

Modelling protection from antimicrobial agents in biofilms through the formation of persister cells

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A mathematical model of biofilm dynamics was used to investigate the protection from antimicrobial killing that could be afforded to micro-organisms in biofilms based on a mechanism of 'persister' cell or phenotypic variant formation. The persister state is a hypothetical, highly protected state adopted by a small fraction of the cells in a biofilm. Persisters were assumed to be generated at a fixed rate, independent of the presence of substrate or antimicrobial agent. Cells were assumed to revert from the persister state when exposed to the growth substrate. Persister cells were assumed to be incapable of growth. The model predicted that persister cells increased in numbers in the biofilm, even though they were unable to grow, accumulating in regions of substrate limitation. In these regions, normal cells failed to grow, but did slowly convert to the persister state. Calculations of persister formation in planktonic cultures predicted that persisters would be present in low numbers in growing cultures, but should accumulate under conditions of slow growth, e.g. very low dilution rates in continuous culture or stationary phase in batch culture. When antibiotic treatment was simulated, bacteria near the biofilm surface were killed, but persisters in the depth of the biofilm were poorly killed. After antibiotic treatment ceased, surviving persister cells quickly reverted and allowed the biofilm to regrow. This modelling study provides motivation for further investigation of the hypothetical persister cell state as an explanation for biofilm resistance to antimicrobial agents.

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INTRODUCTION

When micro-organisms form biofilms, aggregates of cells attached to a surface, they become less susceptible to killing by antimicrobial agents (Stewart & Costerton 2001; Stewart *et al.*, 2000). The tolerance of microbial cells to biocides and antibiotics when growing as biofilms thwarts efforts to control problems associated with bacteria and fungi in such diverse settings as heat exchangers, paper mills, food-processing plants and heart valves. A general explanation for the protection afforded to micro-organisms in biofilms has yet to be discovered.

Among the explanations that have been offered for the reduced susceptibility of micro-organisms in biofilms are slow antimicrobial penetration, slow growth, and the formation of a protected subpopulation of 'persister' cells (Spoering & Lewis, 2001; Stewart & Costerton, 2001). Here we offer a few thoughts on each of these mechanisms before focusing our attention on the last.

Certain antimicrobial agents can fail to penetrate biofilms. This occurs when the antimicrobial is neutralized in the surface layers of the biofilm faster than it diffuses into the

biofilm (Stewart *et al.*, 2000). The rapid reaction required to prevent penetration has only been demonstrated for highly reactive antimicrobials, such as the oxidizing biocides chlorine and hydrogen peroxide. Most antimicrobial agents probably do penetrate the biofilm.

Biofilms are thought to contain cells in a spectrum of growth states from rapidly growing to not growing at all (Brown *et al.*, 1988; Sufya *et al.*, 2003; Xu *et al.*, 2000). This variety of physiological states would be protective because non-growing or stationary-phase cells are known to be less susceptible to antimicrobial challenge. This mechanism surely contributes to biofilm protection from killing by those agents, notably antibiotics, which target macromolecular synthesis.

One of the newest hypotheses for the reduced susceptibility of biofilms to antimicrobials invokes the formation of a special class of protected cells termed persisters (Spoering & Lewis, 2001). Persisters are thought by some to be cells that have differentiated into an inactive, but highly protected state. Persisters might resemble spores in some ways. The persister hypothesis is attractive because it could explain protection from antimicrobial agents of very different chemistries and modes of action. The existence of persister cells is suggested by killing data that indicate most cells in a

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biofilm dying, with a subpopulation that persists even during prolonged exposure or with elevated concentrations of antimicrobial agent (Spoering & Lewis, 2001). The persister population has been estimated to constitute perhaps 0.1–10% of all cells in the biofilm. These cells, it is hypothesized, can survive a catastrophic antimicrobial challenge and reseed the biofilm.

Though we use the term persister in this article, an alternative and possibly synonymous term is phenotypic variant. Phenotypic variants are cells that have spontaneously switched from the wild-type state into a variant state in which the cell exhibits altered phenotypic properties. These properties may include enhanced antimicrobial tolerance (Drenkard & Ausubel, 2002; Balaban *et al.*, 2004).

Previous modelling work on antimicrobials and biofilms has addressed aspects of antimicrobial neutralization and penetration (Chen & Stewart, 1996; Dibdin *et al.*, 1996; Dodds *et al.*, 2000; Nichols *et al.*, 1989; Stewart *et al.*, 1996), slow growth (Roberts & Stewart, 2004; Stewart, 1994) and regrowth (Sanderson & Stewart, 1997; Stewart *et al.*, 1996). No mathematical models of persister formation in biofilms have been described.

The main objective of this study was to investigate whether persister cell formation could confer increased protection from antimicrobial agents to a population of biofilm microorganisms, compared to cells in free aqueous suspension. The comparison to free-floating cells is important. It is intuitively obvious that persister cell formation will reduce the antimicrobial susceptibility of any population, whether biofilm or planktonic. The observation that we seek to explain is that cells in a biofilm are less susceptible than planktonic cells. Can persister formation account for this difference? A second objective of this theoretical investigation was to predict spatial and temporal features of persister dynamics in biofilms. Such predictions might some day guide experimental tests of this protective mechanism.

THEORY

Three cell types and the transformations between these states determined the core of this model. The three cell types were termed live, dead and persister cells. Live cells used substrate, grew according to Monod kinetics, and were taken to be relatively susceptible to killing by antimicrobial agents. When killed through exposure to an antimicrobial agent, live cells were irreversibly converted to dead cells. Dead cells neither consumed substrate nor grew. Persister cells were formed from live cells, were assumed to be incapable of growth, and did not consume substrate. Persister cells could, under the right conditions, revert to live cells. In the persister state, cells were assumed to be much less susceptible to killing by antimicrobial agents than were live cells. When persister cells were killed by an antimicrobial agent, they were irreversibly transformed to dead cells.

We begin by considering the transformations between live and persister cell states. Transformations that produce dead cells will be added subsequently. One can imagine two broad possibilities for how persister formation could afford greater protection to biofilm populations compared to planktonic populations. One possibility is that the frequency of persister formation is higher in a biofilm than it is in a planktonic culture. This would require the existence of some biofilm-specific regulatory mechanism. The other possibility is that persister cells are formed at the same rate in biofilms, but are retained more effectively in biofilms. We have tested the second conjecture in this investigation. No difference in the rate of formation of persister cells in the planktonic and biofilm states was assumed. The rate of persister cell formation was simply taken to be directly proportional to the live cell concentration. The reversion of persister cells to the live cell state was assumed to depend on the presence of the metabolic substrate (Spoering & Lewis, 2001; Sufya *et al.*, 2003). Persister cells exposed to this substrate reverted to live cells faster than persister cells that were deprived of the substrate.

The mathematical form for the cell transformations described above is conveyed below through the material balances derived for planktonic cultures. In a planktonic continuous stirred-tank reactor (chemostat) at steady state, the live cell balance simplifies to

$$D = \mu - k_F + k_R \mu \frac{X_P}{X_L} \quad (1)$$

where X_L is the live cell concentration, X_P is the persister cell concentration, μ is the specific growth rate, and D is the dilution rate. Other parameters are defined in Table 1. From left to right, the terms in this equation represent loss of live cells by flow out of the chemostat, growth, loss of live cells by conversion to persisters, and creation of live cells by reversion of persister cells. The chemostat balance on persister cells yields

$$D = k_F \frac{X_L}{X_P} - k_R \mu \quad (2)$$

Here the terms, from left to right, correspond to flow out of the vessel, persister formation, and persister reversion.

In a planktonic batch culture, the live cell balance is

$$\frac{dX_L}{dt} = \mu X_L - k_F X_L + \mu k_R X_P \quad (3)$$

and the persister cell balance has the form

$$\frac{dX_P}{dt} = k_F X_L - \mu k_R X_P \quad (4)$$

We now address the production of dead cells. In this study, endogenous decay was not considered. Dead cells therefore arose only via the action of an antimicrobial agent. The rate of killing by an antimicrobial agent was assumed to be directly proportional to the concentration of antimicrobial agent. In a batch planktonic culture in which killing is the

Table 1. Parameter values for biofilm modelling

Parameter	Symbol	Units	Value or range
Maximum specific growth rate	μ_{\max}	h^{-1}	0.417
Yield coefficient	Y_{XS}	mg mg^{-1}	0.8
Monod coefficient	K_{S}	mg l^{-1}	0.1
Cell volume fraction	ε_{c}		0.2
Cell intrinsic density	ρ_{x}	mg l^{-1}	3.0×10^5
Steady-state biofilm thickness	L_{f}	μm	10–400
Liquid boundary layer thickness	L_{L}	μm	10
Substrate influent concentration	C_{S}	mg l^{-1}	8
Antibiotic influent concentration	C_{B}	mg l^{-1}	5
Antibiotic dose duration	t_{B}	h	24
Substrate diffusion coefficient	D_{S}	$\text{m}^2 \text{h}^{-1}$	9.67×10^{-6}
Antibiotic diffusion coefficient	D_{B}	$\text{m}^2 \text{h}^{-1}$	1.80×10^{-6}
Biofilm/bulk diffusivity ratio	τ		0.45
Persister formation rate coefficient	k_{F}	h^{-1}	0.0021
Persister reversion rate coefficient	k_{R}		0.1
Live cell death rate coefficient	k_{DL}	$\text{l mg}^{-1} \text{h}^{-1}$	2.08
Persister cell death rate coefficient	k_{DP}	$\text{l mg}^{-1} \text{h}^{-1}$	0
Reactor liquid volume	V	m^3	0.001
Biofilm surface area	A	m^2	0.01
Volumetric flow rate	Q	$\text{m}^3 \text{h}^{-1}$	0.417

only process, the live cell balance would be

$$\frac{dX_{\text{L}}}{dt} = -k_{\text{DL}}X_{\text{L}}C_{\text{B}} \quad (5)$$

where C_{B} is the concentration of antimicrobial agent and other parameters are defined in Table 1.

Killing of persister cells followed the same form, but the disinfection rate coefficient was different, and smaller, than that used for live cells. Persister cell killing in batch culture is described by

$$\frac{dX_{\text{P}}}{dt} = -k_{\text{DP}}X_{\text{P}}C_{\text{B}} \quad (6)$$

The corresponding dead cell balance is

$$\frac{dX_{\text{D}}}{dt} = k_{\text{DL}}X_{\text{L}}C_{\text{B}} + k_{\text{DP}}X_{\text{P}}C_{\text{B}} \quad (7)$$

In the case study examined in this work, the low susceptibility of persister cells was captured by setting the rate of transformation of persister cells to dead cells to zero (i.e. $k_{\text{DP}}=0$).

The basic biofilm model used in this investigation, and its solution, have been described in detail elsewhere (Stewart, 1994; Stewart *et al.*, 1996). The model was based on the conceptual and mathematical formulation derived by Wanner & Gujer (1986). The model described the growth of a uniformly thick biofilm in a continuous-flow stirred-tank reactor – a chemostat with wall growth. Biologically, the system was conceptualized as a single species whose growth rate was determined by the concentration of a single substrate, according to Monod kinetics. Some of the

processes integrated in this model included bulk flow into and out of the reactor, transport of solutes into the biofilm by Fickian diffusion, substrate consumption by the micro-organism, microbial growth, transport of cells within the biofilm by advective displacement, detachment of biomass from the surface of the film, and killing of micro-organisms in the presence of an antibiotic. Macroscopic material balances around the entire reactor vessel were coupled to one-dimensional differential material balances that described processes occurring within the biofilm at the microscale.

Base-case parameter values are summarized in Table 1. For this study, the dilution rate was set to a very large value (417 h^{-1} , 1000 times the maximum specific growth rate of 0.42 h^{-1}). This means that there can be essentially no growth of planktonic cells in the reactor vessel and that the bulk fluid concentration of substrate or antibiotic that the biofilm is exposed to is essentially equal to the influent concentration.

The dynamics of biofilm thickness were determined by the balance of net growth and detachment. In this model, detachment is treated as erosion from the surface of the biofilm at an erosion velocity proportional to the square of the biofilm thickness. The initial biofilm thickness was set to the desired simulation thickness by adjusting the detachment rate coefficient. The value of the detachment coefficient used was the value that yielded the desired steady-state thickness in a simulation in which only live cells were present.

The initial volume fractions of live, persister and dead cells were set to 0.2, 10^{-8} and 10^{-8} , respectively.

RESULTS AND DISCUSSION

A mathematical model of biofilm dynamics predicted that persister cells accumulated in biofilms, even though persisters were unable to grow (Fig. 1). The fraction of persister cells in the biofilm continued to increase over a period of several days. The biofilm thickness was stable during this time (data not shown). The thicker the biofilm was, the greater the fraction of total cells that were persisters (Fig. 1). The fraction of persisters in the biofilm after 10 days ranged from 0.0046 for a 10 μm thick biofilm to 0.33 for a 400 μm thick biofilm.

All of these features can be explained by recognizing that these biofilms are predicted to contain a substrate-depleted region in the lower stratum of the film. In this low-nutrient region, live cells are unable to grow. Live cells continue, however, to spontaneously convert to persister cells. Thus, in the substrate-starved region of the biofilm, persister cells outcompete live cells and accumulate locally. In the surface layers of the biofilm, growth of live cells outpaces their conversion to persister cells, and so live cells dominate. The model predicted exactly this spatial distribution of live and persister cells (Fig. 2a).

The accumulation of persister cells in the depth of a biofilm is analogous to the accumulation of inert or inactive biomass in a biofilm population subject to endogenous decay. Significant fractions of inert biomass in such biofilms have been simulated by classic multispecies biofilm models (Kissel *et al.*, 1984; Wanner & Gujer, 1986; Rittmann & Manem, 1992) as well as newer models (Lapidou & Rittmann, 2004a, b; Picioreanu *et al.*, 2004).

The same mathematical descriptions of persister formation and reversion that were used in the biofilm model can also be solved in a chemostat with no biofilm present. In continuous planktonic culture, the fraction of persisters was predicted to increase as the dilution rate was decreased (Fig. 3). The minimum persister fraction was calculated to occur at the washout dilution rate. For the parameter values

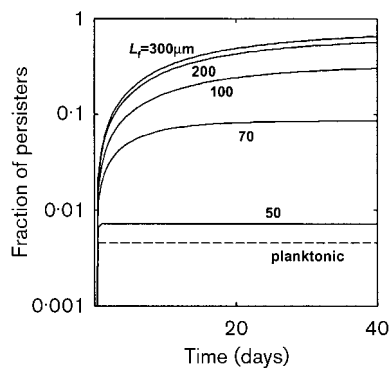


Fig. 1. Accumulation of persister cell in biofilms of various thicknesses (L_f).

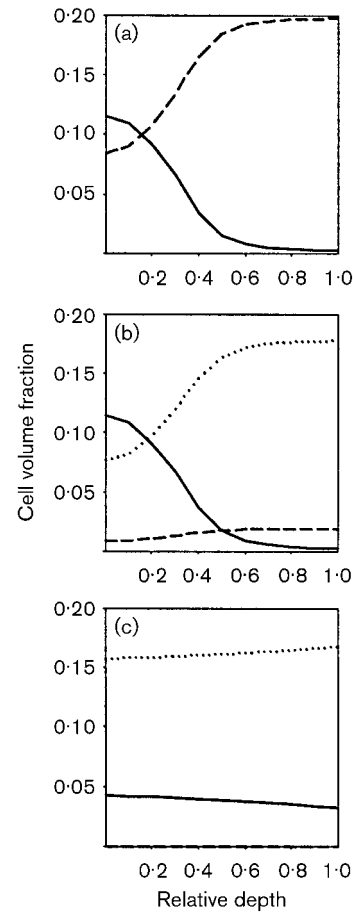


Fig. 2. Persister (solid line), live (dashed line), and dead (dotted line) cell volume fractions as a function of depth in a 3-day-old biofilm. (a) Distribution before antimicrobial dosing; (b) distribution after 0.24 h of antimicrobial exposure; (c) distribution after 24 h of continuous antimicrobial treatment. The biofilm was 100 μm thick in the simulations shown in (a) and (b), and it was 19.4 μm thick in (c). Relative depth is the distance from the substratum divided by the total biofilm thickness.

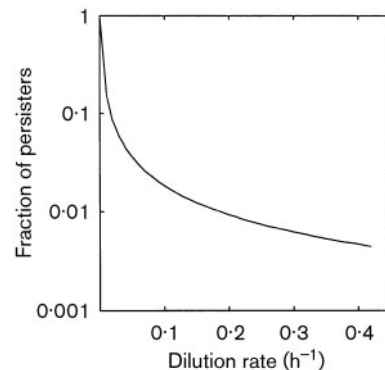


Fig. 3. Fraction of total cells that are persisters in continuous planktonic (chemostat) culture.

used in this study, the minimum persister cell fraction in a chemostat was calculated to be 0.0046. To reach the same level of persisters computed for a 100 μm thick biofilm ($f_p = 0.165$), the dilution rate in the chemostat would have to be just under 0.01 h^{-1} . To reach the same level of persisters computed for a 400 μm thick biofilm, the chemostat dilution rate would need to be 0.0037 h^{-1} . Such low dilution rates are rarely, if ever, applied in experimental studies. These calculations demonstrate, however, that the degree of protection afforded by persister cell formation in a biofilm could be realized in a very slowly growing planktonic culture.

The same conclusion was reached through analysis of persister formation in a planktonic batch culture. In batch culture, the minimum persister cell fraction was calculated to be 0.0046 for the parameter values used in this study. In a simulation of a batch culture, the persister cell fraction increased in stationary phase (Fig. 4). After several days, the persister cell fraction predicted for a stationary-phase batch culture reached levels similar to those computed in biofilms. This result is in agreement with the experimental work of Spoering & Lewis (2001), who presented data suggesting that stationary-phase planktonic cell cultures contain large numbers of persisters.

In all of these calculations, the threshold for persister cell accumulation corresponds to the rate of persister formation being equal to or greater than the growth rate of live cells. This occurs at very low substrate concentrations. For the parameter values used in this investigation, the critical substrate concentration for persister accumulation was $5 \times 10^{-4} \text{ mg l}^{-1}$ (whereas the bulk fluid concentration was 8 mg l^{-1}). Wherever the substrate concentration is less than this critical value, persister cells will outcompete live cells. In thin biofilms, this low level of substrate is never present, and the persister cell fraction tends to the planktonic minimum value. The critical biofilm thickness for persister cell accumulation, using the parameter values in Table 1, was

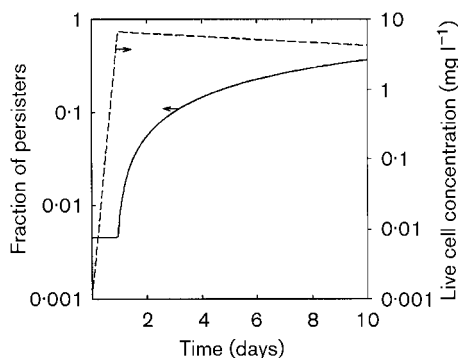


Fig. 4. Fraction of total cells that are persisters (solid line) in batch planktonic culture. Also plotted is the live cell concentration (dashed line). Substrate is depleted in the culture after approximately 1 day.

approximately 50 μm . Any biofilm thicker than this is predicted to allow for persister cells to accumulate.

We wish to emphasize that accumulation and retention of persisters in the biofilm does not depend on specific regulatory mechanisms that increase the rate of persister formation specifically in the biofilm mode of growth. Our results do not disprove such a mechanism, but they do show that it is not necessary to assume such regulation. Our predictions regarding persister competition in biofilms are conservative in that we have assumed that persisters do not grow at all. If persister cells can grow, their ability to compete for space in the biofilm would only be improved.

Persisters continue to accumulate even after biofilm thickness has plateaued (Fig. 1). The time scale for this accumulation, which confers steadily decreasing susceptibility of the biofilm population, was several to many days. This is believed to be the first theoretical description of an explanation for the increased antimicrobial tolerance of ageing biofilms.

When an antimicrobial treatment was simulated, bacteria near the biofilm surface were rapidly killed (Fig. 2b). Persister cells, which were assumed to be invulnerable to the antimicrobial, were unaffected. After prolonged (24 h) antimicrobial exposure, the biofilm contained mostly dead cells and persister cells (Fig. 2c). The remaining persister cells quickly reverted and allowed the biofilm to regrow after antibiotic treatment ceased (Fig. 5). During the day-long antimicrobial exposure, the biofilm thickness decreased from 100 μm to 19.4 μm . This decrease was due to continuing biofilm detachment during a period when growth had been greatly suppressed. Within 24 h after the end of antimicrobial dosing, the biofilm thickness was back

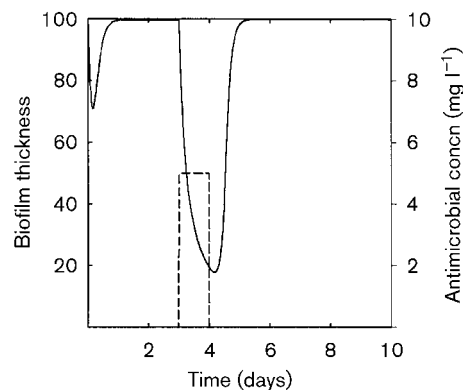


Fig. 5. Regrowth of biofilm containing persister cells after antimicrobial treatment, as reflected in the biofilm thickness (solid line). The bulk fluid concentration of antimicrobial agent is shown as a dashed line. The parameter settings used to perform this simulation were the same as those used to generate Fig. 2. Antimicrobial was dosed for 1 day, beginning on day 3.

to 98 μm , nearly its original thickness. This recovery reflects the rapid growth of the surviving cells.

We conclude that persister formation can, in theory, lead to greater antimicrobial protection in a biofilm compared to a growing planktonic culture. This is because persisters are able to accumulate locally in the biofilm in regions where no, or very little, cell growth is occurring. In these regions, bacteria function as they would in a very slowly growing chemostat culture or in an ageing stationary phase batch culture.

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