A three-dimensional, stochastic simulation of biofilm growth and transport-related factors that affect structure

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Biofilm structural heterogeneity affects a broad range of microbially catalysed processes. Solute transport limitation and autoinhibitor production, two factors that contribute to heterogeneous biofilm development, were investigated using BacMIST, a computer simulation model. BacMIST combines a cellular automaton algorithm for biofilm growth with Brownian diffusion for solute transport. The simulation represented the growth of microbial unit cells in a three-dimensional domain modelled after a repeating section of a constant depth film fermenter. The simulation was implemented to analyse the effects of various levels of transport limitation on a growing single-species biofilm. In a system with rapid solute diffusion, cells throughout the biofilm grew at their maximum rate, and no solute gradient was formed over the biofilm thickness. In increasingly transport-limited systems, the rapidly growing fraction of the biofilm population decreased, and was found exclusively at the biofilm–liquid interface. Trans-biofilm growth substrate gradients also deepened with increasing transport limitation. Autoinhibitory biofilm growth was simulated for various rates of microbially produced inhibitor transport. Inhibitor transport rates affected both the biofilm population dynamics and the resulting biofilm structures. The formation of networks of void spaces in slow-growing regions of the biofilm and the development of columns in the fast-growing regions suggested a possible mechanism for the microscopically observed evolution of channels in biofilms.

INTRODUCTION

Microbial biofilms are communities of bacteria that attach to surfaces and form heterogeneous three-dimensional structures (Stickler, 1999). The functional significance of biofilm structural heterogeneity extends to diverse areas of applied microbiology. In the clinical setting, mature biofilm structure contributes to antibiotic resistance through a variety of mechanisms (Mah & O'Toole, 2001). In the industrial setting, heterogeneous biofilm and aggregate structure affects denitrification processes (de Beer & Schramm, 1999; Schramm et al., 1999), and modelling results indicate that heterogeneous biofilm formation influences the onset of metal corrosion (Picioreanu & van Loosdrecht, 2002). In general, biofilm architecture affects mass transfer in biofilms (Rasmussen & Lewandowski, 1998; Stoodley et al., 1999), and is relevant when considering microbial biofilms as catalysts as well as for managing the efficacy of biocides and compounds that influence biofilm development, e.g. furanones (Ren et al., 2001, 2002).

An approach that has provided new insight into the factors that influence biofilm structure has been the use of cellular automata (CA) simulations. CA simulations of biofilms represent cells as discrete units that replicate stochastically in a two- or three-dimensional domain according to a set of rules, and they are effective at simulating the heterogeneity in biofilms (Pizarro et al., 2001). A general feature of CA models of biofilms is their ability to dynamically generate a range of observed biofilm morphologies using a minimal set of assumptions about cell behaviour; however, computational time constraints have limited most models to two dimensions (Hermanowicz, 1999, 2001; Pizarro et al., 2001). An approach to improving computational efficiency has been to decouple solute transport from stochastic bacterial growth by the use of numerically solved, partial differential equations to describe substrate diffusion (Picioreanu et al., 1998). This method allows the CA model to extend to three dimensions more easily, but does so at the expense of losing heterogeneity in the solute concentration profile. Another new trend in CA biofilm models is an individual-based modelling approach, which allows for variability in each of the cells in the simulation (Kreft et al., 2001). Overall, CA models have been employed...
to simulate several diverse microbial biofilm systems, including a single-species nitrifying biofilm (Picioreanu et al., 1998), a dual-species nitrifying biofilm (Kreft et al., 2001), and an anaerobic biofilm comprising a sulfate reducer and a methanogen (Noguera et al., 1999).

In this work, an individual-based three-dimensional CA model, coupled with discrete Brownian diffusion, was developed. The model, BacMIST (Multi-threaded Independent Solute Transport), uses several techniques to allow for a tractable stochastic solute diffusion simulation in three dimensions in order to retain the effects of local solute concentration heterogeneity. BacMIST simulated two factors that could contribute to the structural heterogeneity in the biofilm. In one scenario, solute transport limitations into the biofilm were considered. Transport limitations affect biofilm structure in various ways, depending on the ecology of the system under consideration. For example, limited oxygen diffusion altered the composition and distribution of microbial populations in nitrifying biofilm-like communities (Vogelsang et al., 2002). Similarly, transport of substrate into membrane-aerated biofilm reactors influenced the size, location and number of active zones within the biofilm (Casey et al., 1999). On the other hand, the population size of sulfate-reducing bacteria near the substratum of a wastewater biofilm remained stable over time as a result of substrate transport limitations (Ito et al., 2002). In a second scenario, the influence of microbially generated autoinhibitor compounds was evaluated. Autoinhibition may result from the microbial synthesis of metabolic end products that produce unfavourable changes in the organism’s environment, such as the lowering of pH upon production of excessive acetate by Escherichia coli growing on glucose (Ingraham & Marr, 1996). Alternatively, the end product itself may also act as an inhibitor, as in the production of methanol by a Pseudomonas sp. growing on methane (Wilkinson et al., 1974). Autoinhibitory compounds are found in several species of surface-colonizing marine bacteria that contribute to biofouling, and have been postulated to function in maintaining bacterial community diversity (Holmstroem et al., 2002). More generally, bacterial programmed cell death, catalysed by autolysins, contributes to developmental processes in a range of diverse bacteria (Lewis, 2000), and may affect biofilm development. The CA model described here accounts for transport of substrate and inhibitors as well as cell death and examines their impact on biofilm structure.

MODEL DESCRIPTION

Reactor geometry. A useful experimental system in which to model biofilm growth and development is the constant depth film fermenter (CDFF). This biofilm reactor was first introduced in 1974, and since then has been improved and developed by Wimpenny and colleagues, who provide a detailed description of the system (Wimpenny et al., 1993). The CDFF approximates a steady state by only allowing biofilm growth in recessed regions of constant depth cut into the surface of a solid plate. A scraper bar constantly passes over the plate surface, removing any biofilm that grows above the tops of the recesses. Fresh growth medium is continuously fed in and distributed over the CDFF surface, aided by the spreading action of the scraper bar, and spent medium is drained out of the apparatus through an outlet port. Since the biofilm is grown in the protected recessed regions, it is not subjected to hydrodynamic forces that can potentially contribute to biofilm detachment and channel formation. This protected geometry also implies that transport processes in the biofilm growth regions of the CDFF are dominated by diffusion.

Simulation domain. Growth simulations performed using BacMIST take place in a three-dimensional spatial domain representing a section of a CDFF recessed region. The domain is discretized into sites on a cubic lattice, and each site or unit cell in the lattice was assumed to be 1 μm³. The domain organization used in this work shares similarities with the CA simulation presented by Hermanowicz (1999), except the domain in this work is in three dimensions. Each of the sites is occupied by either a unit volume of biomass (which could be one or more bacterial cells, possibly embedded in extracellular polymeric substances) or an equivalent volume of liquid. The domain is bounded on one side by a solid surface, the substratum, on which the biofilm grows. The opposite side of the domain represents the top of the recessed region in the CDFF, the ‘shear boundary’. Any cells reaching this point of the domain will be ‘scraped’ from the biofilm and removed from the simulation as they are swept away by passing liquid. In the other two directions, parallel to the growth surface, periodic boundary conditions are implemented. Any cell pushed out of the domain through a periodic boundary will be replaced by an identical cell entering the domain in the same place at the opposite boundary. In this way, the simulation domain represents a repeating unit of an infinite biofilm of finite thickness. For the simulation tests involving structural observation, special care is needed to choose a domain size that would not create an overlapping, aliasing effect. It was empirically determined from numerous trials that a domain size having width and length dimensions greater than height usually gives a more lucid three-dimensional plot, while a cubic domain results in clearer two-dimensional slice images.

Simulated activities. A natural biofilm begins as a small population of cells that have individually adhered to a solid substratum. As these cells grow and divide, their offspring spread over the surface and eventually form an adherent, multicellular contiguous population. Analogously, the biofilm growth simulation begins with a randomly placed population of substratum ‘colonizers’. A file with the descriptions and initial locations of the colonizers must be processed by the simulation before the biofilm growth phase can begin. One colonizing cell is sufficient to initiate a biofilm.

Once the substratum is colonized, the biofilm growth simulation cycles through four classes of behaviours during each time step. (i) Solutes are transported by random-walk diffusion, updating their locations throughout the simulation domain. (ii) Cells consume and produce solutes based on food and inhibitor solute concentrations in their immediate vicinities. As a result, cell growth counters are updated, as are overall solute numbers. (iii) Cells that have consumed enough growth substrate divide. Randomly chosen cells in the population die and lyse. Cells that have moved beyond the shear boundary are also removed from the simulated biofilm. Thus, cell numbers and locations are updated. (iv) The thin interface layer representing the diffusion boundary layer is replenished with solutes to cover the solutes lost in consumption. These behaviours are described in more detail in the following sections. A flow diagram describing the basic simulation is provided in Fig. 1.

Solute transport. Diffusion can be modelled stochastically by a large number of particles undergoing random walks in space. Each random walk consists of a series of equal-length, straight-line movements
interspersed with an equal number of completely random direction changes. This walk simulates the motions exhibited by molecules or small particles which translate due to system internal energy, and which change direction due to collisions with other particles in the same system. While any one particle can be moving in any given direction at a specific time, the net motion of the population of particles will be down the particle concentration gradient.

Each cell in the biofilm growth simulation is a consumer, and possibly a producer, of various solutes. As the biofilm grows and cells are shifted around due to division, the boundary conditions for the solute diffusion problem continuously change. Essentially, the problem is reduced to solute diffusion in three dimensions among a large number of moving sources and sinks. When there are multiple cell types, with different consumption and production patterns, such a problem is extremely complex if implemented using the Fickian description of diffusion. For this reason, random-walk diffusion was chosen as the paradigm for solute transport in the biofilm simulation.

Some simplifications were made in the implementation of random-walk diffusion as the transport mechanism in the biofilm simulation. Instead of describing individual solute molecules, particles represent ‘quanta’ of solute. Each quantum contains some number of solute molecules, so that a relatively small number of quanta of a solute in a simulation lattice cube can represent a moderately high concentration of that solute. Solute diffusivity of a lattice cube not occupied by a biofilm cell is set higher than the diffusivity of a cube containing a biofilm cell. Although data may be lacking in the case of bacterial colonies, diffusion of solutes through tissue and gels has been measured by a number of groups (Berk et al., 1996; Johnson et al., 1996) and is, in general, orders of magnitude lower than diffusion in free solution. An effective diffusivity \( D \) can be defined in a three-dimensional system (Lee et al., 1989):

\[
D = \frac{na^2}{6\Delta t}
\]

where \( n \) is the number of steps taken in the random walk, \( a \) is the length of a single step, and \( \Delta t \) is the time interval over which the \( n \) steps take place (the units of the parameters used in the equations are given in Table 1). A flow diagram describing the implementation of solute transport is provided in Fig. 2(a).

**Microbial consumption and production of solutes.** Following a round of Brownian diffusion, each successful solute particle in the simulation is located somewhere in the active solute domain, and each of these locations is in the interior of a lattice cube of the domain grid. In order for a solute particle to be transferred from the diffusion boundary layer to the biofilm, there must be a concentration gradient for the diffusion. Otherwise, the solute will stay in the boundary layer or diffuse out of the biofilm active domain if the concentration of solute inside the biofilm becomes equal to or greater than the bulk. If a solute quantum is in a grid space occupied by a cell, that cell has an opportunity to act on or be influenced by that solute, depending on the relationship between them. During
the consumption and production phase of the growth simulation, a cell examines all the solutes in its grid space, and acts or does not act on each.

If the cell encounters a quantum of its growth substrate, it may be consumed. Simulated cell consumption of growth substrate is a process first order in cells and first order in substrate. A single cell consuming a single substrate, therefore, does so with probability

\[ k_r \] 

where \( k_r \) is the second-order consumption rate constant, \( \mu C \) and the inverse yield constant, multiplied by an inhibition factor, calculated as follows:

\[ \text{multiplier} = \begin{cases} \text{Factor}^{-1} & \text{if } i > I_{\text{thresh}} \\ 1 & \text{if } i < I_{\text{thresh}} \end{cases} \] (5)

where \( i \) is the number of inhibitor quanta in the cell’s grid space, \( I_{\text{thresh}} \) is the threshold for inhibition specific to this cell–inhibitor pair, and \( \text{Factor} \) is a positive value determining the extent of additional inhibition from each inhibitor quantum over threshold. Since inhibitor decreases the rate constant \( k_{r,i} \), it acts by decreasing a cell’s growth rate.

**Cell division.** Bacterial cells will continue to divide, given adequate nutrients, despite contact with neighbouring cells on all sides. A dividing cell elongates or enlarges, then pinches off into two daughter cells. Since the daughter cells were originally one cell, after division they are adjacent to one another in the biofilm. During the growth and division process, neighbouring cells are pushed out of the way to make room for the new cells, deforming the film matrix (Characklis, 1990a; Gujer & Wanner, 1990). Dividing cells exhibit discrete growth, occupying a single lattice cube of space until division, at which point they divide into two cells, each of which occupies a single lattice cube. One daughter cell remains in the mother cell’s original location. The other daughter cell is placed in a lattice cube adjacent to the first daughter cell, in the direction which offers the least resistance to film deformation (Hermanowicz, 1999). A cell divides in one of 26 directions (1 for each of the 6 faces, 12 edges and 8 vertices of the cube), corresponding to all possible adjacent cubes to the cell. Each direction is checked for free spaces at increasing distances from the dividing cell, and the first direction in which such a free space is found is considered the path of least resistance. If there are several directions which are equally minimal, the cell divides in the direction which such a free space is found is considered the path of least resistance. If there are several directions which are equally minimal, the cell divides in the direction which offers the least resistance to film deformation, one of these is chosen at random.

When a direction has been chosen and division occurs, space is made for the new cell. This is accomplished by pushing the entire line of cells between the dividing cell and the closest free space by one lattice space.
Fig. 2. Flow diagrams for complex events. (a) Solute diffusion. (b) Solute consumption and production. (c) Cell growth and division.
Fig. 3. A pyramidal-dome-shaped colony produced from a single cell. A single cell was placed on a substratum and allowed to grow and divide in the absence of substrate limitations. A 50 µm × 50 µm × 50 µm volume of the simulation domain is shown.

cube in the chosen direction. One lattice cube is thus opened up adjacent to the dividing cell, and the new cell is placed into this lattice cube. A flow diagram describing the implementation of cell division is provided in Fig. 2(c). The formation of a pyramidal-domed colony from a single cell placed on the substratum (Fig. 3) demonstrates the validity of the cell division and placement rules.

As the biofilm cells in the domain rearrange to accommodate new divisions, the diffusion boundary layer evolves to cover the new biofilm structure. At each division, the simulation checks the location of free space that the cell is replacing to see if the 26 spaces in contact are possible boundary layer spaces. If the spaces are adjacent to the outermost biofilm cells, they are assigned as diffusion boundary layer spaces and will be replenished with solutes on the next time step (Fig. 2c). The diffusion boundary space releases its assignment whenever a new cell divides into it or when the space is no longer part of the boundary layer.

Cell death. Individual cell death in a biofilm can occur for different reasons, most of which are poorly understood. Cell death strikes all cells of a species with equal probability, regardless of age, growth rate, location, inhibition status, or other factors. In the biofilm growth simulation, cell death is represented as either complete cell lysis or a dead cell corpus, a user-controllable random selection. A lysed cell is removed from the simulation and leaves a free space (filled with biomass that could be utilized by neighbouring cells) within the biofilm in its former location. A dead cell is a cell that no longer consumes and divides, but still contributes to the structure of the biofilm. Eventually, as new cell divisions put pressure on the dead cell, it breaks down and becomes a free space. Nearby dividing cells can use the free spaces left behind by lysed cells to divide or push adjacent cells into. In both cases, biomass left by lysis may be recycled back to the active domain as available solute. Death rate, as implemented in the biofilm growth simulations, is first order in cell number. The death rate constant, \( k_d \), is uniform for all cells of a given species. A selected cell dies within a time step \( \Delta t \) with probability \( P_d = k_d \Delta t \).

Scaling. The definition of random-walk diffusivity in terms of random-walk parameters, as set forth in equation (1), assumes a large value for \( n \), averaged over the random walks of a large number of particles, and a small value for \( a \), the step size. A realistic diffusivity of the order of \( 10^{-7} \, \text{cm}^2 \, \text{s}^{-1} \), when \( n = 1 \), would require an \( n \) of 10 000. The domain space also needs to be sufficiently large to contain the mean free path for each random walk. Given the large number of solute quanta and the domain size, the total number of iterations required for each \( \Delta t \) would be in the order of \( 1 \times 10^{11} \). Even with a fast computer the simulation time would be much greater than the simulated time.

In order to explore realistic biofilm problems on a desktop computer, we scaled the simulation using dimensional analysis common to reaction-diffusion problems. For example, most reaction-diffusion problems can be characterized by a ratio of the time required for reaction to that required for diffusion, referred to by Characklis (1990b) as a penetration ratio:

\[
\Omega_p = \frac{\text{time constant for diffusion}}{\text{time constant for reaction}} = \frac{L_f^2 / D}{C_{\text{bulk}} / \left( u_{\text{max}} \cdot Y_s / C \cdot \tau_C \right)}
\]

where \( L_f \) is the maximum biofilm depth and \( \tau_C \) is the maximum biofilm density (e.g. 1 cell \( \mu \text{m}^{-3} \). Small values of \( \Omega_p \) indicate that diffusion is faster than solute consumption (production) and that there is minimal transport limitation. Large values of \( \Omega_p \) imply that solute is consumed (removed) more rapidly than it can be replaced by diffusion, and the biofilm is thus transport-limited.

In order to maintain a constant value for \( \Omega_p \) while changing the value of \( D \), we varied the biofilm depth (\( L_f \)). Large values of \( D \) (e.g. \( n = 10000 \)), corresponding to realistic diffusivities, required extremely long simulation times. Small values of \( D \) allowed for shorter simulation times, but corresponded to unrealistic diffusion rates. Using dimensional analysis, one should be able to simulate complex biofilms using small values of \( D \) to speed simulations. To determine if the model was scalable for various values of \( D \) and corresponding values of \( L_f \) at a single value of \( \Omega_p \), the simulation was run for \( n = 10000, 1000, 100, 10 \) (corresponding to diffusivities of \( 10^{-7} \, \text{cm}^2 \, \text{s}^{-1} \) to \( 10^{-3} \, \text{cm}^2 \, \text{s}^{-1} \)) and \( L_f \) of 316, 100, 32 and 10, respectively. The similarity in the simulation results for each pair of values indicates that the lower values of \( n \) and \( L_f \) can be used to simulate realistic diffusivities without significant computational time (Fig. 4). A partial differential equation describing solute diffusion just penetrating through a biofilm (Characklis, 1990b) was solved and plotted as a reference point. The solution equation is

\[
S^* = \frac{x^2}{\Omega_p^2} \cdot \frac{2 x^*}{\Omega_p^2} + 1
\]

where \( S^* \) and \( x^* \) are the non-dimensionalized form of substrate concentration and biofilm depth respectively, and \( \Omega_p \) is set to 1.

Features, assumptions and simplifications in the biofilm growth simulation. Several assumptions were made to facilitate the simulation, and are enumerated below.

1. Cells, or species-specific units of biomass, are discrete and cubical.
2. Extracellular polymeric substances have no special representation and are either neglected or assumed to be uniformly distributed around cells and throughout the biofilm.
3. Solute diffuse in quanta, rather than as single molecules. This discretizes the solute concentrations possible in a lattice grid space.
4. Solute diffuse at a different rate through liquid-filled and cell-filled spaces. Each solute type can have its own diffusion rate through the cell-filled spaces.
5. No additional cell attachment takes place after the initial colonization of a surface by microbial cells. The biofilm population is increased only through cell growth, not through immigration of cells previously suspended in the liquid bulk. Detachment of cells from the biofilm takes place only at the shear layer.

6. Typical simplifications of cell growth are: growth depends on the first-order consumption of a single limiting growth substrate, and a single type of metabolic waste product is synthesized. Forms are assumed for the effects of inhibitors on cell growth. Inhibitors are assumed only to affect growth substrate consumption rates.

7. No active cell migration takes place. Cells do not alter their location in the biofilm, except through displacement by nearby dividing cells. The simplification of non-motility does not apply to all bacterial species or to all strains within a single species, so the simulation may merit modification if applied to a biofilm system composed of a motile organism (e.g., *Pseudomonas*) and a non-motile one (e.g., *Klebsiella*) (Murga et al., 1995; Siebel & Characklis, 1991; Stewart et al., 1997).

8. The diffusion boundary layer can be represented by an interface layer any number of unit cells thick (default one) covering the biofilm. This is done with the assumption of well-mixed bulk and fine calibration of the diffusivity in the interface layer to match that of diffusion boundary layer.

9. The solute diffusion domain can be arbitrarily partitioned in any number of sectors (default in BacMIST is four quadrants), and each can be handled by a separate ‘thread’ process. This multithreaded scheme helps to create the division of labour in BacMIST when run in a multiprocessor, parallel-processing environment.

**SIMULATION EXPERIMENTS**

**Computational resources.** BacMIST was written and optimized in C using Microsoft Visual Studio 6.0 and carried out on a dual 1-4 GHz Intel Pentium 4 Xeon workstation with 1 gigabyte of RAM. Each 50 h transport-limited biofilm simulation took approximately 12 h in real time, and each 150 h autoinhibitory biofilm simulation took approximately 60 h in real time. Visualization of simulated biofilms was done using the graphics capabilities of the software package MATLAB (Version 6.0, The MathWorks), utilizing a colour map consisting of shades of grey.

**Effect of solute transport limitations on biofilm development.** To evaluate the effect of diffusion-related solute transport limitations on biofilm development, three cases of the full biofilm growth simulation in a 40 × 40 × 40 domain with increasing transport limitations were conducted (five replications per case). Consumption and growth rates were fixed for all the transport-limiting cases, while the diffusion step size was varied (Table 2).

**Effect of autoinhibitory product synthesis on biofilm development.** To evaluate the effect of microbial autoinhibition by a metabolic end product on biofilm development, three cases were analysed in a 50 × 50 × 20 domain (five replications per case), showing growth and autoinhibitory product synthesis in a single-species biofilm. The micro-organism consumed a growth substrate, Solute 1, and generated a product, Solute 2, with a stoichiometric ratio of 1. Solute 2 was inhibitory to the growth of the micro-organism, slowing it exponentially with an inverse dependence on the local inhibitor concentration. The diffusion step size of the inhibitor, Solute 2, was varied over a series of simulations; all other parameters remained constant (Table 3). The inhibitor was not present in the

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**Table 2. Values of parameters used in modelling the effect of diffusion-related solute transport**

<table>
<thead>
<tr>
<th>Simulation size: 40x40x40</th>
<th>Unlimited simulation</th>
<th>Full simulation (low transport limitation)</th>
<th>Full simulation (moderate transport limitation)</th>
<th>Full simulation (severe transport limitation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time step Δt (min)</td>
<td>1-0</td>
<td>1-0</td>
<td>1-0</td>
<td>1-0</td>
</tr>
<tr>
<td>Consumption probability $P_c$</td>
<td>–</td>
<td>0-02</td>
<td>0-02</td>
<td>0-02</td>
</tr>
<tr>
<td>Death rate $k_d$</td>
<td>–</td>
<td>0-035</td>
<td>0-035</td>
<td>0-035</td>
</tr>
<tr>
<td>$1/YieldC/S = Y_{SC}$</td>
<td>–</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Diffusion step $a$ (μm)</td>
<td>–</td>
<td>1-0</td>
<td>0-5</td>
<td>0-25</td>
</tr>
<tr>
<td>Penetration ratio $Q_p$</td>
<td>–</td>
<td>1-0</td>
<td>0-5</td>
<td>0-25</td>
</tr>
<tr>
<td>Bulk concn (quanta per cube)</td>
<td>–</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>$μ_{max}$ (h⁻¹)</td>
<td>0-277</td>
<td>(0-277)</td>
<td>(0-277)</td>
<td>(0-277)</td>
</tr>
</tbody>
</table>
bulk liquid, so any inhibitor that diffused out of the biofilm growth domain was swept away and not replaced. This established an inhibitor concentration gradient from the zero-flux boundary at the substratum to a zero-concentration boundary at the interface with the liquid bulk.

### RESULTS

#### Effect of solute transport limitations on biofilm development

Growth substrate transport limitations had a dramatic effect on the growth of single-species biofilms. As cells accumulated and the biofilm thickened, cells far from the bulk liquid, at the solid substratum, saw a reduced growth substrate concentration and consequently consumed less substrate per time step and grew at a slower rate. Slower-growing cells in the biofilm population lowered the overall population growth rate. The growth rate of a population is visualized as the slope of a plot of population size versus time. At large population sizes, the slopes of curves decrease with increasing transport limitations.

BacMIST simulated overall population properties, for example population size, as well as property variations among individual cells. In the case of transport limitations in a single-species biofilm, properties of interest were those which varied significantly over the entire population; for example, the growth substrate concentration in the vicinity of each cell. To visualize property variations, slices from the biofilm were taken parallel or perpendicular to the substratum, and the values of properties of cells within the slice were mapped.

Since all cells in the population were of the same type, and the cells filled the biofilm growth domain, the system reduced to reaction and diffusion in a slab from a constant-concentration source reservoir (the liquid bulk). Thus, the major variation of properties within the single-species biofilm due to transport limitation was expected to be in the direction perpendicular to the substratum. Variations in planes parallel to the substratum were expected to be purely statistical in nature, and unrelated to the transport limitation imposed on the system.

Perpendicular image slices were taken from the biofilms generated by three increasingly transport-limited simulations, and one-dimensional concentration distribution profiles were generated from the slices by the summing of all the solutes across all cells in each layer (Fig. 5). In the least transport-limited biofilm, growth substrate concentration was high throughout the biofilm. There was slight variation in the substrate concentration over the biofilm thickness. In the biofilm with an intermediate level of transport limitation, growth substrate concentration decreased steadily from the top (boundary with the liquid bulk) to the substratum. The concentration slice through the acutely transport-limited biofilm showed that growth substrate concentration rapidly decreased to zero over a short distance into the biofilm from the liquid bulk. Only a small group of cells in the biofilm population were growing, those in layers nearest the liquid bulk boundary.

#### Effect of autoinhibitory product synthesis on biofilm development

Inhibitor transport strongly influenced the growth of the simulated autoinhibited biofilms. In Case 1, where inhibitor exited through the biofilm growth domain at a rapid rate, and therefore did not accumulate significantly, the biofilm grew and filled the domain quickly, much like the non-autoinhibited cases (Fig. 6a). The inhibitor diffusivities for Cases 2 and 3 were significantly lower, by factors of 4 and 16 respectively, than that in Case 1; thus, due to their higher inhibitor concentration, the biofilms for Cases 2 and 3 grew at much slower rates than the biofilm in Case 1 (Fig. 6). Cases 2 and 3 also exhibited a characteristic two-step growth curve. When the biofilm eventually reached the shear layer (approx. hour 61 for Case 2 and approx. hour 110 for Case 3), a second period of rapid growth occurred as the biofilm grew laterally in the domain space. This horizontal growth increased the surface area for inhibitors to exit the system and thus promoted biofilm growth.

The autoinhibition simulations provided new insights into biofilm structure and channel formation. In Case 1, the exit rate of the inhibitor was high and rapid growth was seen throughout the biofilm volume as inhibitors could easily diffuse out of the system. The growth (not pictured) was relatively homogeneous from the substratum to the shear layer, and the structure resembled the pyramidal-dome structure of Fig. 3. The inhibitor concentration distribution was responsible for the column formations becoming more prominent as more inhibitors persisted in the biofilm and inhibited growth (Fig. 7). The developing front edge of the biofilm, when the growth rate was heavily inhibited, often arranged itself into structures that had more available surface area. The slower the inhibitors diffused out, the narrower the structure would have to be to increase surface area/volume (Fig. 8).

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**Table 3. Values of parameters used in modelling the effect of autoinhibitory product synthesis**

<table>
<thead>
<tr>
<th>50x50x20z simulation domain</th>
<th>Case 1</th>
<th>Case 2</th>
<th>Case 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time step (min)</td>
<td>1-0</td>
<td>1-0</td>
<td>1-0</td>
</tr>
<tr>
<td>$k_{LA}$</td>
<td>0-035</td>
<td>0-035</td>
<td>0-035</td>
</tr>
<tr>
<td>$P_{LA}$</td>
<td>0-02</td>
<td>0-02</td>
<td>0-02</td>
</tr>
<tr>
<td>$Y_{1/2}$</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Diffusion step, solute 1 (lattice units)</td>
<td>1-0</td>
<td>1-0</td>
<td>1-0</td>
</tr>
<tr>
<td>Bulk concn, solute 1 (quanta)</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Diffusion step, inhibitor (lattice units)</td>
<td>1-0</td>
<td>0-5</td>
<td>0-25</td>
</tr>
<tr>
<td>Inhibition factor, $S_j$ (quanta)</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Inhibitor threshold, $I_{thresh}$</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Stoichiometric ratio (solute/inhibitor)</td>
<td>1-0</td>
<td>1-0</td>
<td>1-0</td>
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The decrease in the growth rate of cells in Case 2 before the biofilm reached the shear layer in hour 61 (Fig. 6a) was invariably caused by the increasing amount of inhibitors accumulating in the system during this stage (Fig. 7a). Fewer cells could contribute to the dome formation and the biofilm resulted in a mix of peaks and valleys (Fig. 8). The cells that were able to distance themselves from the region of high inhibitor concentration developed into columns that had higher surface area/volume ratios (Fig. 7a) so to minimize the inhibitor accumulation in the column and produce a higher growth rate than the rest of the biofilm. In the Case 3 biofilms, the columns were more visible and resulted from the more stunted growth of the rest of the biofilm due to the high concentration of inhibitors in the local environment (Figs 7b and 8). Only the cells in the very tip of the column contributed to the growth of the biofilm as the cell growth there was less inhibited (Fig. 7b, hour 125). Once the shear layer was reached, the top layers of the column expanded sideways, along the shear layer. This continued until the shear layer was completely covered, and the column turned into a funnel-like structure (Fig. 7b, hour 149). When considering the periodic nature of the domain in the $x$ and $y$ directions, this funnel formation could conceivably link to adjacent funnel formations and together these would resemble a network of channels in the biofilm (Fig. 8).

Fig. 5. Growth substrate concentrations (quanta per lattice cube) in vertical slices through single-species biofilms grown in the full biofilm growth simulation with different levels of solute transport limitation. (a) Images of the slices colour-coded to indicate substrate concentration. The scale on the right indicates the relative substrate concentration: high concentration, black; low concentration, white. (b) One-dimensional concentration profiles generated by summing the solutes across all cells in a particular layer. Transport limitations become more acute from the top to the bottom of the figure, with the bottom pair of slices experiencing severe limitation.

Fig. 6. Time-course of cell growth (a) and total inhibitory product accumulation (b) in the biofilm growth domain. Solid line, Case 1 (Table 3); dashed line, Case 2; dotted line, Case 3.
Fig. 7. Two-dimensional structure of biofilms producing an autoinhibitory product. (a) Moderate inhibitor transport limitation. (b) Severe inhibitor transport limitation. The scales to the right of the solute (column 1) and inhibitor (column 2) concentration plots indicate the relative concentration scale. In the cell structure plots (column 3), the dark squares contain cells, the white squares are empty, the dark grey squares are boundary layer, and the light grey squares represent dead cells.

Fig. 8. Three-dimensional structure of biofilms producing an autoinhibitory product. Left column, Case 2. Right column, Case 3. Top row, prior to reaching shear layer (hour 61 and hour 125 respectively). Bottom row, after reaching shear layer (hour 71 and hour 149 respectively). Large voids can be observed between the columns in the Case 3 biofilm.
DISCUSSION

BacMIST provided new insight into the dynamics of biofilm development that contribute to their structural heterogeneity. In particular, both transport limitations and the production of autoinhibitory metabolites were found to influence the formation of channels and complex structures that occur in mature biofilms. The analyses suggest that transport within and around biofilms is important in the evolution of biofilm morphological features; for example, tower formation. A number of authors have reported biofilms containing fluid-filled microchannels or voids, often in deep layers of the biofilm or at the substratum (de Beer et al., 1994; Lawrence et al., 1991; Lewandowski et al., 1994; Massol-Deya et al., 1995). A porous biofilm understructure would be prone to invasion by flowing fluid, and to the subsequent establishment and widening of a network of channels, easing solute transport limitations. Thus, the structure of a transport-limited biofilm could evolve over time, in response to physical forces as well as the physiological needs of the cells in the biofilm. The porous biofilms of Case 3 (Figs 7 and 8) would be ideal candidates for the formation of towers or pillars of biomass surrounded by freely circulating fluid voids if exposed to fluid shear.

Analysis by BacMIST indicated that the presence of inhibitors may also contribute to the formation of heterogeneous structures in a biofilm. Groups of cells that have more surface area contact with the bulk liquid are more likely to expel the inhibitors and grow more rapidly than cells with less contact with the bulk liquid. Towers arising from these groups of fast-growing cells resemble structures in biofilms observed microscopically (Cowan et al., 2000; Möller et al., 1998). In Cases 2 and 3 of the autoinhibitory simulation, channels formed that featured open, networked paths that separated the rapidly developing and slowly growing portions of the biofilm. The inclined planes and the presence of channels featured in Cases 2 and 3 are consistent with regions observed in deep layers of biofilms (de Beer et al., 1994; Lawrence et al., 1991; Lewandowski et al., 1994; Massol-Deya et al., 1995). Thus, the structure of a transport-limited biofilm could evolve over time, in response to physical forces as well as the physiological needs of the cells in the biofilm. The porous biofilms of Case 3 (Figs 7 and 8) would be ideal candidates for the formation of towers or pillars of biomass surrounded by freely circulating fluid voids if exposed to fluid shear.

Numerous factors that are thought to influence biofilm development and function have yet to be investigated via a stochastic modelling approach. For example, bacterial cell–cell signalling, or quorum sensing, has been demonstrated to play a role in mature biofilm development (Davies et al., 1998). Recently, the CsrA protein, a global regulator of carbon flux, was determined to control the attachment and detachment of bacteria to the substratum. In contrast, the CDFF model may be beneficial for evaluating the role of quorum sensing in mature biofilm development, since the bulk fluid does not convect through the growing biofilm. These local variations, seen on a cell-to-cell scale, contribute to the broad range of cell activity in a biofilm, and may in some cases lead to unexpected system behaviour. Simulated biofilms subjected to different levels of substrate transport limitation varied substantially in their morphology, demonstrating the relevance of this variable to biofilm development. Similarly, the morphology of simulated biofilms that retarded their own growth through autoinhibitory product synthesis was affected by inhibitor accumulation. In these biofilms, slow-growing, inhibited regions were porous, while their faster-growing counterparts exhibited more densely packed biomass.

To date, most CA models of biofilms have been run in two dimensions (Hermanowicz, 2001; Pizarro et al., 2001; Wimpenny & Colasanti, 1997) or in three dimensions using a hybrid approach (Kreft et al., 2001; Picóreanu et al., 1998). Kreft et al. (2001) evaluated a three-dimensional CA model, and kept the depth (z) dimension at two cells in order to improve computation efficiency. Despite the additional burden of the stochastic solute characterization, it was possible to maintain a relatively large three-dimensional domain in BacMIST because of several features. First, a non-dimensional domain scale was used for the analysis of the relationship between the substrate removal rate and diffusivity. Second, there was effective control of substrate tracking, based on the assumption of a pseudo-steady-state solute concentration outside the biofilm. Third, ‘divide and conquer’ algorithms were used that separated the domain into subsections handled by different synchronized threads, and therefore could take advantage of the multi-processor system interface. This methodology could be extended in the future to adopt parallel/distributed computing across many systems, generating a larger domain size. In combination, these features increased the speed of the simulations.

The CDFF that was modelled in this work has been employed in the laboratory to analyse diverse topics in biofilm ecology, including the effect of substratum material on early stage biofilm formation (Morgan & Wilson, 2001), biocide activity (Kinniment et al., 1996; Norwood & Gilmour, 2000) and genetic exchange (Roberts et al., 1999). Recent efforts to investigate the relationship between quorum sensing and biofilm structure development in the laboratory using continuous-flowthrough models have yielded confounding results that have been attributed to hydrodynamic forces (Pürevdorj et al., 2002). In contrast, the CDFF model may be beneficial for evaluating the role of quorum sensing in mature biofilm development, since the bulk fluid does not convect through the growing biofilm.

Spatial gradients in substrate concentration that were perpendicular to the substratum developed in each of the three simulated cases. There was also, however, a detectable amount of local variation in solute concentrations in all directions for each of the examined transport limitations.
detachment of *E. coli* (Jackson et al., 2002); its influence on biofilm structure may be amenable to analysis using a CA approach. Moreover, transport issues continue to be significant in relation to biofilm heterogeneity: the availability of iron was shown to affect the ability of *Pseudomonas aeruginosa* to form cell clusters (Singh et al., 2002). The simple, non-specific framework of BacMIST provided a high degree of flexibility for simulating processes that influenced biofilm development. These included stochastic solute transport, the consumption and production of solutes by cells within the biofilm, cell division, and cell death. Additionally, cells within the biofilm were individualized, allowing for unique responses to their encounters with heterogeneously distributed solutes. Since autoinhibition, multispecies interactions, and communication in microbial consortia most likely take place via solute exchanges, the nature of BacMIST makes it well suited for investigation of biofilm developmental processes. Moreover, the model described should readily accommodate multiple microbial species, allowing for future studies of more complex systems.

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**REFERENCES**


