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Models Related to Growth and Selection of Antibiotic Resistant Bacteria under Drug Exposure

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Patricia Geli*

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Abstract

The worldwide increase of antibiotic resistance and the simultaneous downward trend in development of new antimicrobial drugs has made efforts to prolong the life span of existing antibiotics of utmost importance.

It is known that different dosing-regimens of antibiotics may influence the selection of resistant bacteria. But the nature of variation with different dosing regimens is unknown.

This thesis focuses on mathematical models for the evolution of resistance within the treated host.

We will see that, in spite of simplifications, the models help in identifying key processes behind observed patterns such as selection of resistance, de novo acquired resistance and postantibiotic effects.

KEY WORDS: Antibiotic resistance, Kolmogorov equations, multitype branching processes, penicillin binding proteins, postantibiotic effect, varying environment.

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"We may look back at the antibiotic era as just a passing phase in the history of medicine, an era when a great natural resource was squandered, and the bugs proved smarter than the scientists"

G Cannon. 1995.
Superbug. Nature's Revenge. London: Virgin Publishing.

"All models are wrong, but some are useful"

Box. 1979.
Some problems of statistics and everydaylife: J. Am. Statistical Assoc.

List of papers

This thesis is based on the following papers, which are referred to in the text by their roman numerals:

I P. Geli, S. K. Olofsson, D. I. Andersson, and O. Cars. (2005): Pharmacodynamic Model To Describe the Concentration-Dependent Selection of Cefotaxime-Resistant *Escherichia coli*, Antimicrobial Agents and Chemotherapy, December 2005, p. 5081-5091, Vol. 49, No. 12.

II P. Geli. (2007): Modeling the mechanism of postantibiotic effect, Stockholm University Research Reports in Mathematical Statistics 2007:18.

III P. Geli. (2007): A multi-type branching model with varying environments for for Bacterial Dynamics with Postantibiotic Effect, Stockholm University Research Reports in Mathematical Statistics 2007:19.

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Contents

Biological glossary

Amplification (Gene amplification) The increase in the number of copies of a *gene*. May result from errors in DNA *replication* or *recombination*.

AUC The area under the concentration-time curve over 24 hours.

Bacteria *Cells* of lower form of life without a nuclear *membrane*.

β-lactamase A type of enzyme produced by some bacteria that is responsible for their resistance to beta-lactam antibiotics like penicillins, cephalosporins, cephamycins and carbapenems. These antibiotics have a common element in their molecular structure: a four-atom ring known as a beta-lactam.

C_0 The initial antibiotic concentration of an experiment

cfu *Colony* forming units

Colony A population of cells that are progeny of a single cell.

Drug resistance A term used to describe bacteria which have mutated or changed so they are not affected by an antibiotic that previously killed them or slowed their growth.

Gram negative A classification of bacteria based upon their lack of retention of a certain stain in the laboratory. The staining quality is based on the structure of the cell wall surrounding the bacteria. This structure of the cell wall influences which antibiotics will kill the bacteria. This laboratory staining method was developed by Hans Gram in 1884.

In vitro Refers to a process or reaction occurring in an artificial environment, as in a test tube or culture media.

In vivo Refers to a process or reaction occurring in the living body, human or animal.

MIC The minimum inhibitory concentration. The lowest concentration of antibiotic sufficient to inhibit bacterial growth in vitro

MPC Mutant prevention concentration. The lowest concentration preventing growth of the least susceptible first-step resistant mutant among a large (10^{10} cfu) bacterial population

Mutant An organism or *cell* that has a different inherited characteristic than the remainder of the cells in the population. Usually the result of a change in *DNA* sequence.

Mutation A change in DNA sequence. Usually detected by a sudden and inherited change in an observed characteristic (*phenotype*) of a cell or an organism. However, a mutation may be detected directly by determining a change in the DNA sequence, even though there is no visible characteristic change in the cell or organism. The progeny of the mutant may revert to the previous phenotype, in which case the new mutation is referred to as a reverse mutation or back mutation. A phenotype resulting from a series of two mutations is referred to as two-stage mutation. The rate of mutation may be determined by fluctuation analysis.

PAE See Postantibiotic Effect.

PBP Penicillin Binding Protein.

Pharmacodynamics Describes the effect of the drug and during antibiotic concentration pharmacodynamics means how bacteria are affected by different concentrations of antibiotics and by different lapse of concentrations.

Pharmacokinetics Relates to the disposition of drugs in the body (i.e., their absorption, distribution, metabolism, and elimination).

Phenotype The visible characteristics of a cell or organism, as opposed to genotype, the genetic information of a cell.

Plasmid In bacteria, a circular piece of DNA that is separated from the major (chromosomal) piece of DNA. Plasmids replicate and segregate at cell division idependently of the chromosomal DNA.

PME See Post-MIC Effect.

Postantibiotic Effect (PAE) The phenomenon of continued suppression of bacterial growth after a short exposure of bacteria to antimicrobial agents. The length of the PAE is defined as the difference between the time required for the exposed culture to increase tenfold above the count observed immediately after drug removal and the corresponding time for the unexposed control.

Post-MIC effect Post-MIC effect (PME) describes the effect of sub-MICs on bacteria previously exposed to a continuously decreasing antibiotic concentration and is defined as the difference in time for the counts in cfu of the exposed culture to increase tenfold above the count observed at the MIC level and the corresponding time for the unexposed control.

Selective window The concentration span when the antibiotic concentration is above the MIC of the sensitive strain and below the MIC of the resistant strain.

TEM Is a common name for a group of β-lactamases named after the patient (Temoneira) providing the first sample.

TEM-1 Is the most commonly encountered β-lactamase in gram-negative bacteria. Up to 90% of ampicillin resistance in *E. coli* is due to the production of TEM-1.

TEM-12 Based upon different combinations of changes, currently 140 TEM-type enzymes have been described. TEM-12 which is a mono-mutated variant of TEM-1 is one among them.

1 Introduction

1.1 The development of antibiotic resistance

When antibiotics were introduced for clinical use more than 60 years ago, many believed that this was the final cure for infectious diseases. However, within a few years bacteria controlled by antibiotics had developed resistance to these drugs. Today, antibiotic resistance has become one of the world's most alarming health problems. As organisms become more resistant, treatment options become more limited and treatment failures increasingly likely. It has been estimated that one-fifth of global deaths, representing over 11 million deaths annually, is caused by infectious diseases [31] and a contributing factor to this mortality is treatment failure caused by antibiotic-resistant bacteria.

This worldwide increase of antibiotic resistance and, simultaneously, the downward trend in development of new antimicrobial drugs - has made efforts to prolong the life span of existing antibiotics of utmost importance. Therefore, the need to reverse, or at least minimize the pattern of resistance is therefore essential in order to prolong the life span of existing antibiotics.

1.2 The human microflora

One group of bacteria in which resistance is clinically important are those bacteria that normally colonize hosts asymptomatically (without symptoms). Carriage of these asymptomatic bacteria is often long-lived and is typically not cleared by the immune response of the patient or even by antibiotic treatment. One such bacterium is *Escherichia coli* (*E. coli*), which is an important group of the commensal flora and normally habitats the gut. When translocated from their normal commensal habitat, *E. coli* has the ability to cause extraintestinal infections such as sepsis, wound infections, intra-abdominal abscesses, urinary tract infections (UTIs), and meningitis.

1.3 Mutation frequency

In the bacterial life cycle spontaneous mutations occur at a rate of $10^{-10} - 10^{-9}$ per base pair per generation during DNA replication ([1],[19]).

Considering that *E. coli* bacteria in the feces of a human in one day averages between 100 billion and 10 trillion, and the rate of replication errors that result in resistance mutations, a small number of preexisting resistant mutants are likely to exist in the bacterial population of the normal flora.

1.4 Biological fitness cost and cost compensation

Mutations and acquired resistance genes might confer a biological fitness cost for the resistant bacteria [2]. Experimentally the biological fitness cost of resistance is essentially measured by estimating the relative rates of growth, survival, transmission and clearance of sensitive and resistant bacteria in vitro and in vivo ([8],[28]).

The biological fitness cost associated with antibiotic resistant bacteria has been of special interest according to the general hypothesis that susceptible strains would out-compete resistant isolates in the absence of a selective antibiotic pressure [23]. However, it has been shown that bacteria may rapidly compensate for the loss in fitness genetically by reversion or loss of the resistance gene in which case the bacteria become susceptible or by secondary mutations that restore fitness with maintained resistance ([9],[32],[33],[34]).

1.5 Pharmacokinetics and pharmacodynamics

Pharmacokinetics refers to the way the drug is handled by the body, e.g. absorption, distribution and elimination. Important pharmacokinetic parameters are the peak serum concentration, area under serum concentration curve (AUC) and elimination half-life.

The pharmacodynamic properties determine how an antibiotic affects bacteria. The main pharmacodynamic factor used to express antimicrobial activity is the minimal inhibitory concentration (MIC) of antibiotics.

However, the MIC gives no information on the time-course of the antimicrobial effect. It needs to be combined with studies of how the killing effect relates to the fluctuating drug concentration over time. During the last 20 years, understanding of the interaction between pharmacokinetics and pharmacodynamics (PK/PD) has increased and the PK/PD indices for the efficacy of antibiotics have been defined ([12],[13],[20],[27]).

Some of the most important PK/PD indices are:

- $T > MIC$: The time for which antibiotic concentrations exceed the MIC value.
- C_{max}/MIC: The peak serum concentration divided with the MIC value.
- AUC/MIC: The area under serum concentration curve divided with the MIC value.

The relationship between these parameters are shown graphically in Figure 1.

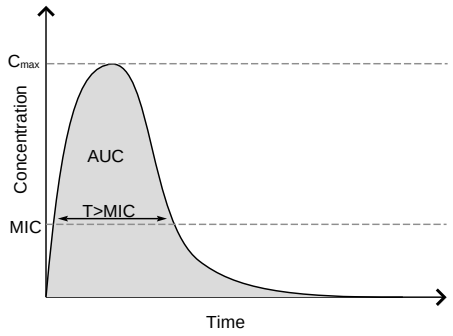

Figure 1: Pharmacodynamic and pharmacokinetic parameters

The pharmacokinetic parameters are interdependent; i.e. an increased dose leads to a higher peak concentration, larger AUC and longer $T > MIC$. Because of this interrelationship, experimental studies (in vivo and in vitro) has been used to try to define the importance of the different parameters for antimicrobial efficacy. In vitro kinetic models are advantageous because the pharmacokinetic parameters, such as the absorption phase, peak concentrations and the drug elimination rate, may easily be varied. Also, by the use of mathematical models, the understanding of these dose-relationships has increased considerably ([5],[10],[21],[37]).

1.5.1 Postantibiotic and post-MIC effects

The postantibiotic effect (PAE) is the phenomenon of continued suppression of bacterial growth after a short exposure of bacteria to antimicrobial agents.

The length of the PAE is defined as the difference between the time required for the exposed culture to increase tenfold above the count observed immediately after drug removal and the corresponding time for the unexposed control.

In vitro, the PAE is typically measured as the delayed bacterial growth after a short on-off exposure to an antibiotic for 1 or 2 h [15]. Such exposure does not reflect the situation in humans under clinical conditions, where bacteria are exposed to antibiotic concentrations that decline only slowly over time, with half-lives of up to several hours [18]. To capture the additional effects from a varying concentration which might at some time fall below the minimum inhibitory concentration (MIC), the term post-MIC effect (PME) is used [26].

The clinical implication of prolonged effects due to exposure of antibiotics lies in the possibility of increasing the intervals between drug administrations,

thus allowing for fewer daily doses without the loss of therapeutic efficacy [14]. Extended dosing intervals of an antimicrobial has several potential advantages, among them reduced cost, less toxicity, and better compliance among outpatients receiving antimicrobial therapy, which in turn reduces the risk for selection of resistance due to suboptimal doses of antibiotics.

1.6 Suboptimal antibiotic dosage regimen - a risk factor for selection of resistance

Selection of resistant subpopulations is one reason for treatment failures when the antibiotic concentration becomes too low at the site of infection to achieve an antibacterial effect on the subpopulation ([16],[17]).

Historically, the dosage regimens (dose, dose interval, length of treatment) of antibiotics have been developed towards optimal efficacy and minimal toxicity and the MIC has traditionally been used as a reference point for the design of the regimens, the goal being to produce tissue concentrations above the MIC at the site of infection. The risk of certain regimens inducing emergence of resistance has rarely been a concern.

When antibiotic concentrations are above MIC levels for susceptible bacteria in a population, but below the MIC levels of resistant subpopulations, the susceptible part of the population population will be killed off, resulting in a totally resistant population, see Figure 2. The concentration span when this selection of a resistant population is possible is called the selective window (SW).

The selection of low-level resistant bacteria in relation to the length of the SWs has been studied in vitro ([6], [29],[30]).

2 Modeling

In recent years, mathematical modeling of infectious diseases has gradually become part of public health decision-making. These are important tools that can be used to understand aspects of the various processes underlying antibiotic resistance and to make predictions ([3],[4],[7],[11],[22], [24],[25],[35]).

A recently published paper, which demonstrates the growing interest in mathematical modeling approaches to evaluate antibiotic resistance [36], showed that the yearly number of publications of antibiotic resistance modeling increased from about 1350 in 1990 to about 6000 in 2006.

Resistance takes place on several levels, both within hosts, in which processes such as the emergence and selection of resistant bacteria are involved, and also between hosts, which describes the processes of the spread

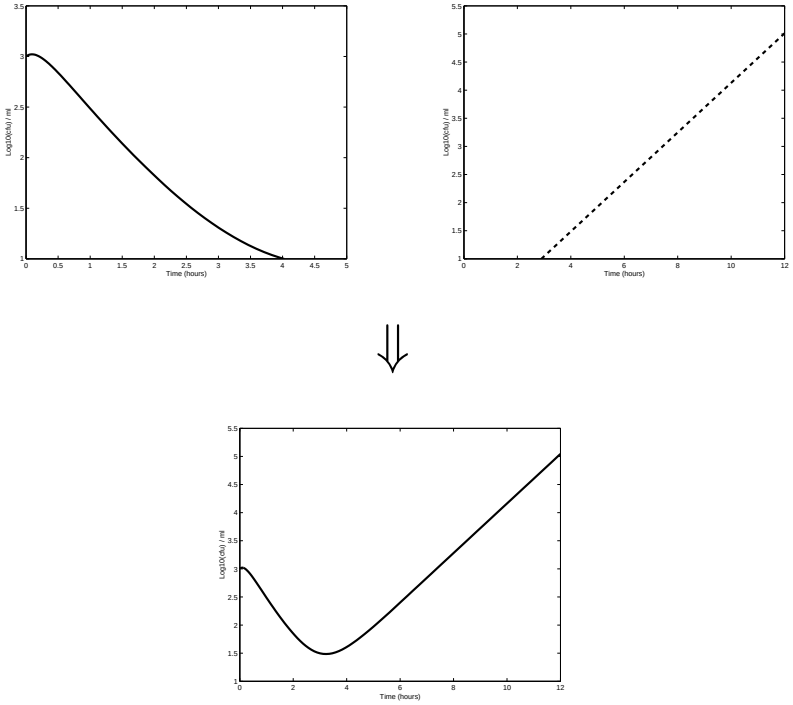

Figure 2: Development of the sensitive (top left), resistant (top right) and total (lower) bacterial populations respectively

of antibiotic resistant bacteria.

This thesis will focus on models describing the processes within hosts, which we will subsequently refer to as within-host models. The models will address the following questions:

- Given a set of pharmacokinetic parameters, what pharmacodynamic outcome will be expected?
- Given a resistant subpopulation, which dosing regimens will be less likely to select for resistance?
- What is the expected number of newborn mutants?
- How does the post-MIC effect vary with the pharmacokinetic parameters?

This thesis consists of three papers, which are described, in brief summary in the following sections.

2.1 Paper I: *Pharmacodynamic model to describe the concentration-dependent selection of cefotaxime-resistant Escherichia coli*

This paper is based on observations from an experimental study made in a so called in vitro kinetic system with two *E. coli*-strains with different susceptibility to cefotaxime. These strains (referred to as parental strains) were exposed to different drug dosing regimens in the in vitro kinetic system. The dosing regimens were controlled by the initial concentration and the half-life of the antibiotic.

The aim of the experimental study was to investigate how the selection of the more resistant parental strain varies with the length of the SW. Both newborn mutants and a PME influenced the outcome of the experimental study. Since there was no possibility to differentiate parental strains from mutant strains, the dynamical relationship between the selection and the SW could not be distinguished. Therefore, a deterministic model that describes the selection of preexisting and newborn mutants as well as the PME as a function of pharmacokinetic parameters was developed.

In this model, parental bacteria grow with a rate $\lambda(t)$ and transforms mutants with a constant rate α. Thus, the number of parental bacteria changes with rate $\lambda(t) - \alpha$.

In order to capture the delayed growth, i.e. the PME in the model, we formulated the growth rate, $\lambda(t)$ as a function of the level of saturation of the bacteria.

Each bacteria is assumed to have penicillin binding proteins (PBPs), which become saturated by antibiotics with a rate $\gamma c(t)$ and are created with a rate β. We assume that a bacterium has the lowest net growth rate (i.e. negative growth) when all PBPs are saturated and conversely the highest growth rate (i.e. positive growth) in the absence of antibiotics when no PBPs are saturated. The interpretation is that a critical proportion of unsaturated PBPs is necessary for cell division.

The mathematical model is fitted to data obtained from one experimental trial and validated through prediction of the outcome for other experimental trials with varying SWs.

2.2 Paper II: *Modeling the mechanism of postantibiotic effect*

The idea of this paper is a direct follow-up to Paper I. Many of the microscopic processes involved in the bacterial dynamics in Paper I, such as the saturation of PBPs, mutations and replications are subject to random

fluctuations and are therefore stochastic. The aim of Paper II is therefore to construct a stochastic model that describes the underlying mechanism for delayed effects on bacterial growth, which in this paper is referred to as PAE.

In the model in Paper II, all bacteria will at each time-point have the same level of saturation resulting in equal growth of the whole bacterial population. In this model, we consider the dynamics of one single bacterium. It is assumed that a bacterium has a fixed total number of PBPs before exposure to any drug effects. Furthermore, in the model, new PBPs are created with an intensity β, unsaturated PBPs become saturated with antibiotics with an intensity $\gamma c(t)$ (with $c(t)$ denoting the antibiotic concentration in the system at time t) and finally PBPs are removed from the bacterium with an intensity μ. Given these intensities, we calculate the probability of a bacterium having a number of unsaturated PBPs and saturated PBPs, respectively, at a time-point t. Hence, each bacterium in the model may have different levels of saturation and therefore different probabilities for cell division. However, since there were no data on this level the model was limited to show some mathematically interesting properties for the probability of saturation given different initial conditions and different concentration lapses of antibiotics.

2.3 Paper III: *A multi-type branching model with varying environment for bacterial dynamics with post-antibiotic effect*

This paper is a direct follow-up from the results in Paper II. The aim is to construct a stochastic model that has the ability to express delayed effects of bacterial growth of a whole population, which could also be compared with real data.

Branching processes is a common class of stochastic models to describe bacterial dynamics assuming that the population consists of only one type of bacteria, each having the same probability for cell division. For this purpose however, we want to capture post-exposure effects of antibiotics. In order to do so, we assume once again that the PAE corresponds to the time required for synthesis of new unsaturated PBPs, sufficient for cell division. We consider a situation with exponentially declining drug exposure in which bacteria have a probability determined in Paper II, with some slight modifications, of getting saturated. The probability of cell division depends on the number of saturated PBPs. It is assumed that a bacterium without any effects of antibiotics lives for a fixed lifetime and gives birth to two new bacteria at the end of its lifetime or dies. Under effects of antibiotics, a bacterium is assumed to undergo a saturation process during its lifetime and at the end of

its lifetime the probability of cell division depends on the level of saturation at reproduction. The more antibiotics, the higher the probability of saturation is and, in turn, the higher the probability is for cell death. For the purpose of this paper it is assumed that PBPs can only become saturated, i.e. no PBPs can be synthesized or removed from the bacterium. Instead, cell division yields offspring, which are less saturated, and eventually bacteria with a sufficient number of saturated PBPs for growth. The extension of the standard branching process model to cover the varying environment of declining drug concentration and multiple types of bacteria due to different saturation levels is called multi-type branching process with varying environment.

Real data (a part of the data used for Paper I) are used for estimating parameters of the model. Numerical results and simulations based on these parameters are presented.

2.4 Conclusions and discussion

The papers of this thesis covers models related to growth and selection of antibiotic resistant bacteria under drug exposure. One main difference is that the model presented in Paper I, as opposed to the models in Paper II and III, is purely deterministic. A second difference is that the model in Paper I cover the dynamics of both preexisting mutants and newborn mutants.

Deterministic models are a wide class of models and the most commonly used class for modeling purposes in the pharmacological literature. The term deterministic means that no randomness is included. This kind of models are useful when drawing conclusions regarding clinical implications based on observations of the mean behavior of a large number of bacteria and trials.

Consider the model in Paper I for describing the growth of bacterial growth dynamics without any influence of mutation,

$$\frac{dP}{dt} = \lambda(t)P(t),$$

where P is the number of bacteria and $\lambda(t)$ is the net growth rate, which is a function of the antibiotic concentration in the system at time t. When $\lambda(t) > 0$, the model will predict a continuous increase in the bacterial count and correspondingly, when $\lambda(t) < 0$, the model will predict a continuous decrease in the bacterial count.

In reality however, bacterial reproduction is a result of random events at random time-points, which results in discrete changes[1] in the counts of bacteria. If the population is large, as when we start the experiments, these

[1]In stochastic models an increase of the total number of bacteria can be one or several

random changes will look smooth and similar to that of the deterministic model.

However, near the turning-point of the bacterial growth curves, the population has often decreased below the detection level of 10 cfu. The remaining antibiotic concentration is no longer effective and hence the deterministic model would in this case, even if the bacterial count is less than one, predict an exponential re-growth of the bacterial population. Compare this with the stochastic model, where this small population would be subject to random phenomena with a chance of dying out, even if there is no concentration of antibiotics left in the system. This difference will result in very different pharmaceutical outcomes and conclusions of the results.

The same principle applies for the component in the model of Paper I considering mutations. Given an experimental trial, this model will always predict a fraction α of mutants in each experiment and hence, lead to a false prediction of resistance in many experiments.

However, the deterministic models were useful in the sense that it made a separate analysis of the various pharmacodynamic and pharmacokinetic factors possible.

2.5 Future research

As all models do, the models presented in this thesis involve some assumptions and simplifications, which might be more or less accurate.

In this thesis, one common factor of the models presented, is that they all have the ability to capture post-exposure effects of antibiotics. However, the models have only been applied to one antimicrobial-organism combination with experimental data that were not originally designed for discovering how the PAE varies with different dosing regimens. Therefore a range of other factors than the PAE might influence the outcome of the data seen from the bacterial experiments. In Paper I, we try take this into account by including the emergence of newborn mutants into the model. However, this has not been done for the stochastic model in Paper III but it would be desirable for the future development of this model. Another limitation of this stochastic model is the low number of PBPs assumed for the simulations of the results. This is at least one tenth of what is realistic and for future research it should be investigated how this assumption affects the PAE. In synergy with data designed for discovering how the PAE varies with some of the remaining questions can perhaps be sorted out.

bacteria, in regards to deterministic models, when the number of bacteria can increase with 1/3 bacterium.

In this thesis, we have been concerned with models, which can be related to how antibiotic resistant bacteria emerge and evolve within an individual host, with and without drug treatment. However, the measures on individual level have consequences for public health by affecting the dynamics of the disease. The papers that have tried to meld the concepts of the dynamics within host to the spread between hosts can be counted on one hand. Future research should therefore be focused on developing theory that melds the concepts from this wide variety of different fields including pharmacology, microbiology population genetics and epidemiology.

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Paper I

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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

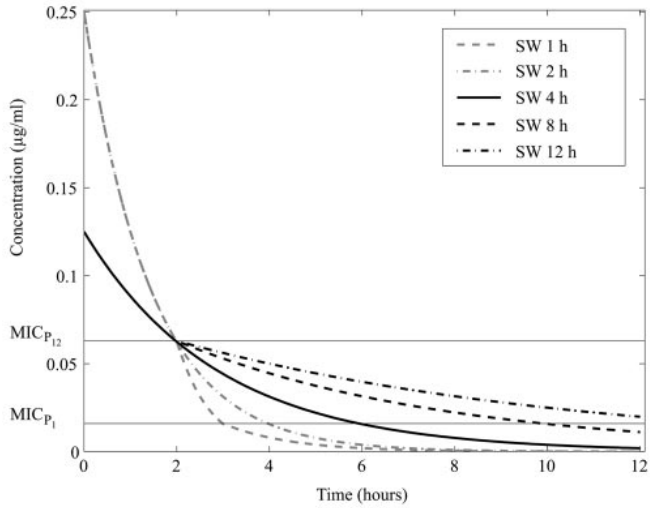


FIG. 1. Concentration profiles of cefotaxime for five selective windows (1, 2, 4, 8, and 12 h) with low C_{max}. MIC_{P1} and MIC_{P12} indicate the MICs of the parental populations TEM-1 and TEM-12, respectively.

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

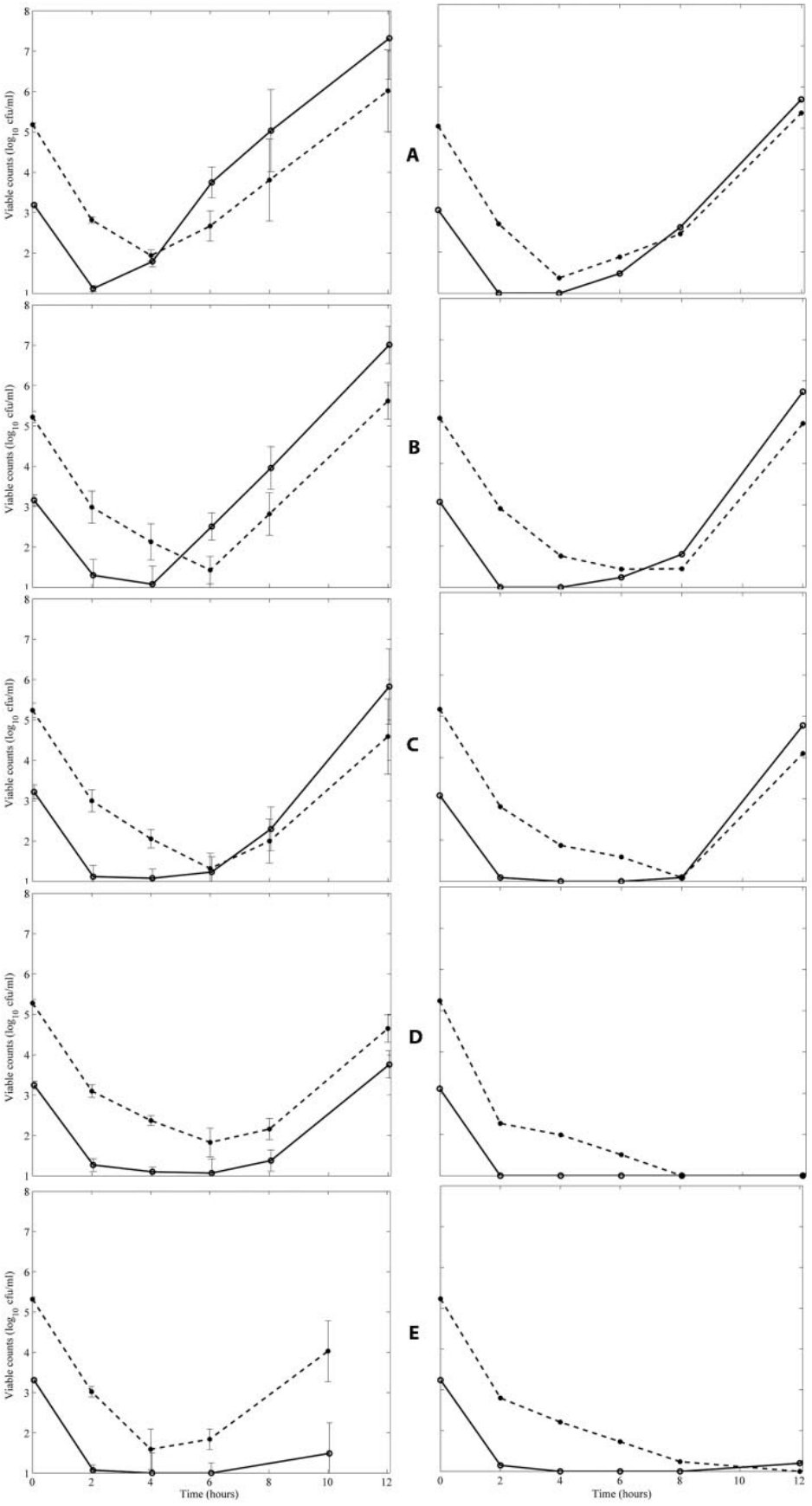
Growth controls. No differences in the growth rates of single cultures of *E. coli* strains TEM-1 and TEM-12 could be observed. Similarly, the competition experiments in the absence of antibiotic showed that the initial ratio (1:1) of the two strains was unchanged after 24 h; also, no differences were 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


A
B
C
D
E
Viable counts ($\log_{10}$ cfu/ml)
Time (hours)
0
2
4
6
8
10
12
1
2
3
4
5
6
7
8

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

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

with

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

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

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

(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:

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

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:

$$\begin{aligned} \frac{dP}{dt} &= \lambda(t)P(t) - \alpha P(t) \\ \frac{dM}{dt} &= \lambda(t)M(t) + \alpha P(t). \end{aligned} \tag{5}$$

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:

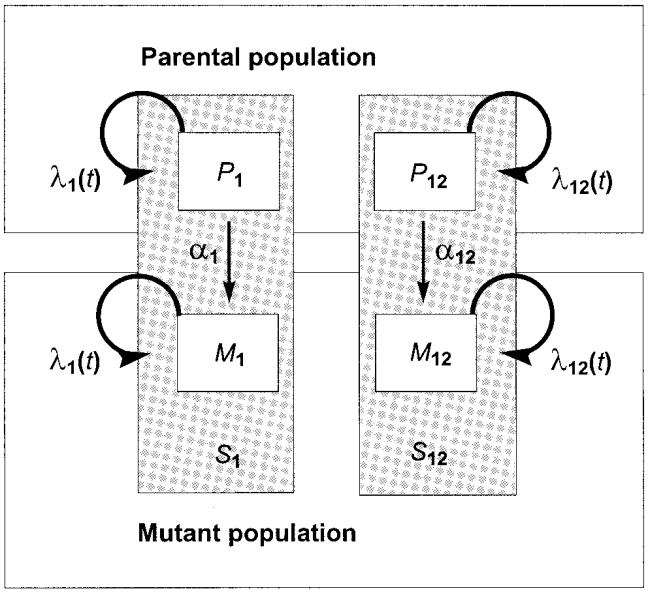


FIG. 3. Illustration of the relative growth rates $\lambda_1(t)$ and $\lambda_{12}(t)$ and mutation rates α_1 and α_{12}. The sums of the parental population and the mutated population, S_1 ($P_1 + M_1$) and S_{12} ($P_{12} + M_{12}$), represent the numbers of bacteria that will be observed during the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

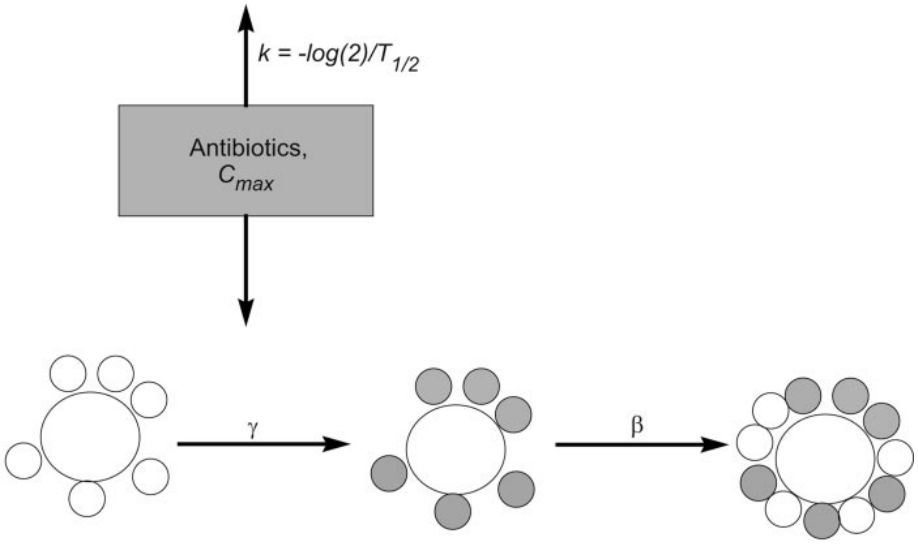


FIG. 4. Modeling of PME. Antibiotic saturation and synthesis of PBPs depends on the initial concentration of drug and the half-life time. The binding rate of antibiotics to PBPs is denoted by γ and the synthesis rate of new PBPs by β. Open circles represent PBPs without bound antibiotic and filled circles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

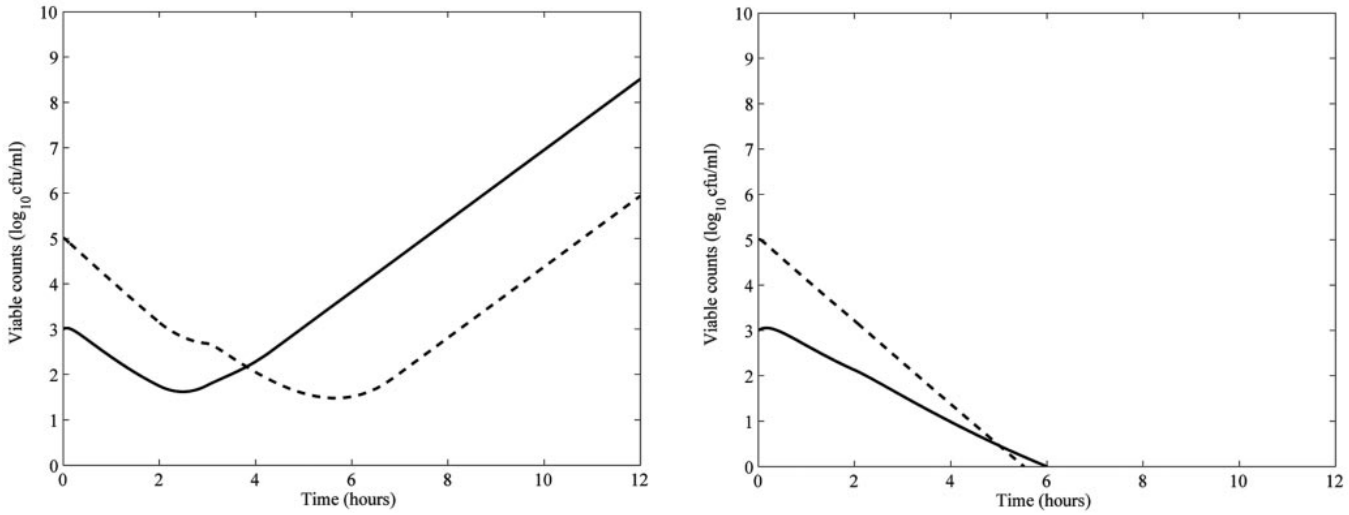


FIG. 5. Predictions of the outcome of the parental strains P_1 and P_{12} for competition assays with *E. coli* strains TEM-1 and TEM-12 with two of the selective windows that were investigated: 1 h (left) and 12 h (right). Dashed line, P_1; solid line, P_{12}.

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

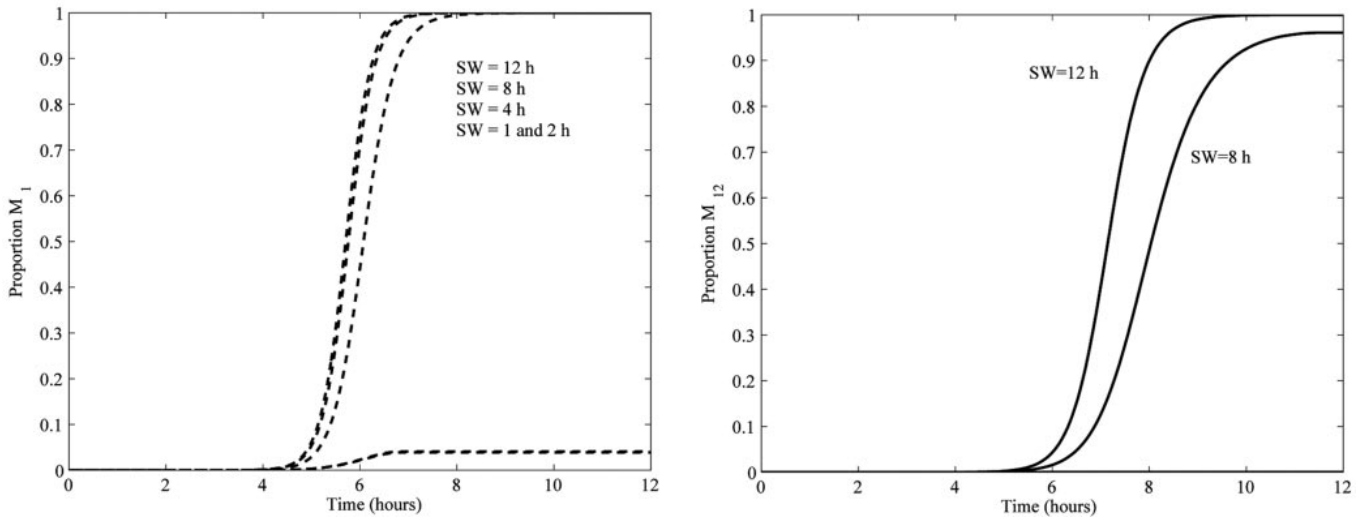


FIG. 6. The proportions of mutants, $M_1/(P_1 + M_1)$ (left), and $M_{12}/(P_{12} + M_{12})$ (right), estimated from the predicted values for the five selective windows that were investigated: 1, 2, 4, 8, and 12 h. Dashed line, strain TEM-1; solid line, strain TEM-12.

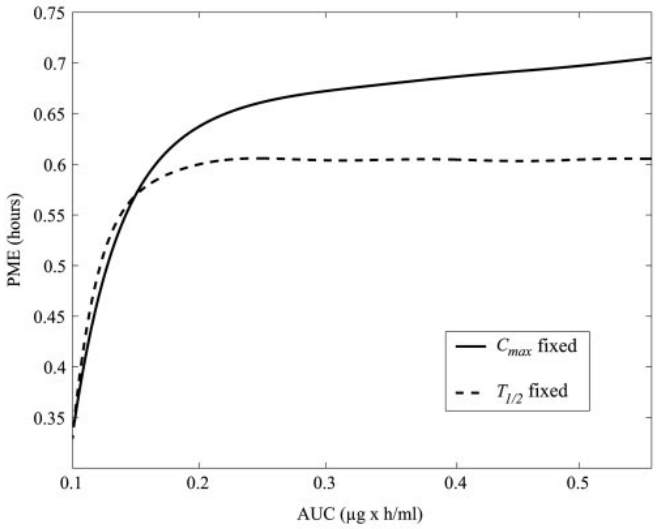


FIG. 7. Predicted PME for two cases yielding the same AUC: constant C_{max} of 0.1 μg/ml and $T_{1/2}$ varying from 0.5 to 5 h; and constant $T_{1/2}$ of 0.5 h and C_{max} varying from 0.1 to 0.8 μg/ml.

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

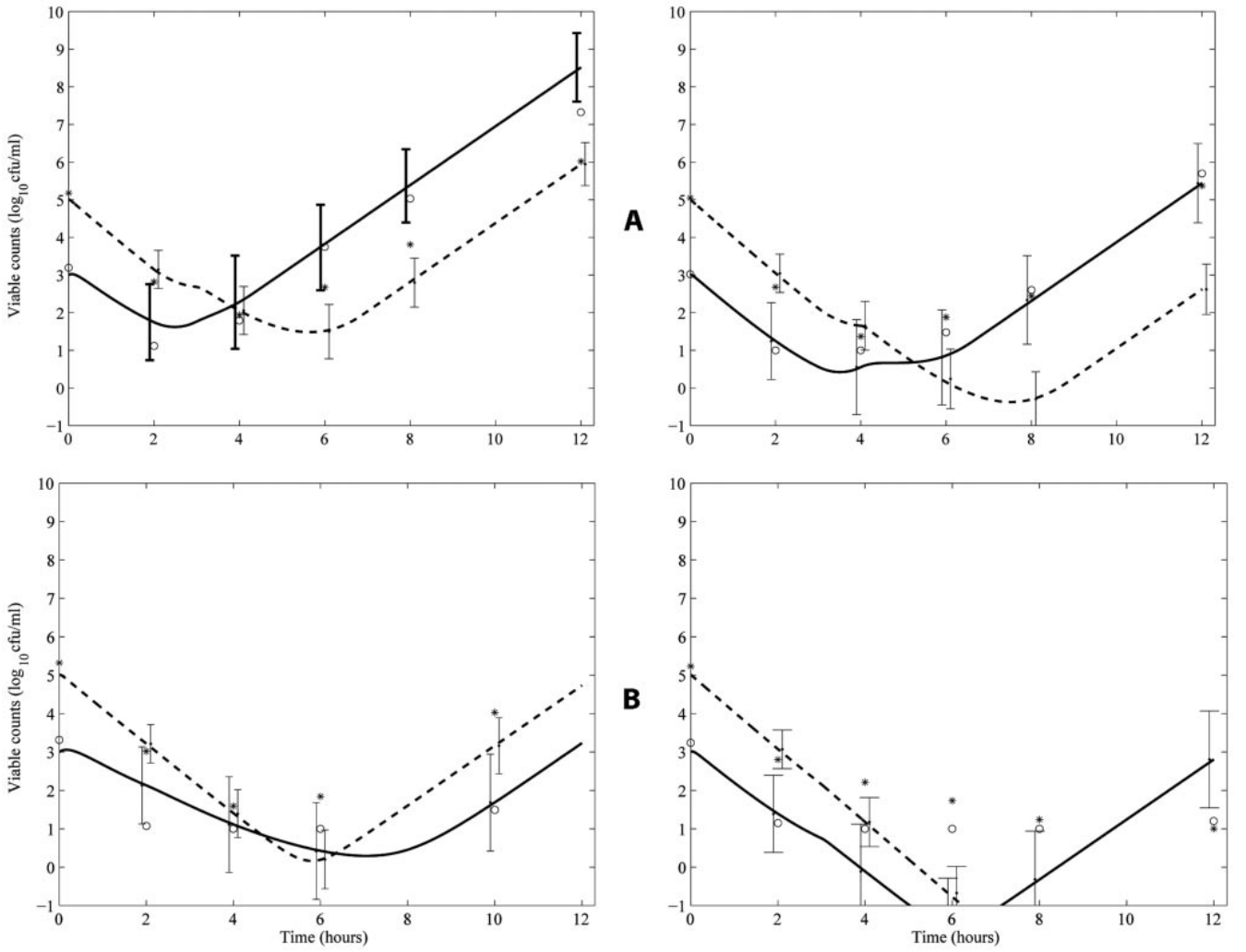


FIG. 8. Predicted and experimental data from competition assays with *E. coli* strains TEM-1 and TEM-12 in the in vitro kinetic model for two selective windows. Shown are data for 1 h (A) and 12 h (B), with low C_{max} (left panel) and high C_{max} (right panel). □, strain TEM-1 observed data; dashed 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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Paper II

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Modeling the Mechanism of Postantibiotic Effect

Patricia Geli*

August 2007

Abstract

In this paper† a stochastic model for describing one of the possible underlying biological mechanisms of postantibiotic effect (PAE) (the delayed regrowth of the bacteria after complete removal of an antibiotic) is formulated. The model is based on the theory of penicillin binding proteins (PBPs), where the PAE is the time required by the bacteria to synthesize new PBPs before growth. Newly synthesized PBPs are unsaturated and becomes saturated under antibiotic pressure and eventually removed.

The model assumes that unsaturated PBPs are attached (synthesized) to a bacterium according to a Poisson process and that these are saturated with an intensity proportional to the antibiotic concentration of the treatment. The calculations and results are divided into three simplifying steps toward a more realistic approach. At first, we assume constant antibiotic concentration and no initial PBPs. Secondly, we assume constant antibiotic concentration, but with an initial set of unsaturated PBPs (no saturated PBPs). Thirdly, we assume exponentially declining antibiotic concentration and the same initial set of unsaturated PBPs.

The stochastic models are solved using a set of Kolmogorov equations and exact solutions with interesting properties can be derived for all three steps. The results are useful for giving a better understanding of the time properties of PAE.

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KEY WORDS: Antibiotic resistance, Kolmogorov equations, penicillin binding proteins, postantibiotic effect.

1 Introduction

The rapid evolution of antibiotic resistance in pathogenic bacteria, due to overuse and misuse of antibiotics, is today a major public health problem. While the antibiotic resistance is increasing, the research for development of new antimicrobial agents is decreasing. As a result, activities to maintain the effect of existing antibiotics and thereby prolong their useful lifespan have a high priority. The knowledge though, of how to use existing antibiotics to minimize the emergence of resistance without compromising efficacy is today inadequate.

The clinical implication of long postantibiotic effects (PAEs) lies in the possibility of increasing the intervals between drug administrations, thus allowing fewer daily doses and thereby potentially reducing treatment costs, increasing patient compliance and decreasing drug exposure ([1],[2]).

In spite of the increasing interest in the PAE as an important parameter for the dosage and frequency of administration of a drug, knowledge of this phenomenon is still incomplete. One possible explanation for the PAE is that it represents the time required for synthesis of new penicillin binding proteins (PBP), before growth of bacteria ([3],[4]).

In this work a stochastic model for describing the dynamics behind PAE is derived. The results are useful for giving a better understanding of the time properties of PAE.

2 Models

The models describe how new PBPs are being created, going from unsaturated to saturated and finally being removed. This process takes place independently for all existing PBPs. We will assume that there are a number of unsaturated PBPs at the start and that new PBPs are created according to a Poisson process during the time of study. The unsaturated PBPs become saturated at a rate that depends on the concentration, while the saturated are removed at a different rate. We will first consider constant concentration and later declining concentration:

$$\begin{cases} c(t) = C_0 & \texttt{(Constant antibiotic concentration)} \\ c(t) = C_0 e^{-kt} & \texttt{(Declining antibiotic concentration)} \end{cases}$$

Assume that PBPs can be in either of two states: 1) unsaturated or 2) saturated with antibiotics. Let $X(t)$ denote the number of PBPs that are unsaturated (in state 1) at time t and $Y(t)$ the number of saturated PBPs

(in state 2) at time t. Furthermore assume that a PBP remains unsaturated for an exponentially distributed time, so that $(X(t), Y(t))$ becomes a Markov process with transition rates as listed in Table 1. A schematic picture of this model is given in Figure 1.

From	To	Rate
(x, y)	$(x+1, y)$	β
(x, y)	$(x-1, y+1)$	$\gamma c(t) x$
(x, y)	$(x, y-1)$	μy

Table 1: Transition rates of the Markov process

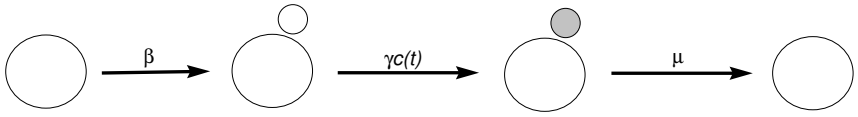

Figure 1: A schematic picture of the model. Initially a new PBP is created (left arrow) with an intensity β. When antibiotics are added to the system, the PBP becomes saturated (middle arrow) with an intensity $\gamma c(t)$ and later the PBP is removed (right arrow) from the bacteria with an intensity μ.

Let $p_{j,k}(t) = P(X(t) = j, Y(t) = k)$ be the joint probability of sizes j and k at time t. The Kolmogorov equations can then be written as

$$\begin{aligned} p_{j,k}(t+\Delta t) &= p_{j,k}(t)[1-(\beta+\gamma c(t) j+\mu k)\Delta t] + p_{j-1,k}(t)[\beta \Delta t] \\ &\quad + p_{j+1,k-1}(t)[\gamma c(t)(j+1)\Delta t] + p_{j,k+1}(t)[\mu(k+1)\Delta t] \\ &\quad + o(\Delta t) \end{aligned} \tag{1}$$

and from this it follows that

$$\begin{aligned} \frac{\partial p_{j,k}(t)}{\partial t} &= -(\beta+\gamma c(t) j+\mu k) p_{j,k}(t) + \beta p_{j-1,k}(t) \\ &\quad + \gamma c(t)(j+1) p_{j+1,k-1}(t) + \mu(k+1) p_{j,k+1}(t) \end{aligned} \tag{2}$$

for $j \geq 1$ and $k \geq 1$.

Consider the probability generating function for $X(t)$ and $Y(t)$ defined as

$$P(s_1, s_2, t) = \sum_{j,k} s_1^j s_2^k p_{j,k}(t). \tag{3}$$

By multiplying both sides in (2) with $s_1^j s_2^k$ and summing over j and k together with the definition in (3), we get the partial differential equation (PDE) for the generating function:

$$\frac{\partial P}{\partial t} = (s_1 - 1)\beta P + (s_2 - s_1)\gamma c(t) \frac{\partial P}{\partial s_1} + (1 - s_2)\mu \frac{\partial P}{\partial s_2} \tag{4}$$

2.1 Constant antibiotic concentration and no initial PBPs

With the initial conditions $X(0) = 0$ and $Y(0) = 0$ which yields $P(s_1, s_2, 0) = 1$, we get the solution to the PDE in (4) as

$$P(s_1, s_2, t) = e^{(s_1-1)\lambda_1(t)+(s_2-1)\lambda_2(t)}, \tag{5}$$

where

$$\begin{cases} \lambda_1(t) = \frac{\beta[1-e^{-\gamma C_0 t}]}{\gamma C_0} \\ \lambda_2(t) = \frac{\beta[e^{-\gamma C_0 t}-1-\frac{\gamma C_0}{\mu}(e^{-\mu t}-1)]}{\gamma C_0-\mu}. \end{cases}$$

This is the product of the generating functions of two Poisson distributions, which means that $X(t)$ and $Y(t)$ are statistically independent random variables.

2.2 Constant antibiotic concentration and an initial set of unsaturated PBPs (no saturated PBPs)

It is more realistic to assume that the initial conditions are $X(0) = n$ and $Y(0) = 0$, which yields $P(s_1, s_2, 0) = s_1^n$.

Since each PBP develops independently, the n unsaturated PBPs that are already in the process from the start and the newly created PBPs can be treated separately, with the latter part following independent Poisson distributions with a bivariate pdf in (5). In order to separate the two parts, let us introduce the notation $(X'(t), Y'(t))$ for PBPs which were created and saturated, respectively after $t > 0$ and $(X''(t), Y''(t))$ for PBPs which were already existing at $t = 0$. Furthermore, with these notations, let $X(t) = X'(t) + X''(t)$ and $Y(t) = Y'(t) + Y''(t)$.

The n unsaturated PBPs that are in the process from the start will move independently with equal probabilities between the different states: From unsaturated to saturated and from saturated to removed. Let $\pi_1(t)$ denote the probability that a PBP that was unsaturated (in state 1) at time 0 will still be unsaturated at time t and furthermore $\pi_2(t)$ the probability that the PBP is instead saturated (in state 2) at time t. Thus, $(X''(t), Y''(t), n - X''(t) - Y''(t))$ will have a trinomial distribution with parameters n, $\pi_1(t)$, $\pi_2(t)$ and $1 - \pi_1(t) - \pi_2(t)$. In this process $(n - X''(t) - Y''(t))$ PBPs are removed.

Now the product of the two parts of probability generating functions for the PBPs present in the process at time 0 and the PBPs arriving after time 0 yields the following distribution

$$P(s_1, s_2, t) = e^{(s_1-1)\lambda_1(t)+(s_2-1)\lambda_2(t)}(1 + (s_1 - 1)\pi_1(t) + (s_2 - 1)\pi_2(t))^n, \tag{6}$$

where the occupation probabilities $\pi_1(t)$ and $\pi_2(t)$ can be derived from the Kolmogorov equation in (2). In this equation the bivariate probabilities $p_{10}(t)$ and $p_{01}(t)$ corresponds to $\pi_1(t)$ and $\pi_2(t)$, respectively. Since we are looking at the process for the n PBPs which started as unsaturated (in state 1), it follows that $\beta = 0$. Hence,

$$\begin{cases} \frac{d\pi_1(t)}{dt} = -\gamma C_0 \pi_1(t) \\ \frac{d\pi_2(t)}{dt} = \gamma C_0 \pi_1(t) - \mu \pi_2(t). \end{cases} \tag{7}$$

From the assumption that the time in the unsaturated state is exponential, it follows with the initial conditions $\pi_1(0) = 1$ and $\pi_2(0) = 0$ that

$$\begin{cases} \pi_1(t) = e^{-\gamma C_0 t} \\ \pi_2(t) = \frac{\gamma C_0}{\mu - \gamma C_0}(e^{-\gamma C_0 t} - e^{-\mu t}). \end{cases}$$

2.3 Exponentially declining antibiotic concentration and an initial set of unsaturated PBPs (no saturated PBPs)

The most realistic model for human kinetics is when we assume that the concentration of an initial dose is declining exponentially rather than being constant.

Again, we can split the problem into two parts: 1) Describing the PBPs existing already at $t = 0$ and 2) describing PBPs that develop at time $t > 0$. It can be shown that the distribution does not change from the result in the previous section, except for different parameters. The probabilities for the already existing PBPs at $t = 0$ are

$$\begin{cases} \pi_1(t) = \frac{e^{\frac{\gamma C_0 e^{-kt}}{k}}}{e^{\frac{\gamma C_0}{k}}} = e^{-C_0 \gamma \int_0^t e^{-ks} ds} \\ \pi_2(t) = e^{-\mu t} \int_0^t \frac{\gamma C_0 e^{\frac{\gamma C_0 e^{-kv}}{k} - v(k-\mu)}}{e^{\frac{\gamma C_0}{k}}} dv. \end{cases}$$

Hence, $(X''(t), Y''(t), n - X''(t) - Y''(t))$ have a trinomial distribution with parameters n, $\pi_1(t)$, $\pi_2(t)$ and $1 - \pi_1(t) - \pi_2(t)$. In order to derive the expressions for $\lambda_1(t)$ and $\lambda_2(t)$, let us introduce

$$c(u, s) = C_0 e^{-k(u+s)} = C_0 e^{-ku} e^{-ks}$$

for the concentration when a PBP created at time u has been exposed to antibiotics during a time s. Furthermore, let

$$C(u, s) = \int_0^s c(u, x) dx = \frac{1}{k} C_0 e^{-ku}(1 - e^{-ks})$$

be the cumulative antibiotic pressure. Now, the density function of the life-time distribution can be written as

$$\gamma c(u,s)e^{-\gamma C(u,s)}$$

and the survival function (the probability that a PBP created at time u is still unsaturated at time T) can be written as

$$e^{-\gamma C(u,T-u)}. \tag{8}$$

Note that $u = 0$ gives $\pi_1(T)$, as defined above.

Integrating (8) over the time period when PBPs are created and multiplying with the intensity, β, yields

$$\lambda_1(t) = \beta \int_0^T e^{-\gamma C(u,T-u)}du = \beta e^{\frac{\gamma}{k}C_0 e^{-kT}} \int_0^T e^{-\frac{\gamma}{k}C_0 e^{-ku}}du. \tag{9}$$

In order to derive $\lambda_2(t)$, we assume that a PBP is created at time $u < T$ and later saturated after time v, where $u + v < T$. The probability that the PBP is still alive at time T, i.e. the probability that a PBP created at time u survives a time $T - u - v$ is then

$$r(u,t) = \int_0^{T-u} e^{-\mu(T-v-u)}\gamma C_0 e^{-ku}e^{-kv}e^{-\frac{\gamma C_0}{k}e^{-ku}(1-e^{-kv})}dv. \tag{10}$$

Again, inserting $u = 0$ in (10) yields $\pi_2(T)$ as defined above.

Integrating (10) over the time period when PBPs are born and multiplying with the intensity, β, yields

$$\lambda_2(t) = \beta \int_0^T r(u,t)du. \tag{11}$$

The solutions $\lambda_1(t)$ and $\lambda_2(t)$ can, as with the constant concentration, be verified by checking that (9) and (11) are solutions to the following differential equation system

$$\begin{cases} \frac{d\lambda_1(t)}{dt} = \beta - \gamma C_0 e^{-kt}\lambda_1(t) \\ \frac{d\lambda_2(t)}{dt} = \gamma C_0 e^{-kt}\lambda_1(t) - \mu\lambda_2(t). \end{cases}$$

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Paper III

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A Multi-Type Branching Model with Varying Environment for Bacterial Dynamics with Postantibiotic Effect

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August 2007

Abstract

In this paper, a multi-type branching process with varying environment is constructed for describing the growth of bacterial populations under treatment of antibiotics. The model captures the phenomenon of delayed growth, postantibiotic effect (PAE).

PAE is the phenomenon of continued suppression of bacterial growth after a short exposure of bacteria to antimicrobial agents.

The clinical implication of long PAEs lies in the possibility of increasing the intervals between drug administrations, thus allowing for fewer daily doses and thereby potentially reducing treatment costs, increasing patient compliance and decreasing drug exposure.

In spite of the increasing interest in the PAE as an important parameter for the dosage and frequency of administration of a drug, knowledge on this phenomenon is still incomplete.

The model is applied to data from an in vitro study with *E. coli* exposed to different dosing regimens of antibiotics.

The model and results provide a common framework to better understand bacterial populations evolving under different selection pressures.

KEY WORDS: Antibiotic resistance, multi-type branching processes, varying environment, penicillin binding proteins, postantibiotic effect.

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1 Introduction

Postantibiotic effect[1] (PAE) is the phenomenon of continued suppression of bacterial growth after a short exposure of bacteria to antimicrobial agents ([3], [20]).

The clinical implication of long PAEs lies in the possibility of increasing the intervals between drug administrations, thus allowing for fewer daily doses without the loss of therapeutic efficacy [5]. Extended dosing intervals of an antimicrobial has several potential advantages, among them reduced cost, less toxicity, and better compliance among outpatients receiving antimicrobial therapy, which in turn reduces the risk for selection of resistance due to suboptimal doses of antibiotics.

The therapy of patients with tuberculosis, is one example where non-compliance with anti-tuberculosis drug therapy has been recognised as a major cause of treatment failure, drug resistance and relapse [2]. Hence, in managing patients with tuberculosis, administration of drugs at intermittent intervals would reduce cost and possibly toxicity of drugs, as well as enhance adherence through greater feasibility of directly observed therapy [10].

Although there is an increasing interest in the PAE as an important parameter for the dosage and frequency of administration of a drug, knowledge on this phenomenon is still incomplete. The aim in this paper is therefore to construct a stochastic model that describes the dynamics of a bacterial population under the influence of different dosing regimens, which correctly takes into account the PAE. The PAE is probably the result of several mechanisms. One explanatory theory for PAE is that it represents the time required for synthesis of new penicillin binding proteins (PBP), before growth of bacteria ([13],[19],[21]). To describe the PBP dynamics, a model previously described in [1] was used. In reality however, PBP dynamics is difficult to observe directly, and hence we have to rely on the data observed in terms of PAE of the bacterial populations. Therefore to capture the PBP dynamics in the dynamics of the bacterial population, a multi-type branching process (MBP) model with varying environments is used.

The PAE is influenced by several factors, including the microorganism, the inoculum (initial population size), the type of antibiotic, the concentration

[1] In vitro, the PAE is typically measured as the delayed bacterial growth after a short on-off exposure to an antibiotic for 1 or 2 h [6]. Such exposure does not reflect the situation in humans under clinical conditions, where bacteria are exposed to antibiotic concentrations that decline only slowly over time, with half-lives of up to several hours [8]. To capture the additional effects from a varying concentration which might at some time fall below the minimum inhibitory concentration (MIC), the term post-MIC effect (PME) is used [14]. For convenience, we will in this paper use the common term PAE to refer to the continued suppression of bacterial growth after any kind of exposure to antimicrobial agents.

of antibiotic, and the duration of exposure [4]. In this paper data from in vitro experiments with *E. coli* subject to different antibiotic dosing regimens of cefotaxime was used to compare and validate the model. The background of the experiments has been described in detail in [12].

Apart from serving as a theoretical framework for understanding the dynamics influence between different dosing regimens (PAE), the model may also be useful to explore optimal dosing regimens. Furthermore, it highlights the importance of taking the stochasticity into account in pharmacokinetic/pharmacodynamic models.

2 Model description

The objective is to construct a model for the bacterial population dynamics under treatment of antibiotics that also explains the phenomenon of delayed regrowth after antibiotics has declined to subinhibitory levels, the PAE.

Branching processes is a convenient class of models for the dynamics of bacterial populations, which consist of only one type of bacteria, each having the same probability for cell division [15]. In order to capture the delayed regrowth of bacteria in the model, we will rely on the theory that the PAE corresponds to the time required for synthesis of new unsaturated PBPs, sufficient for cell division ([13],[19],[21]).

In this paper, we will assume a special case where the probability for cell division of a bacterium depends on the level of saturation of antibiotics. So, assuming that a bacterium has a fixed total number of PBPs, n, there will be $n+1$ different possible levels of saturation in the bacterial population. In other words, the bacterial population will consist of $n+1$ types of bacteria determined by the number of saturated PBPs. The type of bacteria will affect the distribution of the number of offspring and therefore we will describe the reproduction using the theory of multi-type branching processes (MBP).

Let us start by introducing some notation. The population in generation m is characterized by a vector $\mathbf{Z}_m = (Z_{m0}, ..., Z_{mn})$, where Z_{mj} denotes the number of bacteria of type j, i.e. bacteria having j saturated PBPs in the mth generation. Realistically the total number of PBPs (n) is between 100-1000.

Now, the total population size in generation m, $|\mathbf{Z}_m|$, is the sum of the vector components

$$|\mathbf{Z}_m| = Z_{m0} + \ldots + Z_{mn}.$$

Now, let $\xi_{mjk}^{(i)}$ denote the number of offspring of type j in generation m, given by bacterium k of type i. Then, by summing the number of children given by parent k with type i in generation m, the population size of type j in the $(m+1)$th generation is

$$Z_{m+1,j} = \sum_{i=0}^{n} \sum_{k=1}^{Z_{mi}} \xi_{mjk}^{(i)}$$

and the total population size in generation $m+1$ can now be expressed by

$$|\mathbf{Z}_{m+1}| = \sum_{j=0}^{n} Z_{m+1,j}.$$

For a complete model we need a specification of the distribution of the offspring vector for each type distinguished.

Assume that each bacteria lives for a fixed time τ and that there is no overlapping generations. Hence, we consider a process in discrete time at the time points $t = 0, \tau, 2\tau, ...$ One generation of a bacterium will be defined by three phases, saturation, reproduction and distribution of saturated PBPs among its offspring. These three events are described below in more detail.

Before we continue, we will need some notation for the number of saturated PBPs that a bacterium has in each of the three phases. Let us therefore introduce the following stochastic variables:

$Y_1^{(m)}$ = Number of saturated PBPs of one bacterium at the beginning of its generation. Also equal to the type.

$Y_2^{(m)}$ = Number of saturated PBPs at the end of its generation before reproduction.

1. **Saturation process** As long as the antibiotic concentration is positive, we will assume that each generation starts with a saturation process (unsaturated PBP may become saturated). So, given that a bacterium starts with i saturated PBPs, we will have to formulate a probability of having u saturated PBPs at the end of its lifetime (after the saturation process).

   In [1], a stochastic model for the background mechanism of PAE was constructed. This model included the process of synthesis, saturation and death of PBPs as a function of the concentration dynamics. According to this model, newly synthesized PBPs are assumed to be created with an intensity β. PBPs which are unsaturated will eventually

become saturated with an intensity $\gamma c(t)$, where $c(t)$ denotes the antibiotic concentration at time t, and finally saturated PBPs will eventually become removed from the bacteria with an intensity μ. See Figure 1 for a schematic picture of the model.

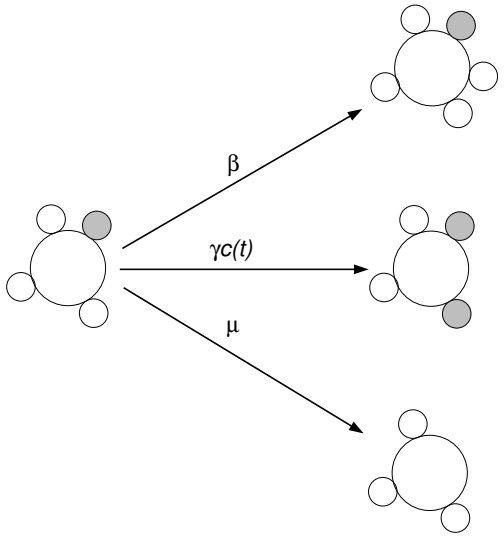

Figure 1: A schematic picture of the general model. PBPs are created (upper arrow) with an intensity β, they become saturated (middle arrow) with an intensity $\gamma c(t)$ and they are removed (lower arrow) from the bacterium with an intensity μ. In this paper it is assumed that only saturation (middle arrow) is possible.

In this paper we will for simplicity consider a special case of the model when there is no new synthesized PBPs and no death of PBPs, only the saturation of already existing PBPs is described. For the further development of the model, the probability of cell division will depend on the level of saturation for in each bacterium. Hence, the constants β and μ are 0. Under these assumptions, we are interested in the probability distribution $p_{iu}(m)$, which denotes the probability that a bacterium with $Y_1^{(m)} = i$ PBPs in generation m, will have $Y_2^{(m)} = u$ after the saturation.

How likely the PBPs are to become saturated after adding antibiotics to the system depends on the dosing regimen of antibiotics, i.e. the concentration at time t expressed by $c(t)$. Each bacterium have a fixed total number of PBPs (n), each assumed to become saturated independently with identical saturation probabilities given the generation. This implies that $p_{iu}(t)$ can be regarded as a result of $n - Y_1^{(m)}$ independent trials, where the probability of having success (i.e. saturation) at each trial is denoted $\pi(t_1, t_2)$. In other words, the number of unsaturated PBPs that have become saturated during one generation is described by the following binomial distribution,

$$(Y_2^{(m)} - Y_1^{(m)})|Y_1^{(m)} = i \sim \text{Bin}(n-i, \pi(m\tau, (m+1)\tau)). \quad (1)$$

Thus,

$$\begin{aligned} p_{i,u}(m) &= P(Y_2^{(m)} - Y_1^{(m)} = u - i | Y_1^{(m)} = i) \quad (2) \\ &= \frac{(n-i)!}{(u-i)!(n-u)!} \pi(m\tau, (m+1)\tau)^{u-i} (1 - \pi(m\tau, (m+1)\tau))^{n-u}, \end{aligned}$$

where $0 \leq i \leq u \leq n$.

Since the concentration of antibiotics is allowed to vary with time, the probability of saturation will also depend on the time t. The dependency on time is described by the following differential equation

$$\frac{d\pi(t_1, t_2)}{dt_2} = -\gamma c(t_2) \pi(t_1, t_2). \quad (3)$$

Let us denote the initial antibiotic concentration added to a system with C_0. The most common dosing regimen of in vitro studies is letting the concentration remain constant throughout the experiment, $c(t) = C_0$. In this case, solving Equation (3), yields

$$\pi(t_1, t_2) = 1 - e^{-\gamma C_0 (t_2 - t_1)}.$$

A more realistic situation, considering the dynamics of human pharmacokinetics is that of exponentially declining concentration.

If instead the initial dose C_0 is declining exponentially so that $c(t) = C_0 e^{-kt}$, the probability of saturation is given by

$$\pi(t_1, t_2) = 1 - \frac{e^{\frac{\gamma C_0 e^{-kt_2}}{k}}}{e^{\frac{\gamma C_0 e^{-kt_1}}{k}}}.$$

This is the case we will consider throughout this paper.

2. **Reproduction**

At the end of the lifetime, a bacterium either reproduces (creating two offspring) or dies (yielding no offspring).

The conditional probability that a bacterium does not reproduce, given that it has $Y_2^{(m)} = u$ saturated PBPs only depends on the number u. It will be given by the following probability

$$q_u = 1 - P(\text{cell division} | Y_2^{(m)} = u)$$

and the probability for reproduction is hence $(1 - q_u)$.

The probability for death is assumed to follow a generalized logistic (also called Richard's) model [16].

$$q_u = k_1 + \frac{k_2}{(1 + se^{-b(u-a)})^{1/s}} \tag{4}$$

The more saturated a bacterium is, the higher the probability will be for cell death.

The motivation for choice of model is the flexibility, which allows us to control the asymptotes, its position and slope. One example of how the generalized logistic curve might look like (with parameters as presented in Section 4) is shown in Figure 2.

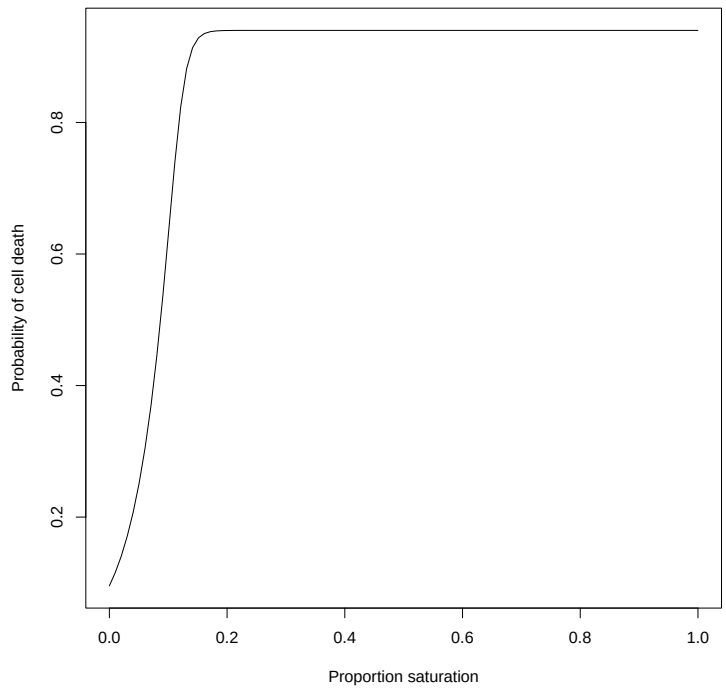

Figure 2: One example of how the generalized logistic curve might look like for an example with parameters as presented in Section 4. The plot shows how the cell death probability varies with the level of saturation.

3. Distribution of saturated PBPs of the parent among its offspring

In case of cell division after the second phase of the cell cycle, the parent dies and produces two offspring having $Y_1^{(m+1)}$ and $Y_2^{(m)} - Y_1^{(m+1)}$ saturated PBPs, respectively and otherwise dies with no offspring produced.

Each offspring will still have a total number n of PBPs, but the number of saturated PBPs of the parent will be randomly distributed among the two offspring. We will denote the conditional probability $r_{u,j}$ of having an offspring with $Y_1^{(m+1)} = j$ saturated PBPs starting its life in generation $m+1$, given that it had a parent with $Y_2^{(m)} = u$ saturated PBPs. We will assume that this follows a hypergeometric distribution. Hence,

$$Y_1^{(m+1)}|Y_2^{(m)} \sim \text{HypGeo}(2n, n, u)$$

which implies that

$$r_{u,j} = P(Y_1^{(m+1)} = j|Y_2^{(m)} = u) = \frac{\binom{2n-u}{n-j}\binom{u}{j}}{\binom{2n}{n}}. \tag{5}$$

with $0 \leq j \leq u \leq n$.

An illustration of how different types of bacteria in the case of three possible types may evolve is shown in Figure 3.

3 Reproduction numbers

We now wish to say something about how the bacterial population will evolve. Many interesting properties describing the population development is determined by the mean reproduction. In Equation (2) we stated that given the size of the preceding generation, the present population size is the sum of the number of offspring of each potential parent and type. Because antibiotic concentration is varying with time, the expectation of these numbers will vary from generation to generation. For this reason, we will introduce a matrix with entry, $\mu_{ij}(m)$, for an i-bacteria's expected number of offspring of type j in generation m. We will refer to this as the mean matrix and denote it by

$$\mathbf{M}(m) = \{\mu_{ij}(m)\}_{i,j=1}^{d}.$$

By the probabilities defined in Section 2 for saturation, reproduction and distribution of saturated PBPs among offspring in Equations (2), (4) and (5), respectively, together with the law of total probability, it follows that

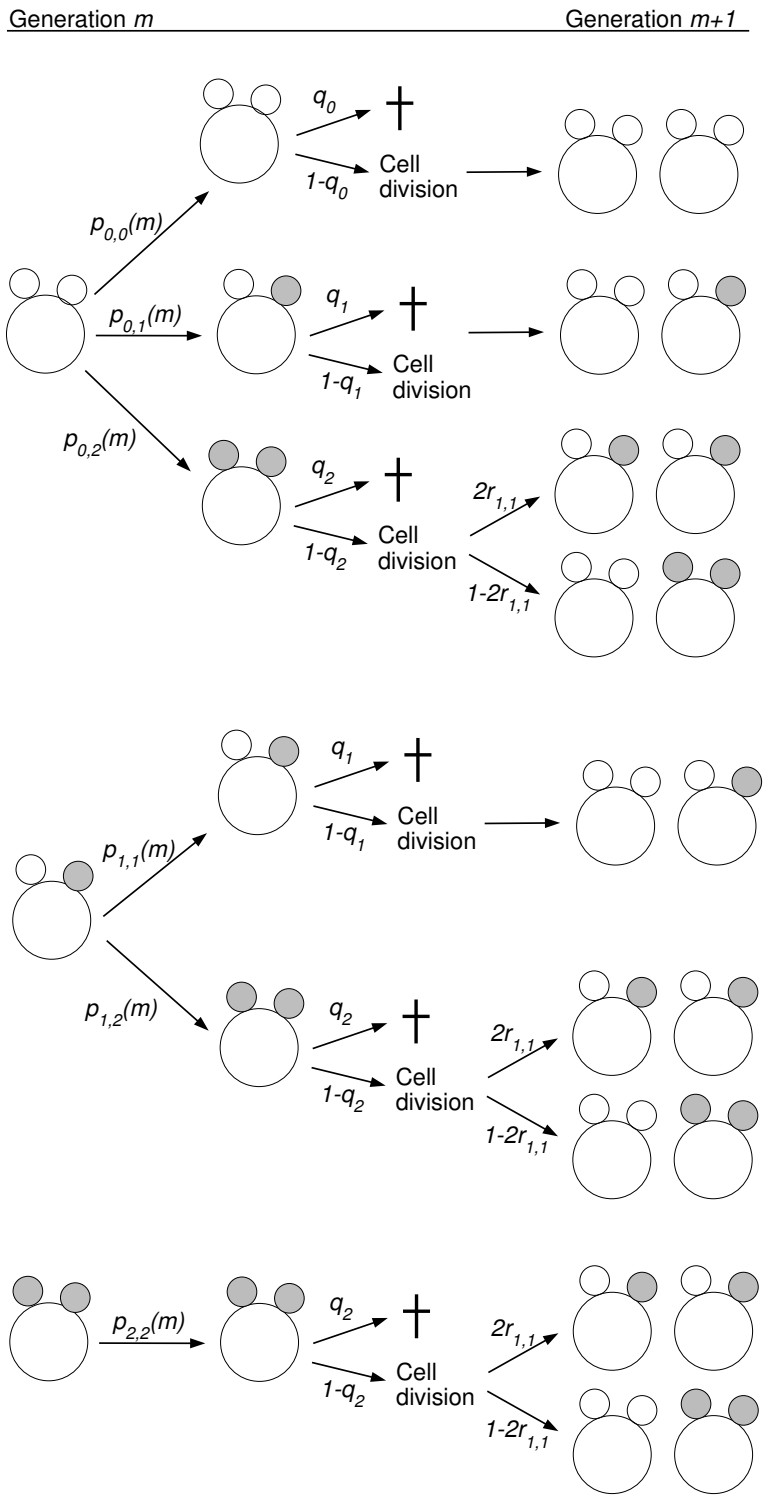

Figure 3: Illustration of possible outcomes from one generation to another for the three different types of bacteria in the case when $n = 2$. Each bacterium lives for a fixed generation life-time and may undergo a change to another type during this time. At the end of the generation the cell either dies with no outcome or generates two offspring.

$$\mu_{i,j}(m) = \sum_{v=i}^{n} p_{i,v}(m)(1-q_v)(r_{v,j} + r_{v,v-j}).$$

The intuitive understanding for the last term, $r_{v,j} + r_{v,v-j}$, in $\mu_{i,j}$ is given by the following: If a parent, which has v saturated PBPs when it splits, gets one child with j saturated PBPs, then the other child must have $v - j$. Also the other way around holds, if a the parent gets one child with $v - j$ saturated PBPs, then the second child must have j saturated PBPs. Hence, we get a contribution to the expected number of j-children both from the probability of having one j-child, $r_{v,j}$, and from the probability of having one $v - j$-child, $r_{v,v-j}$.

Let us introduce the following notation

$$E[\mathbf{Z}_m] = (E[Z_{m0}], \ldots, E[Z_{mn}])^T$$

where T denotes the transpose. Then the relations in vector-matrix form is,

$$E[\mathbf{Z}_m]^T = \mathbf{M}(m)E[\mathbf{Z}_{m-1}]^T$$

and

$$E[\mathbf{Z}_m]^T = \mathbf{M}(m)\mathbf{M}(m-1)\cdots\mathbf{M}(1)E[\mathbf{Z}_0]^T, \tag{6}$$

and since the initial population size, the inoculum, is known, $E[\mathbf{Z}_0]$ can be replaced by $\mathbf{Z}_0$.

4 Data and parameter values

As mentioned in Section 1, there are several factors affecting the PAE. The presence or duration of PAE can differ significantly for specific antimicrobial/organism combinations [6].

The model described in Section 2 can be used for any antimicrobial-organism combination, where the PAE can be defined as above (the time required for synthesis of new PBPs before growth). The parameters in the model will however differ depending on the antimicrobial-organism combination and in order to get any practical use of the model, we have to determine the values of the parameters in the model. For this purpose we use data from a set of in

vitro kinetic experiments with *E. coli* strains which were exposed to different dosing regimens of *cefotaxime*. The data is described in more detail in [12].

In some of the experiments described in [12], the appearance of mutants was shown. Therefore, to avoid the unnecessary impact of other factors not considered in this model, we chose data from the experiments were the mutants were less likely to appear. These experiments will be referred to as, A-F. The initial concentration and half-life associated with each of these trials are presented in Table **??** and in Figure 4 the resulting concentration lapse of the experiments is shown.

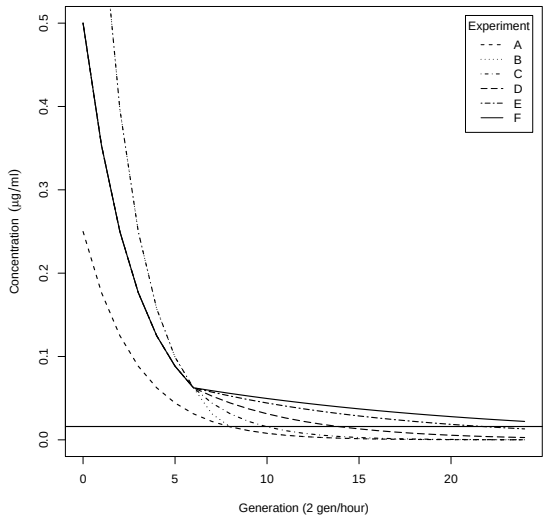

Figure 4: Concentration profiles of cefotaxime for experiments A-E. The horizontal line represents the MIC.

Furthermore, for the purpose of this paper, we only use data for one of these strains (TEM-1). This strain has a minimum inhibitory concentration (MIC) of 0.012 mg/L, which means that the bacterial population, if no PAE is present, would be expected to grow after the antibiotic concentration has declined below this level.

The parameters were estimated using data from experiment C and D, and the experiments E and F were used to validate the model. Additionally, data from experiments without any pressure of antibiotics, see Figure 5 [2] were used for validation. These data gives a check of the maximal growth rate for bacteria without any saturated PBPs.

[2]The time-delay seen in the figure for the growth of bacteria is perhaps a result of the time it takes for bacteria to adapt to the new milieu. The maximal cell division probability is therefore compared to data between 2 and 8 hours.

Table 1: The initial concentration, C_0, the half-life of he concentration, $T_{1/2}$, and the inoculum, Z_{00} (all bacteria are assumed to be unsaturated, and hence of type 0 at the beginning of the experiment), that were used in each of the experiments A-F. The half-life of antibiotics, $T_{1/2}$, is related to the elimination rate, k, by $k < -ln(2)/T_{1/2}$.

Experiment	C_0 (μ g/ml)	$T_{1/2}$ (h)	Z_{00} (log_{10} cfu/ml)
A	0.25	1 (0-12)	5.31
B	1	0.7 (0-3), 0.5 (3-4), 1 (4-12)	5.05
C	1	0.75 (0-3), 1 (3-12)	5.1
D	0.5	1 (0-3), 2 (3-12)	5.17
E	0.5	1 (0-3), 4 (3-12)	5.24
F	0.5	1 (0-3), 6 (3-12)	5.23

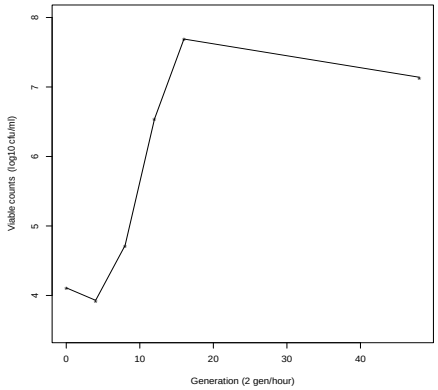

Figure 5: Control curve for growth rate without antibiotics. The time-delay for the growth of bacteria seen between $0-4$ in the graph is perhaps a result of the time it takes for bacteria to adapt to the new milieu. The decrease in the bacterial growth after 24 generations is likely due to limited space in the system.

Due to the extensive amount of time required for numerical calculations with the number of PBPs $n > 10$, the main results will be based on the assumption that $n = 10$, as compared to the realistic number which is between 100-1000.

The parameters γ, k_1, k_2, a, b and s were estimated by minimizing the mean-square errors of the log_{10}-counts of data compared to the corresponding expected value of the bacterial population which was defined in Equation (6). The optimization was made using the optim-routine in R (version 2.5.0) [7].

The following estimates were obtained: $\hat{\gamma} = 1.57$, $\hat{k}_1 = 1.41 \cdot 10^{-5}$, $\hat{k}_2 = 0.94$, $\hat{a} = 1.02$, $\hat{b} = 8.88$ and $\hat{s} = 4.63$. With these estimates the maximal growth

probability $1 - q_0$ achieved from Equation 2 is 0.90, which fits the growth curve of bacteria in absence of any drug effect Figure 5 rather well (fit not shown).

5 Numerical results and simulations

Using the parameters estimated from data from the experimental settings B and C, the expected bacterial growth (based on Equation 6) has been calculated. The results of these calculations are shown in Figure 6.

The data in experiments B, C and D are rather well predicted by the expected outcome of the model. However, in experiments E and F the deviation is larger. In order to get an idea of how much of the deviation that could be explained by the model variance we simulated the outcome ten times.

Experiment A was repeated ten times and in order to investigate how much of the variation seen in the experiments (see Figure 6), we simulated the outcome of the MBP model for the experimental setting A using the parameter estimates from Section 4.

In Figure 7, both data from the ten replicates of experiment A are shown and the result of ten simulations for this experiment are shown.

As seen from the simulated data in Figure 7, the behavior is more or less deterministic before the turning-point to growth. At the turning-point, the counts of the bacterial population are close to the detection limit of 10 bacteria and all variation explained by the model occurs after this turning-point from negative to positive growth. The variation seen in data before the turning-point and partly after the turning-point is perhaps a results of several factors. It is not possible to start with exactly the same inoculum in different repetitions of an experiment. In these repetitions the inoculum varied in the range of $10^{4.94} - 10^{5.35}$ and hence some of the variation before the turning point seen in the data, but not explained by the model is perhaps a result of the varying inoculum. Other variation that might be associated with the experimental methods, such as variation in the initial concentration and the half-life of the concentration, is also not explained by the model.

In another simulation of experiment D (see Figure 8), we see a larger variation after the turning-point as compared to the variation for the simulations of experiment A. In experiment D, the concentration is still relatively high around the turning-point as compared to experiment A and hence the chance that the bacterial population therefore dies out is higher. The bacterial population died out in 7 simulations out of 10 for experiment D, as compared to experiment A in which the bacterial population survived in all simulations.

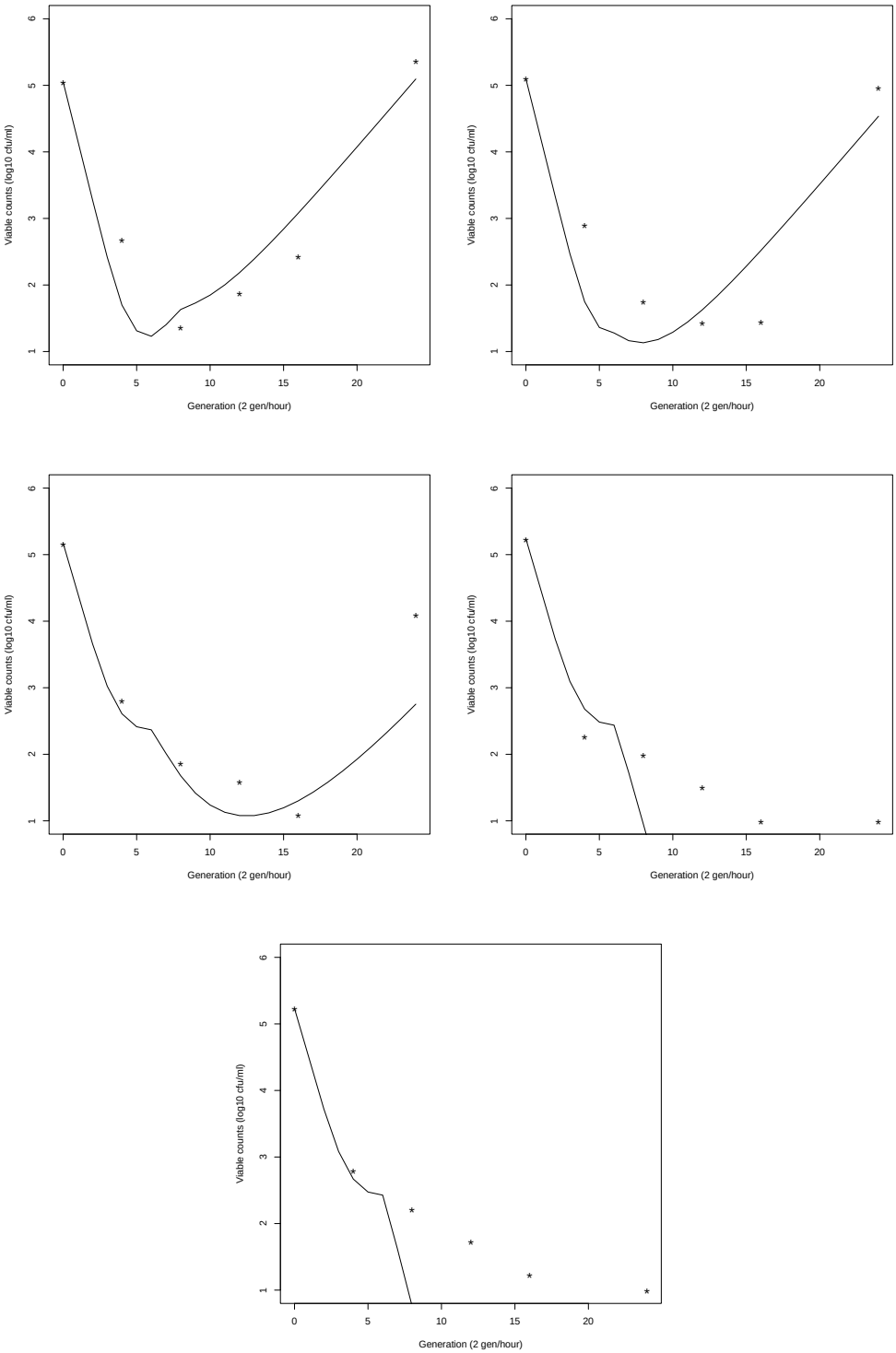

Figure 6: Predicted and experimental data from experimental settings B-F seen from upper left to lower right figure.

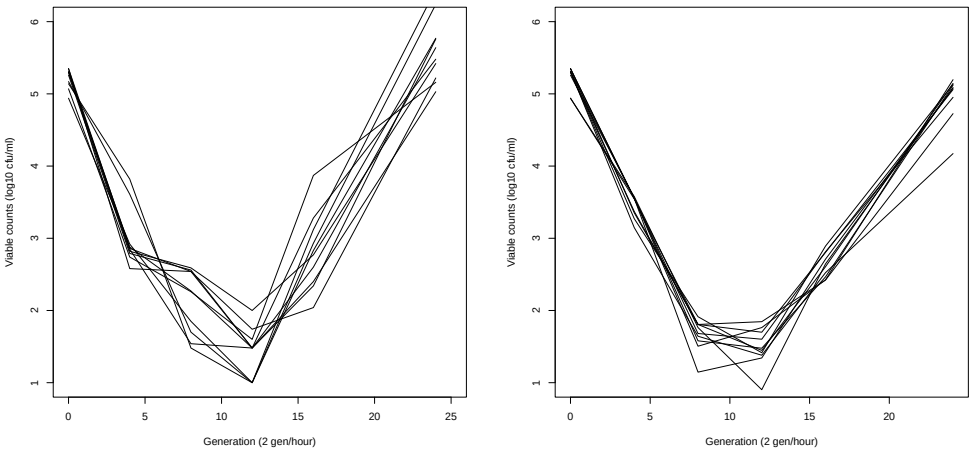

Figure 7: Left: Data from ten repetitions of experiment A. Right: Data from 10 simulated outcomes of experiment A.

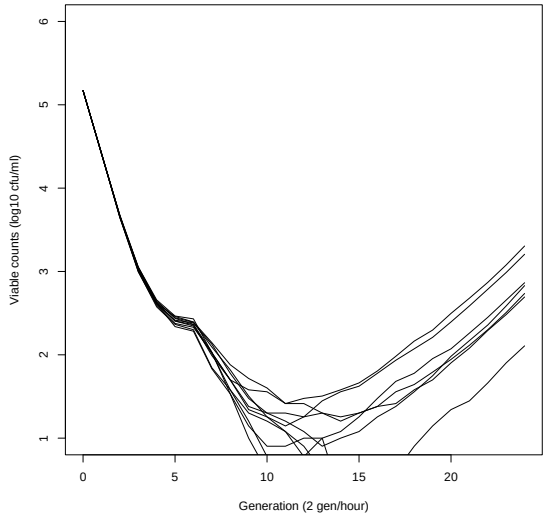

Figure 8: Data from 10 simulated outcomes of experiment D.

6 Discussion

In drug therapy with antibacterial drugs, effective dosage strategies are needed to maintain target drug effects. Mathematical models for the relationship between drug dose and drug effect, so called pharmacokinetic/pharmacodynamic (PK/PD) models, has been studied extensively over the years ([9], [11], [17]).

In this study, we have presented a stochastic model for describing the growth of bacterial populations under treatment of antibiotics, which also captures the phenomenon of delayed growth, the PAE. The model is a multi-type branching process with varying environments and the bacterial growth in this model depends on the saturation of PBPs, which in turn depends on the antibiotic concentration in the system. The main difference from previously published papers on PK/PD models is 1) the stochasticity and 2) the possibility of a delayed effect, PAE. To our knowledge, this is the first stochastic model presented for describing the phenomena PAE.

The model was constructed to be used for any antimicrobial-organism combination, where the PAE can be explained by the theory that the PAE represents the time required for synthesis of new PBPs before growth. However, in this paper, data from a set of in vitro kinetic experiments with *E. coli* strains which was exposed to different dosing regimens of *cefotaxime* was used to test and validate the model.

These experiments were not originally designed for discovering how the PAE varies with different dosing regimens and therefore a range of other factors than the PAE might influence the outcome of the data seen from the bacterial experiments.

In the case of no PAE, the bacterial population would grow immediately after concentrations have declined below the so called MIC. By subtracting the times for which MIC was reached in the experiments A-F with the times of the tuning point as predicted by the model for the corresponding experiments, showed PAEs ranging from -45 minutes to +1 hour in experiments A-D. In experiments E and F, the bacterial populations were predicted to die out before the concentration had declined below the MIC.

There are several explanations for this outcome. In some experiments described in newborn mutations that affected the outcome was seen. The reason for choosing the experiments B-F was that the initial dose of antibiotics was increased to a level above the MICs for the newborn mutants, which would make these variants less likely to be represented in the bacterial population. Despite the increased dose of antibiotics, still newborn mutants were observed for experiment D. Newborn mutants would yield an earlier re-growth of a subpopulation as compared to what would be predicted by the model and could explain the negative PAE predicted for this experiment.

Also, variation arising from the performance of the experiments might influ-

ence the outcome. One source of variation is the initial concentration. The mean initial concentration had a coefficient of variation of 11%. The affect of this variation on the expected length of the PAE has not been investigated further in this paper.

Irrespective of these factors, the simulations showed that the PAEs seen for different experiments are in the range of could be expected just as a result of random variation. As deterministic differential equations has been the mainstay of PK/PD modeling [18], we want to highlight the importance of taking the random fluctuations of the microscopic processes underlying PBP dynamics, mutations and replications into account when modeling. In the limit where the number of bacteria in a PK/PD system is large, these random fluctuations are of negligible magnitude compared with the average bacteria and the deterministic description provides an approximation with negligible error. On the other hand, the deterministic description can lose its validity when the bacterial population becomes small: which is the case after the turning-point where bacteria typically are below the limit of detection for viable counts of 10 cfu/ml.

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