_{0-24}/MPC ratios of at least 5.88 and 85.1 h and steady-state ratios of at least 7.11 and 87.1 h, respectively.

DISCUSSION

The purpose of this study was to examine the relationship between pharmacokinetics and the evolution of resistance in bacterial populations. We chose to do this with *S. aureus* and ciprofloxacin in an in vitro system. The system allowed us to expose the bacteria to several different pharmacokinetic pro-

files and to monitor changes in bacterial subpopulations, MICs, efflux, and mutations in the topoisomerase genes. Other investigators have examined the pharmacodynamics of fluoroquinolones (47, 48) and elucidated various mechanisms of resistance in *S. aureus* (23), but to our knowledge, we are the first to examine the interplay of pharmacokinetics, resistance mechanisms, and the subpopulation dynamics underlying the evolution of fluoroquinolone resistance in these bacteria.

The starting cultures used in our in vitro system experiments were mainly comprised of ciprofloxacin-susceptible bacteria possessing silent mutations or wild-type sequences in the QRDRs of *grrA/B* and *gyrA/B*, as expected (8, 16, 50, 56). However, minor subpopulations with low-level ciprofloxacin resistance were recovered on agar containing 0.5 to 2.0 μ g/ml from the starting cultures at frequencies previously reported for first-step *S. aureus* mutants (21, 24). Genotyping of our variants revealed heterogeneity in their fluoroquinolone resistance determinants, consistent with the classic belief that mutations occur randomly during nonselective bacterial growth. Some variants possessed the point mutations (corresponding to S80F, S80Y, or A116P) in the QRDR of *grrA* that are known to cause low-level fluoroquinolone resistance in genetic studies and associated with resistance in clinical isolates (15, 42, 50, 56, 59). Other resistant subpopulations did not have mutations in the QRDR of either *grrA/B* or *gyrA/B*. Resistance among these variants may have been due to mutations outside the recognized topoisomerase gene QRDRs or to efflux (26, 39, 44). The two- to fourfold lowering of the ciprofloxacin MICs in the presence of reserpine suggests that a reserpine-sensitive efflux pump, possibly NorA, was present in some bacteria in the starting populations. This finding is in concordance with a previous report describing reserpine-sensitive efflux systems in a majority of *S. aureus* clinical strains (22).

The pattern of bacterial killing and regrowth in the in vitro system varied with the pharmacokinetic profile simulated and the bacterial strain tested. As $C_{avg\ ss}$ increased, the initial bacterial killing rate approached a maximum, and the rate of regrowth decreased. The emergence of ciprofloxacin resistance also varied in different pharmacokinetic environments. The smallest increases in MIC were noted when static ciprofloxacin concentrations were maintained just above the MIC. In these

experiments, ciprofloxacin concentrations were maintained within the MSW the entire time. The low concentrations appeared to enrich variants with low-level resistance but apparently did not provide sufficient selective pressure for evolution toward higher levels of resistance. The greatest MIC increases were observed with the simulated clinical dosing regimens (400 mg every 8 and 12 h) providing $C_{\text{avg ss}}$ values which fell between the MIC and the MPC for both strains. Within 24 h, low-level resistant variants present in the starting cultures became the predominant population; their numbers increased, and *grlA/grlA* double mutants appeared in many cases by 96 h. A less pronounced increase in ciprofloxacin MIC was noted with the simulated pharmacokinetic profile of 750 mg every 12 h, which produced a value for $C_{\text{avg ss}}$ near the MPC. In this case, selective enrichment of resistant subpopulations occurred, but no mutations in the *gyrA* QRDR were detected. Resistance to ciprofloxacin did not develop in pharmacokinetic environments in which concentrations exceeded the MPC over the entire dosing interval. Instead, viable counts declined to the limit of detection, and *grlA* mutants that were present in the starting populations appeared to be eliminated. These findings suggest that fluoroquinolone dosage regimens designed to eradicate topoisomerase mutants and other low-level resistant variants typically present in *S. aureus* cultures may prevent the emergence of resistance.

Our work supports the concept that resistant subpopulations of *S. aureus* are selectively enriched when ciprofloxacin concentrations fall within the MSW, but unlike other investigators we did not find a clear relationship between the time that antimicrobial levels remained within the MSW and the degree of resistance that occurred (17). For instance, high-level resistant variants appeared in a fluctuating environment (400 mg every 12 h) with a percentage of time in the MSW during the first 24-h period ($T_{0-24 \text{ MSW}}$) of $\geq 96.9\%$, but did not appear in a continuous-infusion regimen with a $T_{0-24 \text{ MSW}}$ of 100%. These findings suggest that, in addition to T_{MSW} , other pharmacokinetic parameters, possibly including the $C_{\text{avg ss}}$ within the MSW, are also important in the emergence of resistance. Although others have proposed that selection is more intense in fluctuating than static antimicrobial environments (4), we cannot draw conclusions about the influence of exposure pattern on the evolution of resistance with our data. This would require a different study design in which the effects of static and fluctuating pharmacokinetic profiles producing identical values for $C_{\text{avg ss}}$ are simulated and compared.

Our data showed that resistance did not occur with the $C_{\text{max}}/\text{MIC}$ ratio of 47.2 and the $\text{AUC}_{24}/\text{MIC}$ ratio of 584 h afforded by the dosing simulation of 2,800 mg every 12 h but did occur with the $C_{\text{max}}/\text{MIC}$ ratios up to 13.4 and $\text{AUC}_{24}/\text{MIC}$ ratios up to 159 h provided by other regimens. The latter findings do not support the suggestion made by others that $C_{\text{max}}/\text{MIC}$ ratios of ≥ 8 to 12 and $\text{AUC}_{24}/\text{MIC}$ ratios of ≥ 125 h are required for the eradication of bacteria and the prevention of resistance (6, 18, 35, 45, 49, 55a). This observation, however, is based on only five simulated pharmacokinetic profiles. Additional experiments using a wider range of concentrations and incremental changes in $C_{\text{max}}/\text{MIC}$ or $\text{AUC}_{24}/\text{MIC}$ ratios would be needed to fully evaluate these pharmacodynamic concepts and determine optimal values for our bacteria in the in vitro system.

Bacteria with high-level resistance and *grlA/grlA* double mutations appeared at the end of some of our simulated regimens. The origin of these bacteria is unclear. It is unlikely that the appearance of these mutants at 96 h was simply due to selection, because we were unable to detect them in any of the 24 starting cultures. The probability that double mutants would arise from wild-type bacteria should be very low (10^{-15} to 10^{-17}) since the frequency of our *grlA* mutations was 10^{-6} to 10^{-8} , and the frequency of mutations in *gyrA* may be even lower ($\sim 10^{-9}$) (24). However, mutation frequencies may change in the presence of ciprofloxacin, since the fluoroquinolone is known to be mutagenic (13).

High-level resistant variants with *gyrA* mutations may have originated from *grlA* mutants in the starting populations. In our experiments, *grlA/grlA* mutants recovered at 96 h were always preceded by low-level resistant variants in the starting cultures that had the same *grlA* mutation. This is consistent with previous reports showing that sequential mutations in *grlA* and *gyrA* of *S. aureus* result in incremental increases in MIC (15, 24).

Although stepwise mutations in fluoroquinolone targets may explain the evolution of resistance during some dosage regimen simulations, some high-level resistant bacteria had no mutations in the QRDRs of *grlA/B* or *gyrA/B*. Others have also reported resistant *S. aureus* variants lacking the expected mutations in the QRDRs of target genes (12, 43). In one of our experiments we found a mutation (corresponding to A176G) outside the traditional QRDR that has recently been reported to be associated with fluoroquinolone resistance (25). However, other high-level resistant bacteria recovered during some of our experiments did not have mutations outside the conventional QRDR of *grlA* or *grlB*, suggesting that mutations in an unrecognized determinant(s) may have contributed to the high-level resistance. In addition, the reserpine screening studies suggest that efflux may be responsible, in part, for the emergence of some resistant bacteria. The relative contributions of target mutations and efflux in the evolution of resistance cannot be elucidated without additional ciprofloxacin uptake and genetic studies.

Although we cannot explain the exact pathways taken by the bacteria in their evolution from susceptibility or low-level resistance to high-level resistance, bacteria recovered at the end of the 96-h experiments with the same MIC often had different mutations in the QRDRs of the topoisomerase genes and different levels of efflux. These differences occurred in several cases even though the bacteria had been exposed to the same simulated ciprofloxacin regimen. This suggests that the bacteria follow heterogeneous pathways of evolution and that the sequence of resistance mechanisms employed is not predetermined.

Of note, ciprofloxacin-resistant variants recovered from the in vitro system experiments maintained their resistant phenotype after seven passages on ciprofloxacin-free agar. Furthermore, the MICs for highly resistant bacteria that emerged during a 96-h experiment did not change even when the bacteria were maintained in the in vitro system for an additional 4 days without exposure to ciprofloxacin. These findings are consistent with the observation that susceptible bacteria readily evolve to resistance but rarely revert to a susceptible pheno-

type (33). For this reason, it is essential to prevent resistance from occurring in the first place.

The in vitro system has several characteristics that may have influenced our results. Resistant variants may have been more likely to appear because of the poorly described effects of cellular crowding, relative anoxia (37), and the absence of an immune system. Conversely, resistant bacteria may have been less likely to emerge because drug distribution in the peripheral compartment of the in vitro system was uniform and did not mimic the gradients often present within tissues (2, 3).

Despite potential limitations of the in vitro system, it is interesting to speculate on the clinical relevance of our in vitro findings. Of all the ciprofloxacin environments simulated in our system, the clinical pharmacokinetic profiles (400 mg every 8 or 12) were most likely to result in the emergence of *S. aureus* variants with high-level resistance and mutations in *grrA* and *gyrA*. This may explain, in part, the high prevalence of *grrA/gyrA* double mutant genotypes reported in collections of fluoroquinolone-resistant *S. aureus* clinical isolates (50, 56). These findings suggest that novel approaches to fluoroquinolone dosing are needed.

Our results differ from those of others who examined the killing and regrowth of *S. aureus* exposed to ciprofloxacin in in vitro systems (6, 17, 31, 35). The discrepancies between our results in which resistance (often high level) emerged and those of others in which resistance did not emerge may be due to factors such as the type of in vitro system used, phenotypic and genotypic heterogeneity of the bacteria tested, methods of drug administration, and the duration of the experiments.

Our work suggests that careful attention should be paid to population size, genotypes and phenotypes of resistant subpopulations, experiment duration, and profiles of drug exposure when examining the evolution of fluoroquinolone resistance in an in vitro system. Bacterial numbers must be sufficiently large ($>1/\text{resistance frequency}$) to ensure that resistant variants are present. Resistant subpopulation and mechanistic studies should be considered because of the genotypic and phenotypic heterogeneity in the resistance determinants observed in some bacterial populations. Experiments must be of sufficient duration to allow for the emergence of resistant variants since their appearance may be delayed if there is a fitness cost associated with resistance (1). The concentration-time profiles simulated with the in vitro system should mimic as closely as possible the patterns observed in vivo since small concentration differences may exert strong selection pressure. In the case of antimicrobial agents administered by intermittent infusion, the drug should be infused and not administered as a bolus into the central compartment to allow concentrations to accumulate until a steady state is reached. We propose that a consensus method for conducting studies of antimicrobial resistance in in vitro systems would be beneficial. If standards are adopted, data from different studies might be pooled, enhancing their utility. Standardization of pharmacodynamic terminology has already been proposed (38).

We evaluated the emergence of resistance when test strains were exposed to five different ciprofloxacin concentration-time profiles in the in vitro system. To fully characterize the relationship between pharmacokinetics and the emergence of resistance, additional concentration-time profiles should be sim-

ulated using dose escalation and fractionation approaches. Unfortunately, in vitro system studies of resistance are lengthy and resource and labor intensive. This limits the number of concentration-time profiles that can reasonably be simulated. The usefulness of the in vitro system might be enhanced if it were paired with a pharmacodynamic model integrating pharmacokinetic, subpopulation, and resistance mechanism data. The pharmacodynamic model could then be used to predict the fate of bacterial populations as a function of antimicrobial concentration. We demonstrate the potential utility of pharmacodynamic modeling in predicting the emergence of ciprofloxacin resistance in *S. aureus* populations in a companion paper (9).

ACKNOWLEDGMENTS

This work was supported, in part, by Public Health Service grant GM-066072 (to M.E.E.) from the National Institute of General Medical Sciences and by a grant from the Society of Infectious Diseases Pharmacists. Jeff Campion was supported by the American Foundation for Pharmaceutical Education and the University of Kentucky Research Challenge Trust.

We thank Robert P. Rapp for many helpful discussions of this work and Glenn W. Kaatz (Wayne State University School of Medicine) for kindly supplying the *S. aureus* control strains for ciprofloxacin efflux screening studies.

REFERENCES

- Andersson, D. I., and B. R. Levin. 1999. The biological costs of antibiotic resistance. *Curr. Opin. Microbiol.* **2**:489–493.
- Baquerio, F., and M. C. Negri. 1997. Selective compartments for resistant microorganisms in antibiotic gradients. *BioEssays* **19**:731–736.
- Baquerio, F., M. C. Negri, M. I. Morosini, and J. Blázquez. 1998. Antibiotic-selective environments. *Clin. Infect. Dis.* **27**(Suppl. 1):S5–S11.
- Baquerio, F., M. C. Negri, M. I. Morosini, and J. Blázquez. 1997. The antibiotic selective process: concentration-specific amplification of low-level populations, p. 93–111. In S. B. Levy (ed.), *Antibiotic resistance: origins, evolution, and spread*. John Wiley & Sons, New York, N.Y.
- Blaser, J. 1985. *In-vitro* model for simultaneous simulation of the serum kinetics of two drugs with different half-lives. *J. Antimicrob. Chemother.* **15**(Suppl. A):125–130.
- Blaser, J., B. B. Stone, M. C. Groner, and S. H. Zinner. 1987. Comparative study with enoxacin and netilmicin in a pharmacodynamic model to determine importance of ratio of antibiotic peak concentration to MIC for bactericidal activity and emergence of resistance. *Antimicrob. Agents Chemother.* **31**:1054–1060.
- Blondeau, J. M., X. Zhao, G. Hansen, and K. Drlica. 2001. Mutant prevention concentrations of fluoroquinolones for clinical isolates of *Streptococcus pneumoniae*. *Antimicrob. Agents Chemother.* **45**:433–438.
- Brockbank, S. M. V., and P. T. Barth. 1993. Cloning, sequencing, and expression of the DNA gyrase genes from *Staphylococcus aureus*. *J. Bacteriol.* **175**:3269–3277.
- Campion, J. J., M. E. Evans, and P. J. McNamara. Pharmacodynamic modeling of ciprofloxacin resistance in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.*, in press.
- Catchpole, C., J. M. Andrews, J. Woodcock, and R. Wise. 1994. The comparative pharmacokinetics and tissue penetration of single-dose ciprofloxacin 400 mg i.v. and 750 mg po. *J. Antimicrob. Chemother.* **33**:103–110.
- Diekema, D. J., M. A. Pfaller, F. J. Schmitz, J. Smayevsky, J. Bell, R. N. Jones, M. Beach, and the SENTRY Participants Group. 2001. Survey of infections due to *Staphylococcus* species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY antimicrobial surveillance program, 1997–1999. *Clin. Infect. Dis.* **32**(Suppl. 2):S114–S132.
- Dong, Y., X. Zhao, J. Domagala, and K. Drlica. 1999. Effect of fluoroquinolone concentration on selection of resistant mutants of *Mycobacterium bovis* BCG and *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **43**:1756–1758.
- Drlica, K., and X. Zhao. 1997. DNA gyrase, topoisomerase IV, and the 4-quinolones. *Microbiol. Mol. Biol. Rev.* **61**:377–392.
- Evans, M. E., and W. B. Titlow. 1998. Selection of fluoroquinolone-resistant methicillin-resistant *Staphylococcus aureus* with ciprofloxacin and trovafloxacin. *Antimicrob. Agents Chemother.* **42**:727.
- Ferrero, L., B. Cameron, and J. Crouzet. 1995. Analysis of *gyrA* and *grrA*

- mutations in stepwise-selected ciprofloxacin-resistant mutants of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **39**:1554-1558.
16. Ferrero, L., B. Cameron, B. Manse, D. Lagneaux, J. Crouzet, A. Famechon, and F. Blanche. 1994. Cloning and primary structure of *Staphylococcus aureus* DNA topoisomerase IV: a primary target of fluoroquinolones. Mol. Microbiol. **13**:641-653.
 17. Firsov, A. A., S. N. Vostrov, I. Y. Lubenko, K. Drlica, Y. A. Portnoy, and S. H. Zinner. 2003. In vitro pharmacodynamic evaluation of the mutant selection window hypothesis using four fluoroquinolones against *Staphylococcus aureus*. Antimicrob. Agents Chemother. **47**:1604-1613.
 18. Forrest, A., D. E. Nix, C. H. Ballow, T. F. Goss, M. C. Birmingham, and J. J. Schentag. 1993. Pharmacodynamics of intravenous ciprofloxacin in seriously ill patients. Antimicrob. Agents Chemother. **37**:1073-1081.
 19. Fournier, B., and D. C. Hooper. 1998. Mutations in topoisomerase IV and DNA gyrase of *Staphylococcus aureus*: novel pleiotropic effects on quinolone and coumarin activity. Antimicrob. Agents Chemother. **42**:121-128.
 20. Gibaldi, M., and D. Perrier. 1982. Pharmacokinetics, 2nd ed. Marcel Dekker, Inc., New York, N.Y.
 21. Gilbert, D. N., S. J. Kohlhepp, K. A. Slama, G. Gurnkemeier, G. Lewis, R. J. Dworkin, S. E. Slaughter, and J. E. Leggett. 2001. Phenotypic resistance of *Staphylococcus aureus*, selected *Enterobacteriaceae*, and *Pseudomonas aeruginosa* after single and multiple in vitro exposures to ciprofloxacin, levofloxacin, and trovafloxacin. Antimicrob. Agents Chemother. **45**:883-892.
 22. Gootz, T. D., and K. E. Brighty. 1998. Chemistry and mechanism of action of the quinolone antibacterials, p. 29-80. In V. T. Andriole (ed.), The quinolones, 2nd ed. Academic Press, San Diego, Calif.
 23. Hooper, D. C. 2001. Emerging mechanisms of fluoroquinolone resistance. Emerg. Infect. Dis. **7**:337-341.
 24. Hori, S., Y. Ohshita, Y. Utsui, and K. Hiramatsu. 1993. Sequential acquisition of norfloxacin and ofloxacin resistance by methicillin-resistant and -susceptible *Staphylococcus aureus*. Antimicrob. Agents Chemother. **37**:2278-2284.
 25. Ince, D., and D. C. Hooper. 2001. Mechanisms and frequency of resistance to gatifloxacin in comparison to AM-1121 and ciprofloxacin in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **45**:2755-2764.
 26. Ince, D., and D. C. Hooper. 2000. Mechanisms and frequency of resistance to premarloxacin in *Staphylococcus aureus*: novel mutations suggest novel drug-target interactions. Antimicrob. Agents Chemother. **44**:3344-3350.
 27. Ito, H., H. Yoshida, M. Bogaki-Shonai, T. Niga, H. Hattori, and S. Nakamura. 1994. Quinolone resistance mutations in the DNA gyrase *gyrA* and *gyrB* genes of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **38**:2014-2023.
 28. Kaatz, G. W., and S. M. Seo. 1997. Mechanisms of fluoroquinolone resistance in genetically related strains of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **41**:2733-2737.
 29. Kaatz, G. W., S. M. Seo, and C. A. Ruble. 1993. Efflux-mediated fluoroquinolone resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **37**:1086-1094.
 30. Kaatz, G. W., S. M. Seo, and C. A. Ruble. 1991. Mechanism of fluoroquinolone resistance in *Staphylococcus aureus*. J. Infect. Dis. **163**:1080-1086.
 31. Kang, S. L., M. J. Rybak, B. J. McGrath, G. W. Kaatz, and S. M. Seo. 1994. Pharmacodynamics of levofloxacin, ofloxacin, and ciprofloxacin, alone and in combination with rifampin, against methicillin-susceptible and -resistant *Staphylococcus aureus* in an in vitro infection model. Antimicrob. Agents Chemother. **38**:2702-2709.
 32. Lettieri, J., M. C. Rogge, L. Kaiser, R. M. Echols, and A. H. Heller. 1992. Pharmacokinetic profiles of ciprofloxacin after single intravenous and oral doses. Antimicrob. Agents Chemother. **36**:993-996.
 33. Levin, B. R., M. Lipsitch, V. Perrot, S. Schrag, R. Antia, L. Simonsen, N. M. Walker, and F. M. Stewart. 1997. The population genetics of antibiotic resistance. Clin. Infect. Dis. **24**(Suppl. 1):S9-S16.
 34. Maniatis, T. 1994. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, New York, N.Y.
 35. Marchbanks, R. C., J. R. McKiel, D. H. Gilbert, N. J. Robillard, B. Painter, S. H. Zinner, and M. N. Dudley. 1993. Dose ranging and fractionation of intravenous ciprofloxacin against *Pseudomonas aeruginosa* and *Staphylococcus aureus* in an in vitro model of infection. Antimicrob. Agents Chemother. **37**:1756-1763.
 36. Miles, A. A., S. S. K. Misra, and J. O. Irwin. 1938. The estimation of the bactericidal power of the blood. J. Hyg. **38**:732-749.
 37. Morrissey, I., and J. T. Smith. 1994. The importance of oxygen in the killing of bacteria by ofloxacin and ciprofloxacin. Microbios **79**:43-53.
 38. Mouton, J. W., M. N. Dudley, O. Cars, H. Derendorf, and G. L. Drusano. 2002. Standardization of pharmacokinetic/pharmacodynamic (PK/PD) terminology for anti-infective drugs. Int. J. Antimicrob. Agents **19**:355-358.
 39. Muñoz-Bellido, J. L., M. A. A. Manzanares, J. A. M. Andrés, M. N. G. Zufiaurre, G. Ortiz, M. S. Hernández, and J. A. García-Rodríguez. 1999. Efflux pump-mediated quinolone resistance in *Staphylococcus aureus* strains wild type for *gyrA*, *gyrB*, *grlA*, and *norA*. Antimicrob. Agents Chemother. **43**:354-356.
 40. National Committee for Clinical Laboratory Standards. 1999. Methods for determining bactericidal activity of antimicrobial agents. Approved guideline M26-A, vol. 19. National Committee for Clinical Laboratory Standards, Wayne, Pa.
 41. National Committee for Clinical Laboratory Standards. 2003. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A6, vol. 23. National Committee for Clinical Laboratory Standards, Wayne, Pa.
 42. Ng, E. Y., M. Trucksis, and D. C. Hooper. 1996. Quinolone resistance mutations in topoisomerase IV: relationship to the *flqA* locus and genetic evidence that topoisomerase IV is the primary target and DNA gyrase is the secondary target of fluoroquinolones in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **40**:1881-1888.
 43. Pan, X. S., P. J. Hamlyn, R. Talens-Visconti, F. L. Alovero, R. H. Manzo, and L. M. Fisher. 2002. Small-colony mutants of *Staphylococcus aureus* allow selection of gyrase-mediated resistance to dual-target fluoroquinolones. Antimicrob. Agents Chemother. **46**:2498-2506.
 44. Piddock, L. J., Y. F. Jin, M. A. Webber, and M. J. Everett. 2002. Novel ciprofloxacin-resistant nalidixic acid-susceptible mutant of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **46**:2276-2278.
 45. Preston, S. L., G. L. Drusano, A. L. Berman, C. L. Fowler, A. T. Chow, B. Dornseif, V. Reichl, J. Natarajan, and M. Corrado. 1998. Pharmacodynamics of levofloxacin. A new paradigm for early clinical trials. JAMA **279**:125-129.
 46. Sanger, F. 1981. Determination of nucleotide sequences in DNA. Science **214**:1205-1210.
 47. Schentag, J. J., A. K. Meagher, and A. Forrest. 2003. Fluoroquinolone AUC breakpoints and the link to bactericidal killing rates. Part 1: in vitro and animal models. Ann. Pharmacother. **37**:1287-1298.
 48. Schentag, J. J., A. K. Meagher, and A. Forrest. 2003. Fluoroquinolone AUC breakpoints and the link to bactericidal killing rates. Part 2: human trials. Ann. Pharmacother. **37**:1478-1488.
 49. Schentag, J. J., D. E. Nix, and M. H. Adelman. 1991. Mathematical examination of dual individualization principles. Relationships between AUC above MIC and area under the inhibitory curve (AUC) for cefmenoxime, ciprofloxacin, and tobramycin. DICP **25**:1050-1057.
 50. Schmitz, F. J., M. E. Jones, B. Hofmann, B. Hansen, S. Scheuring, M. Luckefahr, A. Fluit, J. Verhoef, U. Hadding, H. P. Heinz, and K. Kohrer. 1998. Characterization of *grlA*, *grlB*, and *gyrB* mutations in 116 unrelated isolates of *Staphylococcus aureus* and effects of mutations on ciprofloxacin MIC. Antimicrob. Agents Chemother. **42**:1249-1252.
 51. Shah, A., J. Lettieri, L. Kaiser, R. Echols, and A. H. Heller. 1994. Comparative pharmacokinetics and safety of ciprofloxacin 400 mg i.v. thrice daily versus 750 mg po twice daily. J. Antimicrob. Chemother. **33**:795-801.
 52. Shah, A., J. Lettieri, and D. Nix. 1995. Pharmacokinetics of high-dose intravenous ciprofloxacin in young and elderly and in male and female subjects. Antimicrob. Agents Chemother. **39**:1003-1006.
 53. Spellberg, B., J. H. Powers, E. P. Brass, L. G. Miller, and J. E. Edwards, Jr. 2004. Trends in antimicrobial drug development: implications for the future. Clin. Infect. Dis. **38**:1279-1286.
 54. Takahashi, H., T. Kikuchi, S. Shoji, S. Fijumura, A. Binte Lutfor, Y. Tokue, T. Nukiwa, and A. Watanabe. 1998. Characterization of *gyrA*, *gyrB*, *grlA*, and *grlB* mutations in fluoroquinolone-resistant clinical isolates of *Staphylococcus aureus*. J. Antimicrob. Chemother. **41**:49-57.
 55. Tanaka, M., Y. Onodera, Y. Uchida, and K. Sato. 1998. Quinolone resistance mutations in the *GrlB* protein of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **42**:3044-3046.
 - 55a. Thomas, J. K., A. Forrest, S. M. Bhavanani, J. M. Hyatt, A. Cheng, C. H. Ballow, and J. J. Schentag. 1998. Pharmacodynamic evaluation of factors associated with the development of bacterial resistance in acutely ill patients during therapy. Antimicrob. Agents Chemother. **42**:521-527.
 56. Wang, T., M. Tanaka, and K. Sato. 1998. Detection of *grlA* and *gyrA* mutations in 344 *Staphylococcus aureus* strains. Antimicrob. Agents Chemother. **42**:236-240.
 57. Wright, D. H., G. H. Brown, M. L. Peterson, and J. C. Rotschafer. 2000. Application of fluoroquinolone pharmacodynamics. J. Antimicrob. Chemother. **46**:669-683.
 58. Wright, D. H., V. K. Herman, F. N. Konstantinides, and J. C. Rotschafer. 1998. Determination of quinolone antibiotics in growth media by reversed-phase high-performance liquid chromatography. J. Chromatogr. B Biomed. Sci. Appl. **709**:97-104.
 59. Yamagishi, J., T. Kojima, O. Yoshihiro, K. Fujimoto, H. Hattori, S. Nakamura, and M. Inoue. 1996. Alterations in the DNA topoisomerase IV *grlA* gene responsible for quinolone resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **40**:1157-1163.
 60. Yoshida, H., M. Bogaki, S. Nakamura, K. Ubukata, and M. Konno. 1990. Nucleotide sequence and characterization of the *Staphylococcus aureus* *norA* gene which confers resistance to quinolones. J. Bacteriol. **172**:6942-6949.
 61. Zhao, X., and K. Drlica. 2001. Restricting the selection of antibiotic-resistant mutants: a general strategy derived from fluoroquinolone studies. Clin. Infect. Dis. **33**(Suppl. 3):S147-S156.