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Pharmacodynamic Model To Describe the Concentration-Dependent Selection of Cefotaxime-Resistant *Escherichia coli*

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Antibiotic dosing regimens may vary in their capacity to select mutants. Our hypothesis was that selection of a more resistant bacterial subpopulation would increase with the time within a selective window (SW), i.e., when drug concentrations fall between the MICs of two strains. An in vitro kinetic model was used to study the selection of two *Escherichia coli* strains with different susceptibilities to cefotaxime. The bacterial mixtures were exposed to cefotaxime for 24 h and SWs of 1, 2, 4, 8, and 12 h. A mathematical model was developed that described the selection of preexisting and newborn mutants and the post-MIC effect (PME) as functions of pharmacokinetic parameters. Our main conclusions were as follows: (i) the selection between preexisting mutants increased with the time within the SW; (ii) the emergence and selection of newborn mutants increased with the time within the SW (with a short time, only 4% of the preexisting mutants were replaced by newborn mutants, compared to the longest times, where 100% were replaced); and (iii) PME increased with the area under the concentration-time curve (AUC) and was slightly more pronounced with a long elimination half-life ($T_{1/2}$) than with a short $T_{1/2}$ situation, when AUC is fixed. We showed that, in a dynamic competition between strains with different levels of resistance, the appearance of newborn high-level resistant mutants from the parental strains and the PME can strongly affect the outcome of the selection and that pharmacodynamic models can be used to predict the outcome of resistance development.

The rapid evolution of antibiotic resistance in pathogenic bacteria, combined with a decreasing interest from the pharmaceutical industry in developing new antibiotics, has created a major public health problem (34, 48). As a result, activities to maintain the effects of existing antibiotics and thereby prolong their useful life span have a high priority. However, the knowledge of how to use existing antibiotics to minimize the emergence of resistance without compromising efficacy is today inadequate.

Among the most frequently used antibiotics are β-lactams, such as penicillins and cephalosporins (26). β-lactams interrupt the synthesis of the bacterial cell wall by forming a covalently bound complex with penicillin-binding proteins (PBPs), which are enzymes important in the final process of cell wall formation in bacteria (43, 44). The ability to produce TEM-β-lactamases is the main mechanism for β-lactam resistance in enteric gram-negative bacteria. The β-lactamase enzymes inactivate penicillins and other β-lactams by hydrolyzing the β-lactam ring (24). The first plasmid-mediated β-lactamase enzyme, TEM-1, was described shortly after the introduction of ampicillin for clinical use (6). Horizontal transfer of resistance genes led to a rapid interspecies spread of resistance, and today, TEM-1 is the most prevalent plasmid-mediated β-lactamase found in gram-negative organisms (40, 41, 47). Antibiotic pressure has selected for over 130 TEM-1 β-lactamase mutants with expanded hydrolytic capacities and activities against a variety of β-lactam antibiotics, including monobactams, carbapenems, and extended-spectrum cephalosporins (25, 42). TEM-12 is a descendant of the TEM-1 enzyme and differs in a single substitution of arginine for serine at position 164 (22, 45). As a monomutated β-lactamase, TEM-12 expresses an only slightly increased hydrolytic activity for cefotaxime. The most efficient TEM variants, which confer high-level resistance to cefotaxime, diverge from the native enzyme in several amino acids (4).

The growth of resistant subpopulations during treatment of a patient initially infected with susceptible bacteria presents an important problem. A number of in vitro studies have examined the effect of different dosing regimens in order to suppress the resistant subpopulations (1, 10, 23, 31, 35). A study by Negri et al. (31) revealed that low antibiotic concentrations can affect the selection of bacterial populations that show only small differences in susceptibility. Their work was based on a competition assay with *Escherichia coli* strains expressing different plasmid-borne variants of TEM-β-lactamase enzymes. Negri detected a range of cefotaxime concentrations, a selective window, at which the selection of the strain with highest level of resistance was most intense. The experiments, however, were performed with static antibiotic concentrations in culture. Since antimicrobial therapy usually results in fluctuating drug concentrations in the patient, the selection process during treatment can be expected to differ from that in models with static antibiotic concentrations. Therefore, the outcome of the static model is difficult to apply on an individual patient level. In our study using a kinetic model, the selective window

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(SW) was defined as the concentration range between the MICs of two strains.

The purposes of this study were as follows: (i) use an in vitro kinetic model to study the selection of cefotaxime-resistant *E. coli* for different time periods within the SW, and (ii) construct a general mathematical model that describes the expected changes in the bacterial population as a function of pharmacokinetic parameters. Our hypothesis was that a longer time within the SW would increase the selection of the more resistant strain, when two strains were competing in the model. Unlike earlier studies examining the efficacy of various dosing regimens in preventing the emergence of resistance, this model incorporates the selection of both preexisting and newborn mutants and any potential post-MIC effect (PME). The PME is the period when regrowth is delayed even after antibiotic concentrations have fallen below the MIC (13, 21) and, like the in vivo postantibiotic effect, includes the effects of subinhibitory concentrations (9, 27). The model provides a convenient theoretical framework to understand experimental data and a theoretical basis for optimal dosing regimens, in order to maintain efficacy while simultaneously preventing the emergence of resistance.

MATERIALS AND METHODS

Bacterial strains, growth controls, and media. The bacterial strains used in this study were a pair of *E. coli* strains, REL606(pBGTEM-1) and REL607(pBGTEM-12), kindly provided by Negri (31). The nonconjugative plasmids pBGTEM-1 and pBGTEM-12, constructed by Negri (31), carry the $bla_{\text{TEM-1}}$ and $bla_{\text{TEM-12}}$ β-lactamase genes, respectively. The original strains, *E. coli* B REL606 and REL607, have been used in previous studies (17, 18). REL606 is unable to grow on L-arabinose (Ara^-) and forms red colonies on tetrazolium-arabinose agar, while REL607 (Ara^+) forms pink colonies (18, 31). These chromosomal markers allowed identification of the two strains in a mixed population.

Growth rates were determined for strains REL606(pBGTEM-1) and REL607(pBGTEM-12) separately in tubes. These strains will be referred to as TEM-1 and TEM-12 in this paper. Competition experiments were performed in the in vitro kinetic model (described below) with an initial 1:1 ratio of the competing strains. In addition, the competition experiments were performed with an inverse pair, REL607(pBGTEM-1) and REL606(pBGTEM-12), to confirm the neutrality of the plasmids and the arabinose marker of the host bacteria.

The plasmids have kanamycin resistance as a selective marker; hence, the strains were maintained on Columbia agar (Acumedia Manufacturers, Inc., Baltimore, MD) plates supplemented with 30 μg kanamycin/ml. The liquid medium used for bacterial growth was Mueller-Hinton broth (Difco Laboratories, Detroit, MI), and the solid medium in the assays was tetrazolium-arabinose indicator agar (18). The bacteria were grown at 35°C, and liquid cultures were incubated without shaking.

Antimicrobial agents. Cefotaxime powder was obtained from Aventis (Stockholm, Sweden) and was dissolved in 1 ml sterile distilled water to a concentration of 10 mg/ml. Fresh stock solutions were prepared on the day of use and diluted in Mueller-Hinton broth.

Susceptibility testing. The MICs of cefotaxime for the native strains were determined by a macrodilution technique according to CLSI (formerly NCCLS) standards (30) and were done in triplicate on separate occasions. The MICs for the strains containing TEM-1 and TEM-12 were 0.016 and 0.063 μg/ml, respectively, and these MIC values were used for the study design.

To detect the appearance of novel resistant mutants during exposure to cefotaxime, colonies were taken from the 24-h samples and analyzed with Etest on Columbia agar plates according to the instructions by the manufacturer (AB Biodisk, Solna, Sweden). The Etest method resulted in slightly lower MICs for the parental strains, 0.012 μg/ml for TEM-1 and 0.032 μg/ml for TEM-12, than with the macrodilution technique.

Determination of antibiotic concentrations. The initial cefotaxime concentrations in the in vitro kinetic experiments were determined with a microbiological agar diffusion method. Plates with tryptone-glucose agar, pH 7.4, were seeded with a standardized inoculum of *Escherichia coli* MB3804. Antibiotic standards and samples from the experiments were applied to agar wells at a volume of 30 μl, and the plates were incubated overnight at 35°C. All assays were made in triplicate and the correlation coefficient for the standard curves was always ≥0.99.

TABLE 1. C_{max} and $T_{1/2}$ values

SWs	C_{max} (μg/ml)	$T_{1/2}$ (h)[a]
1 h	0.25	1 (0–2), 0.5 (2–3), 1 (3–24)
2 h	0.25	1 (0–24)
4 h	0.125	2 (0–24)
8 h	0.125	2 (0–2), 4 (2–24)
12 h	0.125	2 (0–2), 6 (2–24)
1 h	1	0.75 (0–3), 0.5 (3–4), 1 (4–24)
2 h	1	0.75 (0–3), 1 (3–24)
4 h	0.5	1 (0–3), 2 (3–24)
8 h	0.5	1 (0–3), 4 (3–24)
12 h	0.5	1 (0–3), 6 (3–24)

[a] Numbers in parentheses are the time periods (h) for the respective $T_{1/2}$.

In vitro kinetic model. The in vitro kinetic model used in this study has been described earlier (12, 21). It consists of a spinner flask (110 ml) with an open bottom that was placed on a holder with an outlet connected to a pump (P-500; Pharmacia Biotech, Uppsala, Sweden). A filter membrane with a pore size of 0.45 μm was supported by a metal rack between the flask and the holder, impeding the dilution of bacteria. A magnetic stirrer ensured a homogenous mixing of the culture and prevented membrane pore blockage. The spinner flask had two side arms: one with a silicone membrane inserted to enable repeated sampling and another connected to plastic tubing from a vessel containing fresh medium. The medium was drawn from the culture vessel, through the filter, at a given rate by the pump. Fresh medium was sucked into the flask at the same rate by the negative pressure built up inside. Antibiotic added to the flask was diluted according to the first-order kinetics according to equation 3 in the mathematical model. The apparatus was placed in a thermostatic room at 35°C during the experiment.

Study design: selective windows. Competition assays were performed with various times within the SW, i.e., time periods when the concentration of cefotaxime is below the MIC for TEM-12 but above the MIC for TEM-1. The flask was prepared with broth and the desired initial antibiotic concentration (C_{max}, Table 1) and was installed in the thermostatic room (35°C). Bacteria from 6- to 7-h broth cultures were added to the flask to create a culture mixture of TEM-1 and TEM-12 at a proportion of 99:1. The initial bacterial concentrations of TEM-1 and TEM-12 β-lactamase-producing strains were 10^5 CFU/ml and 10^3 CFU/ml, respectively. The time that the concentrations exceeded the MIC ($T >$ MIC) for the TEM-12 strain was 2 h in all SWs, while $T >$ MIC for the TEM-1-producing strain was varied. The elimination half-life ($T_{1/2}$) in the kinetic model was adjusted accordingly and, if needed, changed during the experiments to obtain SWs of 1, 2, 4, 8, and 12 h (Table 1 and Fig. 1), and the experiments were run for 24 h. Samples of 200 to 400 μl were withdrawn at different time points and treated with penicillinase type IV (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) for 20 min to prevent antibiotic carryover. Dilutions of the samples were then seeded on tetrazolium-arabinose indicator plates, and after 24 h at 35°C, the pink and red colonies were counted. The limit of detection for viable counts was 10 CFU/ml. The strains were easily discriminated in all experiments except for the 24-h sample in two SWs with increased C_{max} where there was heavy growth of TEM-12 (see Results). The experiments were repeated five times except SW 2 h, for which 10 separate experiments were performed for estimation of parameters in the statistical model. MIC determinations were performed with Etest as described in Materials and Methods.

Selective windows with increased dose of cefotaxime. The experimental design described above was repeated using a fourfold higher C_{max}. In these experiments, $T_{1/2}$ was simulated to attain a $T >$ MIC of 3 h for the TEM-12-producing strain and SWs of 1, 2, 4, 8, and 12 h. The kinetics used in this set of experiments are shown in Table 1. Experiments were performed twice for each SW, and possible changes in cefotaxime susceptibility were detected with Etest as previously described.

Characterization of high-level cefotaxime-resistant mutants. The MICs of cefotaxime, chloramphenicol, and tetracycline were determined with Etest for parental and mutated strains TEM-1 and TEM-12, as well as for *E. coli* MG1655 and *E. coli* LM201 (*ompF*<>FRT; derived in *Escherichia coli* MG1655, the

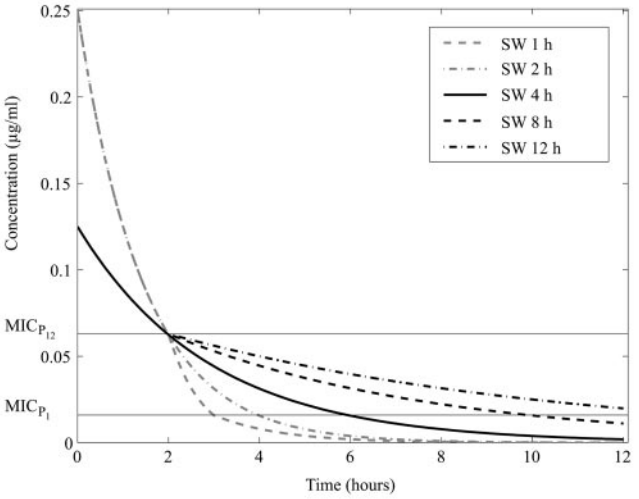


FIG. 1. Concentration profiles of cefotaxime for five selective windows (1, 2, 4, 8, and 12 h) with low C_{max}. MIC_{P1} and MIC_{P12} indicate the MICs of the parental populations TEM-1 and TEM-12, respectively.

Δ*ompF* has been generated by homologous recombination technology). For PCR amplification and DNA sequencing of *ompF*, DNA was prepared from parental and mutated strains TEM-1 and TEM-12 using the Wizard genomic DNA purification kit (Promega, Madison, WI). The primer sequences used for PCR and sequencing were constructed from the *ompF* gene of *E. coli* K12: 1F (5′-CGTGAGATTGCTCTGGAAGG-3′), 3R (5′-CTCAACCTCTTGGCA ACGGTA-3′), 2F (5′-TCGTACTTCAGACCAGTAGC-3′), 5R (5′-ACGGTG AAAACAGTTACGGT-3′), 4F (5′-ATTGATTTGAGTTTCCCCTTTA-3′), and 6R (5′-TGACGGTGTTCACAAAGTTCC-3′). PCR was carried out in 20-μl volumes containing 1 μM forward and reverse primers, 0.5 μl DNA sample, and 5 mM Mg^{2+} (3 mM for primers 4F and 6R). The reactions were run in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA) and the following temperature profile was used: initial denaturation at 95°C for 30 s; 30 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 7 min. For primers 4F and 6R, the annealing temperature was 53°C. The PCR products were purified with a GFX-DNA purification kit (Amersham Biosciences, Uppsala, Sweden). A BigDye Terminator v 1.1 cycle sequencing kit (Applied Biosystems) was used for sequencing, and the analysis was performed with an ABI 3100 Genetic Analyzer, a multicolored-fluorescence-based DNA analyzing system. The parental and mutated *E. coli* strains TEM-1 and TEM-12 were also tested for organic solvent tolerance as previously described by Komp Lindgren et al. (16).

RESULTS

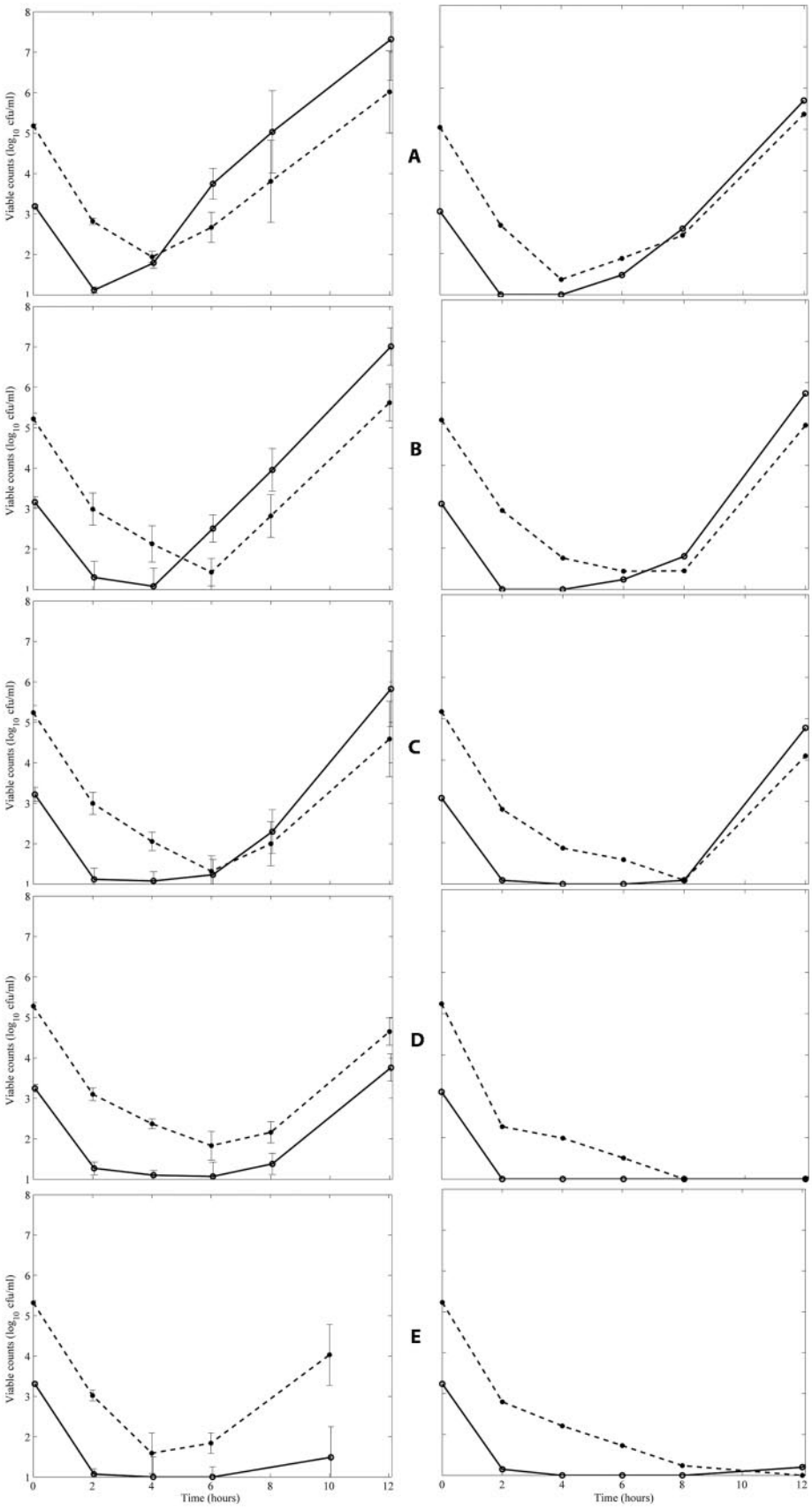
Growth controls. No differences in the growth rates of single cultures of *E. coli* strains TEM-1 and TEM-12 could be observed. Similarly, the competition experiments in the absence of antibiotic showed that the initial ratio (1:1) of the two strains was unchanged after 24 h; also, no differences were noted for the inverse pair. This confirmed previous published results (17, 31) showing that the plasmids and the arabinose genetic markers are neutral.

Selective windows. The mean initial cefotaxime concentrations were within 10% of expected values (coefficient of variation, 11%). When TEM-1 and TEM-12 were mixed at a proportion of 99:1 in the in vitro kinetic model and challenged with cefotaxime to obtain different times within the SW, an increase in the proportion of the TEM-12-producing strain was observed in the SWs for 1, 2, 4, and 8 h (Fig. 2A to D, left panels). Since regrowth of both strains was apparent already after 12 h, results only up to this time point are shown in the graphs. In the first three SWs (1, 2, and 4 h) there was a clear dominance of TEM-12 but, unexpectedly, the selection of TEM-12 appeared to be less effective in the SW of 8 h, and in the SW of 12 h, TEM-1 was selected (Fig. 2E, left panel).

Two phenomena were discovered that influenced the outcome of the selection. First, for the two longest times within the SWs (8 and 12 h), strain TEM-1 recovered several hours before the cefotaxime concentration had decreased to the MIC. This unexpected growth was most likely due to a new acquired resistance that was detected for the TEM-1-harboring strain. The strain repeatedly attained a high-level resistance (MIC = 0.094 to 0.19) in the SWs of 8 and 12 h, and occasionally, in the SW of 4 h. In contrast, strain TEM-12 retained its original MIC throughout most of the experiments; a decrease in cefotaxime susceptibility (MIC = 0.19 to 0.50) was only noted a few times for the experiments with long times within the SW, presumably due to the lower bacterial inoculum. A second phenomenon affecting the selection model was a PME. This was most apparent for the TEM-12-producing strain, again as a consequence of the lower rate of newly formed mutants for this strain. For TEM-12, $T >$ MIC was fixed in the five SWs, but although subinhibitory concentrations were attained after 2 h, suppression of bacterial growth persisted. The PME for the TEM-12-harboring strain was most pronounced for long times within the SW; no PME was detected for the shortest time of 1 h.

Selective windows with increased concentration of cefotaxime. To minimize the selection of high-level resistant mutants, experiments were performed with increased C_{max} (Fig. 2A to E, right panels). In these experiments, growth of strain TEM-1 was reduced and possibly prevented in the SW of 8 and 12 h. Since the TEM-12-producing bacteria were in dominance, potential colonies of the TEM-1 strain could not be separated in the mixed population. Thus, they were scored as zero growth. No increase in MICs was seen for strain TEM-1 colonies except in one of the two 4-h SWs (MIC = 0.094). With a high antibiotic concentration, the growth of newly formed mutants of TEM-1-producing bacteria was prevented. As a result, selection of the TEM-12-producing strain was increased in the SW of 8 and of 12 h. Bacterial regrowth of TEM-12 was noted after 12 h in these two SWs (Fig. 2D to E, right panels), and at 24 h, TEM-12 had grown more than 7 log CFU, while TEM-1 was undetectable. With even higher concentrations of cefotaxime (4 μg/ml) both *E. coli* strains could be completely eliminated (data not shown).

Characterization of high-level cefotaxime-resistant mutants appearing in the competition experiments. The high-level cefotaxime-resistant mutants that appeared showed MICs of chloramphenicol and tetracycline that were four times higher than for the parental strains. This finding suggested that cefotaxime, chloramphenicol, and tetracycline resistance were caused by an inactivation of a transport function or activation of an efflux system. For example, an *ompF* mutation could cause the cefotaxime-resistant phenotype (31). To examine this possibility, the MICs of the high-level cefotaxime-resistant strains were compared with the MICs for two isogenic *E. coli* strains, one wild type and one with a deletion in *ompF*. However, the defined *ompF* mutation had a much smaller effect on the MICs for chloramphenicol and tetracycline than did those



in our mutants, suggesting that the mutations were not in *ompF*. In addition, DNA sequencing of the *ompF* gene in parental and mutated strains TEM-1 and TEM-12 revealed no changes. To further investigate the high-level cefotaxime resistance, the organic solvent tolerance was measured, a phenotype associated with overexpression of the transmembrane AcrAB-TolC multidrug efflux pump (46). However, none of the tested bacteria were tolerant to cyclohexane.

Mathematical model. Since there was no simple mathematical relationship between the selection and the time within the SW, a mathematical model of pharmacokinetics and bacterial population dynamics was constructed, with the aim to predict how the time within the SW affects the selection and/or the emergence of resistance. A technical description of the model has previously been published as a Master's thesis from Stockholm University (11).

(i) Pharmacokinetics. The elimination of the initial concentration of antibiotics, C_{max}, follows first-order kinetics with a elimination rate $k(t)$ that changes at some time points depending on the experimental setting. Thus

$$\frac{dC}{dt} = -k(t)C(t) \quad (1)$$

with

$$k(t) = \begin{cases} 0 \le k_0 < t_1 \\ t_1 \le k_1 < t_2 \\ k_2 \ge t_2, \end{cases} \quad (2)$$

where t_1 is the time point when the elimination rate k_0 was changed to k_1, and t_2 is the time point when k_1 was changed to k_2. The elimination rates and time points used are defined in Table 1. Solving the differential equation yields

$$C(t) = \begin{cases} C_{max}e^{-k_0 t} & 0 \le t < t_1 \\ C_{max}e^{-k_0 t_1 - k_1(t - t_1)} & \text{if } t_1 \le t < t_2 \\ C_{max}e^{-k_0 t_1 - k_1(t_2 - t_1) - k_2(t - t_2)} & t \ge t_2. \end{cases} \quad (3)$$

(ii) The basic model of population dynamics. The parental strains are denoted by *P*. Changes in these populations will, in a simple model, depend only on the net growth rate $\lambda(t)$ (can be negative or positive), i.e., the rate of cell division minus kill rate due to antibiotics, as follows:

$$\frac{dP}{dt} = \lambda(t)P(t). \quad (4)$$

However, to make the model more realistic, the appearance of mutants and the PME were included.

Extensions of the model. (i) Appearance of mutants. It was assumed that mutations occurred with a constant rate α during the whole experimental period. Thus, the number of parental bacteria decreases at the same rate as mutants occur. Mutants are denoted by *M*, which adds the following term to the basic model:

$$\frac{dP}{dt} = \lambda(t)P(t) - \alpha P(t)$$
$$\frac{dM}{dt} = \lambda(t)M(t) + \alpha P(t). \quad (5)$$

Here the net growth, $\lambda(t)$, depends on the concentration at each time point and will explain some of the PME observed in the experiments. Figure 3 shows the rates that determine the population dynamics. The sums of parental and mutant populations, *S*, are the numbers of bacteria that are observed in the experiments.

(ii) PME. The modeling of the PME assumed that bacterial killing with antibiotics and regrowth of the population depend on both the antibiotic saturation and the synthesis of PBPs (19, 43, 49). Let $B(t)$ denote the number of unsaturated PBPs at time *t*, and let B_{max} denote the maximal number of PBPs before the inclusion of any drug effect. Then the changes in the relative number of unsaturated PBPs, $Q = B(t)/B_{max}$, can be illustrated by Fig. 4. The figure shows that PBPs are saturated by antibiotics with a rate γ and are synthesized with a rate β. This can be expressed as:

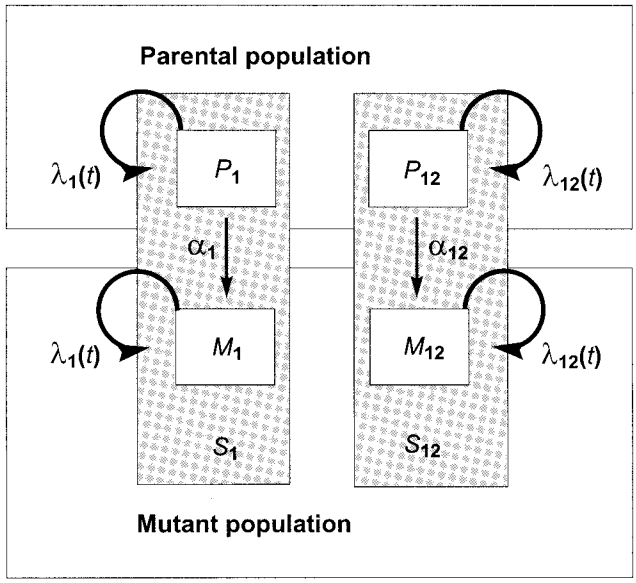


FIG. 3. Illustration of the relative growth rates $\lambda_1(t)$ and $\lambda_{12}(t)$ and mutation rates α_1 and α_{12}. The sums of the parental population and the mutated population, S_1 ($P_1 + M_1$) and S_{12} ($P_{12} + M_{12}$), represent the numbers of bacteria that will be observed during the experiments.

FIG. 2. Competition assays with *E. coli* strains TEM-1 and TEM-12 exposed to cefotaxime in the in vitro kinetic model. Five selective windows were investigated: 1 h (A), 2 h (B), 4 h (C), 8 h (D), and 12 h (E). In the first series (low C_{max}; left panels) $T >$ MIC was varied for TEM-1 (3, 4, 6, 10, and 14 h), and fixed (2 h) for TEM-12. Each graph displays means of 5 experiments with the exception of SW 2 h, which shows means based on 10 experiments. The bars represent standard deviations. In the second series of SWs (high C_{max}; right panels) the cefotaxime doses were four times higher to prevent the emergence of high-level resistant mutants. $T >$ MIC was varied for strain TEM-1 (4, 5, 7, 11, and 15 h), and fixed (3 h) for strain TEM-12. Each graph displays the means of two experiments. Solid line, strain TEM-12; dashed line, strain TEM-1.

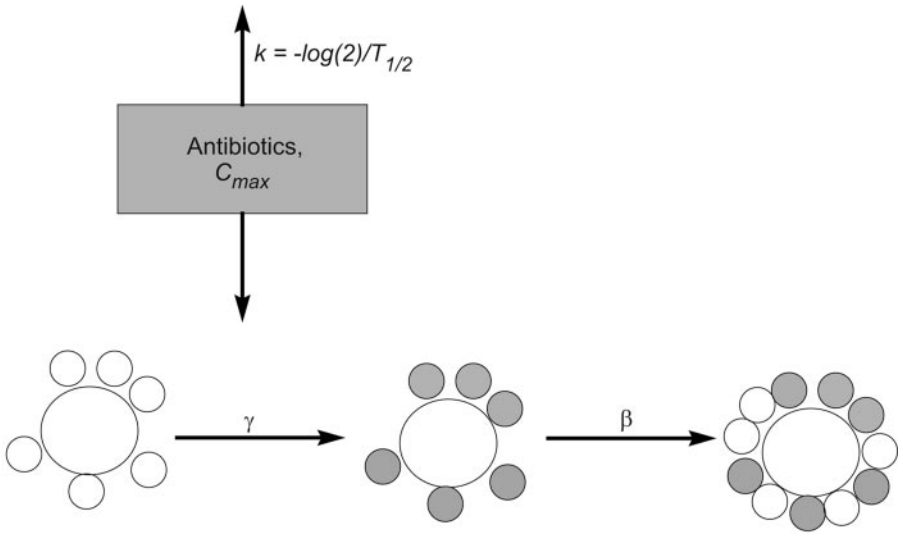


FIG. 4. Modeling of PME. Antibiotic saturation and synthesis of PBPs depends on the initial concentration of drug and the half-life time. The binding rate of antibiotics to PBPs is denoted by γ and the synthesis rate of new PBPs by β. Open circles represent PBPs without bound antibiotic and filled circles represent PBPs to which antibiotic is bound.

$$\frac{dQ}{dt} = \beta - \gamma C(t)Q(t). \tag{6}$$

The changes in the population are assumed to be proportionally dependent with a constant ν on the changes in PBP, meaning that:

$$\lambda(t) = \nu Q(t) + \lambda_{\min}. \tag{7}$$

The minimum bacterial net growth rate, $\lambda_{\min}$, can be negative under antibiotic pressure and is assumed to be present when all PBPs are saturated. Conversely, in the absence of antibiotics when no PBPs are saturated, a maximal growth rate of bacteria, $\lambda_{\max}$, is present. In this case $Q(t) = 1$, and $\lambda(t)$ reduces to $\lambda(t) = \lambda_{\max}$ and $\nu = \lambda_{\max} - \lambda_{\min}$.

In the case of no PME, the stationary concentration, i.e., the concentration at which bacteria are neither killed nor able to grow (28), is expected to be equivalent to the MIC. In other words, the net growth $\lambda(t)$ equals zero when the concentration at time point t is equal to the MIC. Furthermore, if the number of unbound PBPs at the moment when the concentration has reached the MIC (C_{MIC}) is denoted by Q_{MIC}, equation 7 yields the following relationship:

$$\lambda(0) = \nu Q_{\mathrm{MIC}} + \lambda_{\min} = 0$$

$$\Rightarrow Q_{\mathrm{MIC}} = -\frac{\lambda_{\min}}{\lambda_{\max} - \lambda_{\min}}. \tag{8}$$

It was furthermore assumed that the dynamics of PBPs was much faster than the dynamics of the concentration. Hence, from equation 6,

$$Q(t) \approx -\frac{\beta}{\gamma C(t)}. \tag{9}$$

Equations 8 and 9 now give the following differential equation system:

$$\begin{aligned}
\frac{dQ_P}{dt} &= \beta\left(1 + \frac{\lambda_{\max} - \lambda_{\min}}{\lambda_{\min} C_{\mathrm{MIC}_P}} C(t)Q_P(t)\right) \\
\frac{dP}{dt} &= ((\lambda_{\max} - \lambda_{\min})Q_P(t) + \lambda_{\min})P(t) - \alpha P(t) \\
\frac{dQ_M}{dt} &= \beta\left(1 + \frac{\lambda_{\max} - \lambda_{\min}}{\lambda_{\min} C_{\mathrm{MIC}_M}} C(t)Q_M(t)\right) \\
\frac{dM}{dt} &= ((\lambda_{\max} - \lambda_{\min})Q_M(t) + \lambda_{\min})M(t) + \alpha P(t).
\end{aligned} \tag{10}$$

Depending on which strain we refer to, the parameters will

TABLE 2. Parameter estimates of the model[a]

P_1 and M_1		P_{12} and M_{12}	
Parameter	Estimate	Parameter	Estimate
$\lambda_{\max}$	1.8 h^{-1}	$\lambda_{\max}$	1.8 h^{-1}
$\lambda_{\min}$	−2.3 h^{-1}	$\lambda_{\min}$	−2.3 h^{-1}
α_1	$8.19 \cdot 10^{-10}$ h^{-1} ($8.19 \cdot 10^{-10}$, $8.37 \cdot 10^{-10}$)	α_{12}	$1.42 \cdot 10^{-9}$ h^{-1} ($1.42 \cdot 10^{-9}$, $1.42 \cdot 10^{-9}$)
β_1	1.00 h^{-1} (0.99, 1.012)	β_{12}	0.77 h^{-1} (0.76, 0.78)
MIC_{P_1}	0.0070 μg/ml (0.0056, 0.0084) (0.012)	$\mathrm{MIC}_{P_{12}}$	0.037 μg/ml (0.036, 0.038) (0.032–0.048)
MIC_{M_1}	0.19 μg/ml (0.15, 0.23) (0.094–0.19)	$\mathrm{MIC}_{M_{12}}$	0.50 μg/ml (0.49, 0.51) (0.19–0.50)

[a] Values within first set of parentheses per entry are confidence intervals for the estimates, and values within second set are observed MICs.

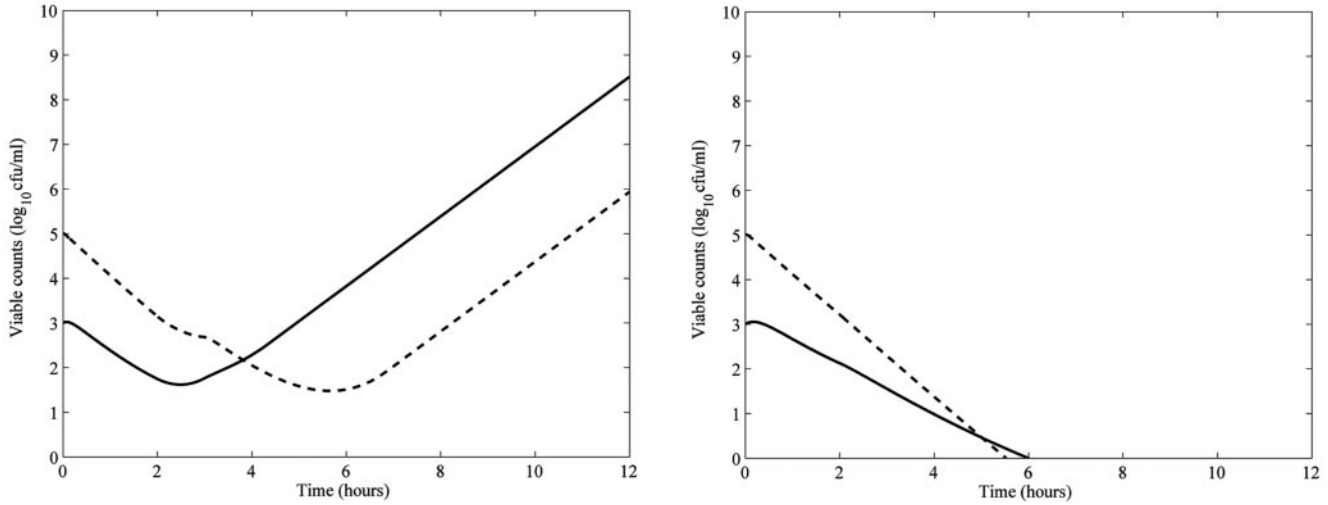


FIG. 5. Predictions of the outcome of the parental strains P_1 and P_{12} for competition assays with *E. coli* strains TEM-1 and TEM-12 with two of the selective windows that were investigated: 1 h (left) and 12 h (right). Dashed line, P_1; solid line, P_{12}.

differ (see parameter estimates in Table 2). The differential equation 10 has no analytical solution and was therefore solved numerically using Matlab 6.5.

Parameter estimation. Since the model defined in equation 10 is deterministic, it does not encompass the uncertainty due to measure or stochastic variation. However, by specifying a model for the underlying probability mechanism, inference about the parameters can be achieved. Since the variance in data increased with the number of CFU, a variant of weighted least-squares regression that takes the heteroscedasticity into account was chosen (see Appendix). For the parameter estimation, data from 10 independent experiments with SW of 2 h were used. Estimates are shown in Table 2.

Note that the MICs were estimated as unknown parameters for all strains. Therefore these estimates of MICs can be compared to those measured by Etest (Table 2). The difference between measured data and estimates from the model is small, which provides a validation of the model.

Prediction of the selection and the proportion of mutants. Predictions of the outcomes of the parental strains indicate that the selection of parental TEM-12 increases with the time within the SW (1, 2, or 4 h), as long as the level of antibiotics is low enough to allow regrowth of this strain (Fig. 5). Thus, our theory holds for the parental strains. Since the proportion of mutants appears to increase with the time within the SW (Fig. 6) it will no longer be possible to see a relationship between selection and time within SW. The proportion of mutant TEM-12 organisms becomes high later than mutant TEM-1, which is due to the initially smaller inoculum of parental TEM-12.

PME. Generally, the PME increases with area under the concentration-time curve (AUC), but the shape of the AUC is

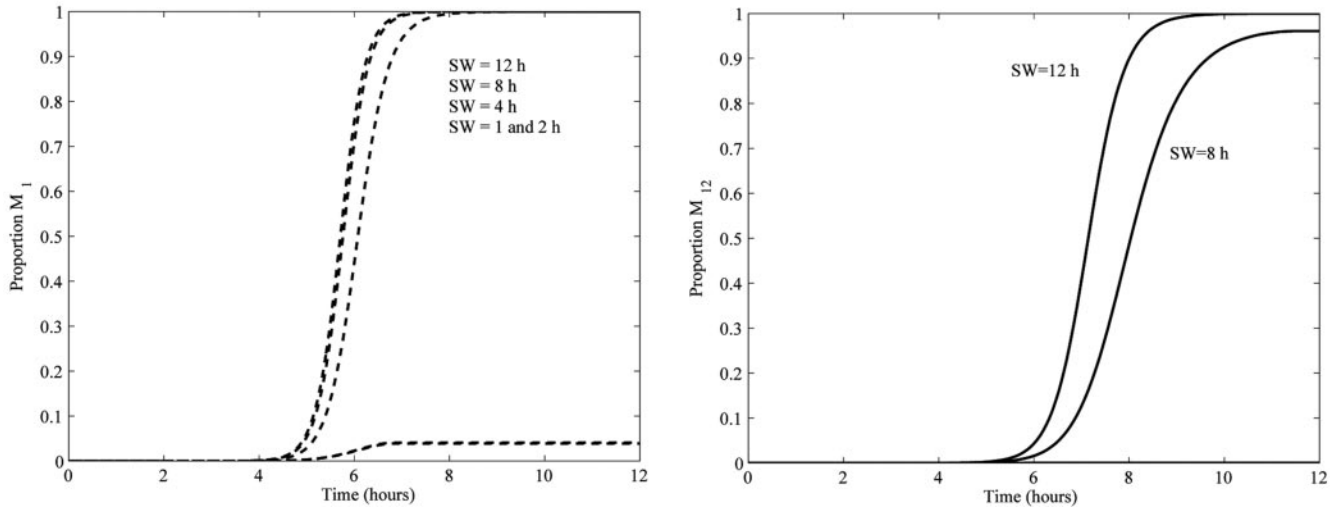


FIG. 6. The proportions of mutants, $M_1/(P_1 + M_1)$ (left), and $M_{12}/(P_{12} + M_{12})$ (right), estimated from the predicted values for the five selective windows that were investigated: 1, 2, 4, 8, and 12 h. Dashed line, strain TEM-1; solid line, strain TEM-12.

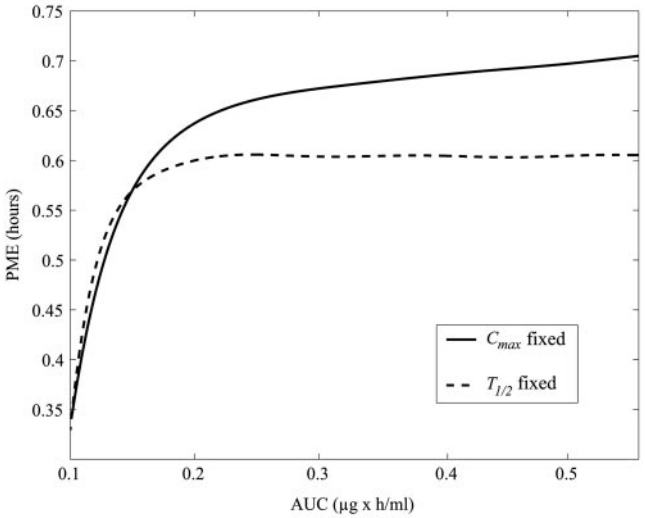


FIG. 7. Predicted PME for two cases yielding the same AUC: constant C_{max} of 0.1 μg/ml and $T_{1/2}$ varying from 0.5 to 5 h; and constant $T_{1/2}$ of 0.5 h and C_{max} varying from 0.1 to 0.8 μg/ml.

also important. Thus, increasing the AUC by varying the $T_{1/2}$ will have a greater impact on the PME than the corresponding increase in C_{max} (Fig. 7).

Prediction of parental and mutant populations. To examine the validity of the model, the outcomes for the four other time periods within the SW (1, 4, 8, and 12 h), with low and high C_{max}, were predicted and compared with experimental data. Observed data were well included by a 95% prediction interval. Predictions for SW of 1 and 12 h are shown in Fig. 8.

DISCUSSION

An important strategy to reduce antibiotic resistance development is the implementation of drug dosing regimens that minimize the appearance of resistance mutants without compromising efficacy. In this context, realistic pharmacodynamic models that allow prediction of the effect of the dosing regimens are helpful. Most published models describe the relationship between the net growth rate of a bacterial population and the antibiotic concentration using an E_{max} model (2, 3, 5, 7, 14, 20, 29, 32, 33, 37) but do not include the appearance of resistant mutants during drug exposure or the post-MIC effect.

Here, we present a pharmacodynamic model that, in contrast to previous models, includes rates for the occurrence of mutants and the saturation and synthesis of PBPs. Thus, the model can be used to predict the selection of both preexisting and newborn mutants as well as the effect of any potential PME. By reestimating parameters, the model can be used for predictions of pathogens and antibiotics other than *Escherichia coli* and cefotaxime.

From an earlier study, it was expected that the selection of the more resistant parental strain (TEM-12) would increase with the time within the selective window (31). In concordance with this hypothesis, our experimental data showed a high dominance of the TEM-12 strain in the 1-, 2-, and 4-h SWs. However, when SWs of 8 and 12 h were tested, the selection of TEM-12 actually decreased and in the SW of 12 h, the low-resistance parental strain (TEM-1) was selected. The lack of correlation between the strength of selection and the time within the SW was a result of emergence of newborn high-level resistant mutants and the influence of PME. The experiments demonstrated that the TEM-1 strain repeatedly attained a high-level resistance in the SWs of 8 and 12 h, and occasionally in the SW of 4 h. The mutants that appeared from the TEM-1 strain had MICs about 12 times the original MIC, a resistance level higher than for the parental TEM-12 strain. This explains why selection of the TEM-12 strain was decreased for longer times within the SW. Increasing the initial cefotaxime concentration four times prevented the growth of new mutants from TEM-1 in almost every experiment. This led to an increased selection of TEM-12 in SW of 8 and 12 h, which better concurs with the hypothesis that longer time within the selective window increases selection of the more resistant parental strain (TEM-12).

To validate the model, we compared the predicted outcome with observed data. The predictions were found satisfactory regarding both the selection of preexisting and newborn mutants and PME, despite the fact that the following simplifications were made. First, the mutants were assumed to appear with a constant rate, and not randomly. Second, there was no fitness cost associated with the high-level mutants. The latter simplification does not alter the prediction for which strain is selected, but it influences the amount of the selection and may explain the increased deviance between observed data and predicted outcomes in experiments. Finally, a fundamental difference between our model and antimicrobial treatment in patients is the lack of a host immune response in the model. Thus, in vivo, the antimicrobial efficacy and potency of drugs are assisted by immune factors. To increase the predictive power of future refined pharmacodynamic models, relevant immunological parameters should be included.

In a situation where antibiotic concentrations are declining and newborn high-level resistant mutants are formed, the outcome becomes complex and will strongly depend on the concentration that prevents growth of the most resistant strain. Obviously, if drug concentrations are continuously maintained above this concentration, no resistant mutants will appear. Importantly, even shorter time periods above this concentration can effectively prevent appearance of newborn mutants (see Results and Fig. 2, right panels). The issue of suppression of resistant subpopulations has also been addressed by Jumbe et al., who used a mathematical model to calculate an AUC/MIC ratio that amplified a mutant subpopulation in vivo as well as a ratio that prevented the emergence of resistance (15). Here we showed that if drug concentrations are lower and are maintained in the selective window, selection of the more resistant parental strain (TEM-12) as well as mutants from both parental strains will occur and increase with longer time within the SW. Using a fixed AUC, selection will be minimized using a high-dose, short-elimination half-life regimen rather than a low-dose, long half-life regimen. With regard to the PME, it can vary with the pharmacokinetic profile (8, 9, 21). In our model, with a fixed AUC the PME is slightly more pronounced, with a long half-life rather than a short one. Thus, although a long PME would allow extended dosing intervals with preserved efficacy, it would also promote resistance.

In conclusion, our experimental data and mathematical modeling show that in a dynamic competition between strains

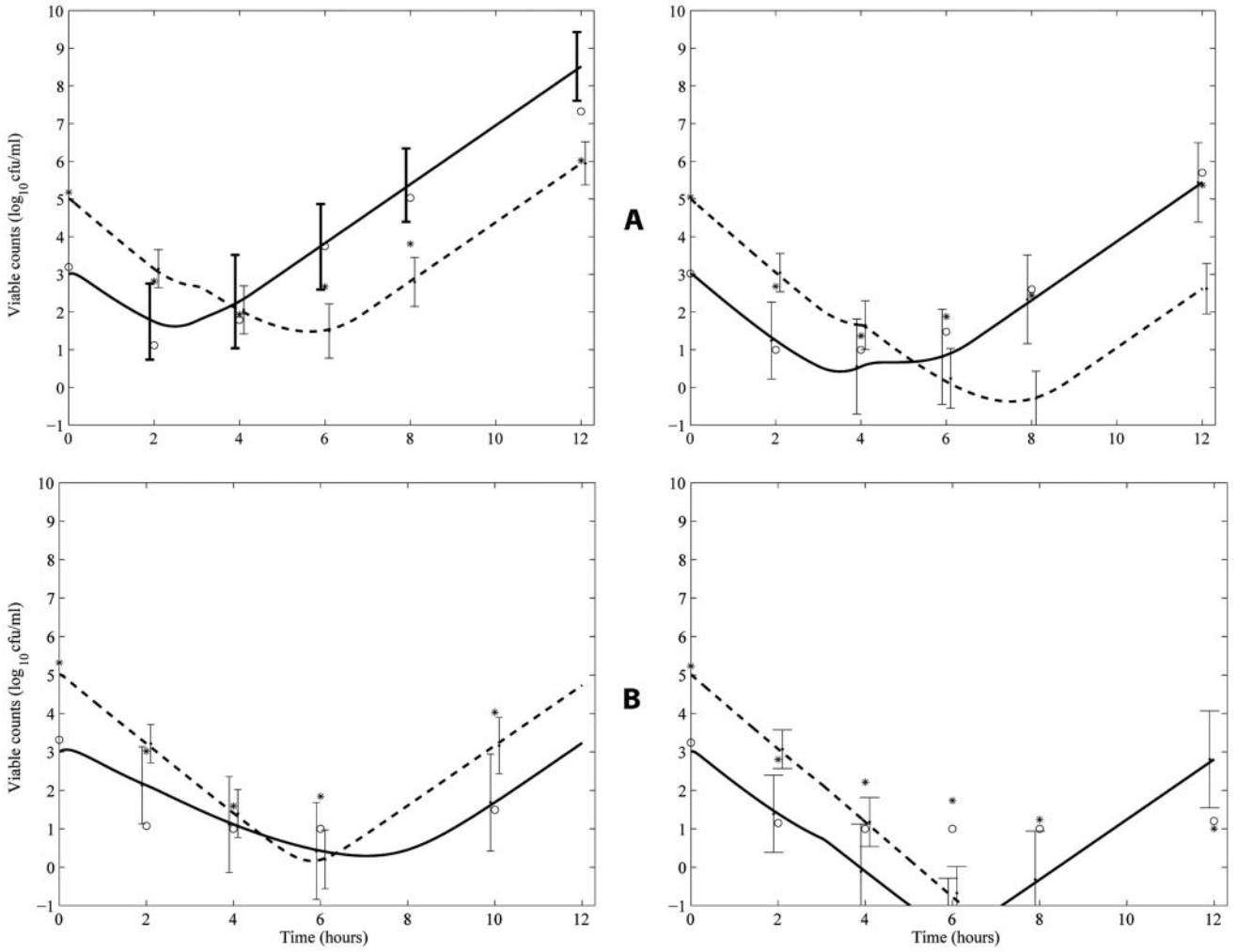


FIG. 8. Predicted and experimental data from competition assays with *E. coli* strains TEM-1 and TEM-12 in the in vitro kinetic model for two selective windows. Shown are data for 1 h (A) and 12 h (B), with low C_{max} (left panel) and high C_{max} (right panel). *, strain TEM-1 observed data; dashed line, TEM-1 predicted data; ○, strain TEM-12 observed data; solid line, TEM-12 predicted data. The bars correspond to 95% predictive intervals.

with different levels of resistance, the appearance of newborn high-level resistant mutants from the parental strains and the post-MIC effect can strongly affect the outcome of the selection. Thus, it is important that pharmacodynamic models incorporate biologically relevant parameters to allow more realistic predictions of resistance development.

APPENDIX

Estimation of parameters in the mathematical model. The sum of the parental and mutant strains, $S(t_j)$, was in equation 8 defined as a function of the unknown parameters α, β, C_{MIC_P}, and C_{MIC_M}. Since the complete probability mechanism was too complicated to specify a full likelihood, a quasi-likelihood approach (36) to achieve robust inference was used. This means that only the mean and variance functions have to be specified, instead of the probability structure. The conditional mean and variance functions of $S(t_j)$, given $S(t_{j-1})$, were achieved by assuming that $S(t_j)$ followed a branching process (39). That means, let $S(t_j)$, for $j = 0, 1, 2, 3, 4$, or 5, denote the number of bacteria at the time points t_0, t_1, t_2, t_3, t_4, or t_5 (0, 2, 4, 6, 8, or 12) and for the experimental setting with five time points (0, 2, 4, 6, 10. If each single bacterium in generation zero, S_0, produces new bacteria with a mean μ and variance σ^2, the total number of offspring will depend on the size of the previous generation. Thus, the size of the jth generation is

$$S_j = \sum_{i}^{S_{j-1}} Z_i, \tag{A1}$$

where Z_i is the number of offspring to the ith bacteria of generation $j - 1$.

Furthermore, the variance for the size of the jth generation is

$$\mathrm{Var}(S_n) = \sigma^2 \mu^{n-1}\left(\frac{\mu^n - 1}{\mu - 1}\right), \tag{A2}$$

for $\mu \neq 1$. Now, assume that there are n generations between time point t_j and t_{j-1}. Since each bacterium produces offspring with a mean μ in each generation, the conditional mean of the number of bacteria at time point t_j given the number at time point t_{j-1} is

$$E[S(t_j) \mid S(t_{j-1})] = S(t_{j-1})\mu^n = \theta(t_j) \tag{A3}$$

and the conditional variance,

$$\mathrm{Var}(S(t_j) \mid S(t_{j-1})) \approx \theta(t_j)\sigma^2 \frac{\mu^n}{\mu(\mu-1)}, \quad \text{(A4)}$$

for $\mu \neq 1$. Set

$$c = \sigma^2/\mu(\mu-1). \quad \text{(A5)}$$

Then, Gaussian approximation (38) gives that

$$E[\log_{10}(S(t_j)) \mid S(t_{j-1})] \approx \log_{10}\theta(t_j) \quad \text{(A6)}$$

and

$$\mathrm{Var}[\log_{10}(S(t_j)) \mid S(t_{j-1})] \approx \frac{c}{\log(10)^2 S(t_{j-1})}. \quad \text{(A7)}$$

It follows that $S(t_j)$ given $S(t_{j-1})$ is approximately normally distributed with the mean as in equation A6 and variance as in equation A7, and hence the quasi-likelihood function (not presented) for the conditional number of bacteria can be derived. To estimate the parameters, the quasi-likelihood function was based on 10 independent experiments with SW of 2 h and maximized by solving the score function numerically using Matlab version 6.5.

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