Microbial motility involvement in biofilm structure formation – a 3D modelling study

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Abstract A computational model explaining formation of mushroom-like biofilm colonies is proposed in this study. The biofilm model combines for the first time cell growth with twitching motility in a three-dimensional individual-based approach. Model simulations describe the tendency of motile cells to form flat biofilms spreading out on the substratum, in contrast with the immotile variants that form only round colonies. These computational results are in good qualitative agreement with the experimental data obtained from *Pseudomonas aeruginosa* biofilms grown in flowcells. Simulations reveal that motile cells can possess a serious ecological advantage by becoming less affected by mass transfer limitations. Twitching motility alone appears to be insufficient to generate mushroom-like biofilm structures with caps on stalks. Rather, a substrate limitation-induced detachment of motile cells followed by reattachment could explain this intriguing effect leading to higher-level biofilm structure.

Keywords Biofilm structure; mathematical model; migration; mushroom formation; *Pseudomonas aeruginosa*; twitching motility; type IV pili

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Introduction

It is recognised that biofilm structure formation is a complex process involving several mechanisms occurring at very different temporal and spatial scales. The essential physico-chemical processes determining the three-dimensional (3D) biofilm structure formation have already been introduced in mathematical models. Processes such as solute and cell transport, metabolic reactions, hydrodynamics, biomass growth and detachment have been integrated in spatially multidimensional models of biofilm development in time (e.g. Picioreanu *et al.*, 2004; Xavier *et al.*, 2005). Although very successful, this modelling approach has focused almost exclusively on the importance of physical and chemical factors on biofilm development. Biology has been included only through general kinetic and stoichiometric parameters: specific rate constants, saturation and inhibition coefficients and nutrient/biomass yields. This is certainly justified for the majority of engineering applications where only global nutrient and biomass fluxes need to be known. For medical and all biology-oriented fields, this approach is, however, too simplistic. For understanding the full importance of these mechanisms in living systems, biology, chemistry and physics must be seen together. It is, therefore, of vital importance to include specific biological processes in a comprehensive computational model.

Development of complex structures in biofilms may be directed by regulated cellular motility and cellular responses to the nutrient conditions. Hypothetically, the development of mushroom-like biofilm colonies of *Pseudomonas aeruginosa* (PA) is determined by two independent activities involving a first stage of stalk development influenced by the nutritional conditions and a second stage of cap formation dependent on twitching

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motility and possibly influenced by nutrient gradients and quorum sensing. Twitching motility is a type of bacterial surface translocation, which does depend on flagella and where twitching movements of individual cells appear as small, intermittent jerks that often change the direction of movement (Wall and Kaiser, 1999). Conceptual models for PA biofilm formation suggest either: (i) the requirement of motility in microcolony formation by aggregation (O'Toole and Kolter, 1998) and the subsequent formation of larger microcolony structures via a maturation process (e.g. Costerton *et al.*, 1999); or (ii) initial microcolony formation by clonal growth, and twitching motility thereafter causing spreading over the substratum and preventing the formation of larger microcolony structures in the flat and mature biofilm (Klausen *et al.*, 2003b). Microcolonies forming by aggregation are not clonal while those forming by growth are clonal, with important evolutionary consequences (Klausen *et al.*, 2006). These contrasting hypotheses are quantitatively evaluated in this work by a computational model.

Methods

Since biofilm development clearly occurs differently under different nutritional and environmental conditions, the model is built by extending an existing biofilm modelling framework (Xavier *et al.*, 2005). Briefly, this model framework includes growth of individual microbial cells as a function of calculated spatial distribution of diverse solute concentrations (e.g. substrates and products). The 3D distribution of solutes results from simultaneous transport (e.g. diffusion) and reaction processes. Microbial growth is followed by cell divisions and then cell redistribution by a pushing algorithm (Kreft *et al.*, 1998). Moreover, cells can also attach to and detach from the biofilm according to a specified set of quantitative rules. To focus on the effect of motility, the growth kinetics is assumed to be following a simple Monod dependency of growth rate on lumped substrate concentration (saturation kinetics, with parameters in Table 1).

The novelty and, at the same time, the challenge presented in this study consists of devising adequate methods to simulate the role of microbial migration (motility) in biofilm formation. As twitching motility is a process involving single cells or small groups of cells, the natural way to represent this is by an individual-based modelling (IbM) approach (Kreft *et al.*, 2001). Including twitching motility of individual bacteria in the existing biofilm modelling framework can be carried out in several ways. The straightforward approach is to allow individual cells to move randomly when either in

Table 1 Model parameters for the standard case simulations 1, 2 and 3

Parameter	Value	Units
System dimensions		
Size of computational domain	$200 \times 200 \times 200$	μm^3
Solutes parameters		
Bulk concentration of substrate	40	$gCOD/m^3$
Diffusion coefficient of substrate	4.5×10^{-6}	$m^2\ day^{-1}$
Mass transfer boundary layer thickness	20	μm
Microbial parameters		
Maximum specific growth rates of wild-type and pilA mutant	15	day^{-1}
Substrate half-saturation coefficient for wild-type	20	$gCOD/m^3$
Substrate half-saturation coefficient for pilA mutant	20	$gCOD/m^3$
Yield biomass on substrate	0.2	gCOD/gCOD
Initial total number of cells	10	
Initial ratio of motile to immotile cells	1:1	
Maximum mass of a cell at division	1.5×10^{-11}	$gCOD/m^3$
Maximum biomass density in a cell	150,000	$gCOD/m^3$
Migration speed of wild-type (0 for pilA mutant)	5, 10 and 30	$\mu m/h$

contact with the substratum surface or in contact with other cells (i.e. on the biofilm surface). The migration speed can be varied and can also be expressed as a function of nutrient levels.

Results and discussion

Base case: motility and growth

By this simple motility mechanism, part of the experimental results in Klausen *et al.* (2003b) can be qualitatively explained. Three crucial experiments with PA biofilms grown in flowcells were chosen to be reproduced by the computational model for comparison between the biological experiments and the model results. A first case consists of biofilm development from an initial 1:1 mixture of differently tagged (yellow and cyan fluorescent proteins, yfp and cfp) but otherwise identical immotile PA cells (pilA mutants). A second case involves biofilm formation from a 1:1 mixture of immotile cells (pilA mutants tagged with cfp) and wild-type motile cells (tagged with yfp). Finally, the third case studies biofilm formation from an initial (1:1) mixture of differently tagged motile wild-type cells.

Model simulations have shown that motile cells have the tendency to form flat biofilms spreading out on the substratum, whereas immotile variants form only round colonies. These computational results are in good qualitative agreement with the experiments by Klausen *et al.* (2003b), at least for the first two days of biofilm development (Figure 1).

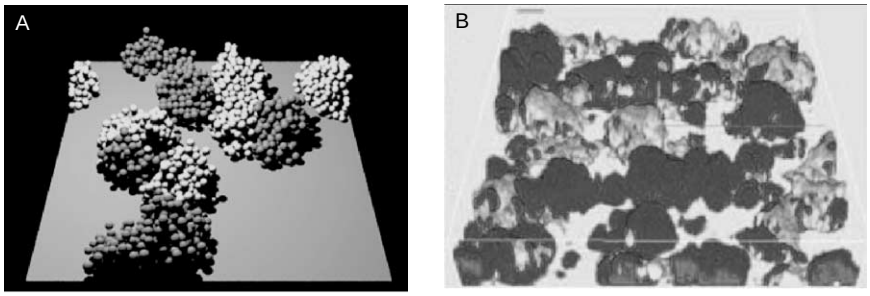
Moreover, model simulations revealed that motility has a serious effect on cell proliferation rate. Owing to the ability of wild-type motile cells to cover larger areas of substratum with a thin blanket-like biofilm, they become less affected by mass transfer limitations. As shown by Picioreanu *et al.* (1998), the flux of substrate transferred to a flat biofilm is higher than that transferred to an irregularly shaped (here, half-round colonies) and patchy biofilm. This results in a higher growth rate of cells from the flat and thin biofilm than of those from round colonies, the latter easily becoming substrate-limited in the colony core. By comparing the numbers of cells of each type, it is clear that the motile cells gain a competitive advantage over the immotile cells (Table 2); not only that the motile cells are more numerous, but also the total number of cells in the biofilm is larger when motile cells are present. The results can also be compared in terms of the time progress of the surface-based biomass density on the flat support (Figure 2). The initial period of exponential growth, not significantly affected by substrate limitations, is followed by another stage of linear growth, where cells grow only in a superficial layer where substrate is available. Figure 2 clearly shows that in a biofilm formed by non-migrating cells, the cells become substrate-limited sooner (case 1: after $\sim$0.7 days), compared with a biofilm formed by motile cells (case 3: after $\sim$1.2 days). These qualitative results will be compared in a quantitative way with experimental observations by use of an automated image analysis system (e.g. COMSTAT).

The speed at which cells migrate is a tuneable model parameter. As expected, the area covered by the motile cells in the same time interval is larger at increased migration speeds (results shown in Figure 3). In accordance with the earlier observations, the wider spreading over the substratum surface gives cells better access to substrate and, consequently, a larger number of motile cells proliferated at higher migration speeds (Table 2).

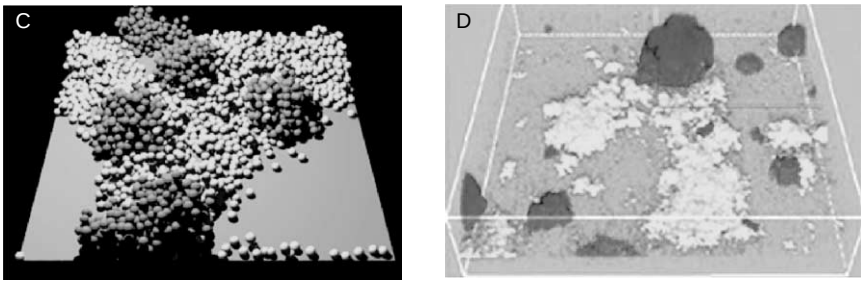
Cap-formation case: motility, growth, detachment and attachment

Although the simple motility/growth model reproduces the early biofilm development trends observed by confocal laser scanning microscopy in flowcells, explanation of the later biofilm development stages is much more challenging. Mushroom-like structures are consistently observed in these PA experiments after about 4–5 days (Klausen *et al.*,

Case 1- Biofilm development from a 1:1 mixture of yellow and blue fluorescent pilA mutant (immotile) *Pseudomonas* cells



Case 2- Biofilm development from a 1:1 mixture of yellow fluorescent wild-type (motile) and blue fluorescent pilA mutant (immotile) *Pseudomonas* cells



Case 3- Biofilm development from a 1:1 mixture of yellow and blue fluorescent motile wild-type *Pseudomonas* cells

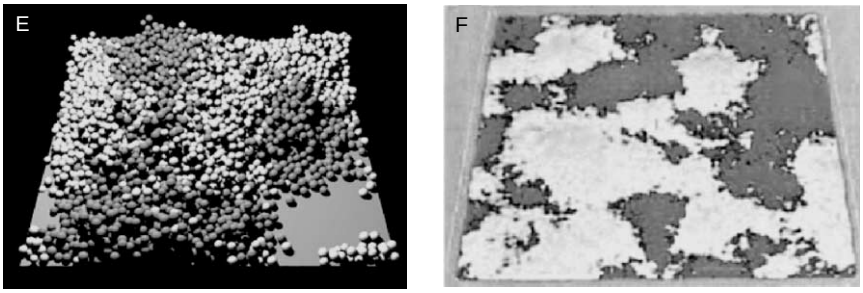


Figure 1 Model results (left) and experimental observations (right) in three cases of PA biofilm formation involving motile and immotile cells. Experimental results from Klausen *et al.* (2003b). Table 2 (cases 1, 2 and 3) indicates the number of motile and immotile cells present in the simulated biofilms after 33 hours. The experimental pictures in cases 1, 2 and 3 are acquired in 4-days, 1-day and 1 day-old biofilms, respectively. Animated simulation results are available at: http://www.biofilms.bt.tudelft.nl/motility_WST/index.html

Table 2 Numbers of wild-type motile and immotile pilA mutant PA cells present after 33 hours in different simulated cases

Case description	Type 1	Type 2
Case 1: yellow and blue fluorescent pilA mutant (immotile)	Immotile 1073	Immotile 1063
Case 2: yellow fluorescent wild-type (motile, 5 μm/h) and blue fluorescent pilA mutant (immotile)	Motile 1298	Immotile 1146
Case 3: yellow and blue fluorescent wild-type (both motile, 5 μm/h)	Motile 1701	Motile 1609
Case 4: as Case 2, but migration speed 10 μm/h	Motile 1630	Immotile 1010
Case 5: as Case 2, but migration speed 30 μm/h	Motile 1788	Immotile 1076

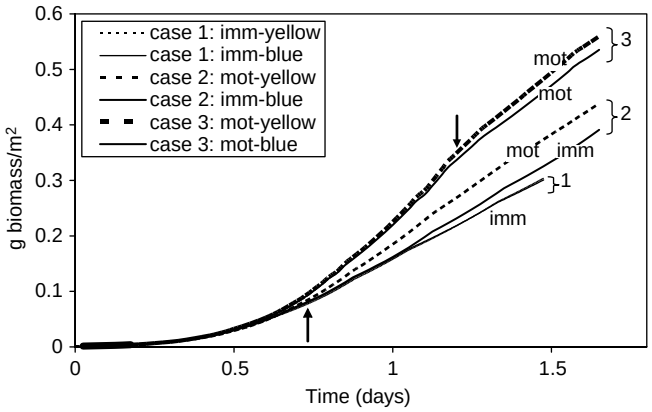


Figure 2 Biomass accumulated on the substratum, as resulted from simulated cases 1, 2 and 3. Arrows indicate the approximated time when severe substrate limitation leads to a linear biomass growth

2003a, b). Several hypotheses could explain the formation of these caps on top of the roughly hemispherical regular colonies. Cell motility is certainly involved, but there is no definitive evidence whether it is twitching or swarming, and whether it is a single cell activity or a social activity of a group of cells. This implies that when only single cell motility is considered (such as in the model presented here), the cells reaching the top of the immotile colonies will continue to migrate such that a true cap will never be formed. One could further argue that chemotaxis plays a role in cap formation because the biofilm tops are exposed to higher substrate concentrations, towards which the motile cells can be directed. We also implemented a chemotactic motility mechanism, but this actually leads to formation of strings of cells (filaments), generally with a vertical orientation. Another possibility, however, involves cell detachment from the biofilm when nutrient conditions are less favourable or just suddenly changing (Sauer *et al.*, 2004; Purevdorj-Gage *et al.*, 2005; Klausen *et al.*, 2006) and subsequent random reattachment. A model version implementing this hypothesis seems to describe the experimental observations better than the other hypothesis.

To describe the phenomenon of mushroom-like biofilm colony formation by computational means, a modified version of the model was created including cell attachment and detachment. However, this is not sufficient: time lapse CLSM imaging of biofilms shows that after a certain time (1–2 days), the colonies formed by immotile cells eventually cease to grow (see Figure 6 in Klausen *et al.*, 2003b). It is unclear what causes this growth stop; nevertheless, we need to model the effect, which can be achieved by assuming that immotile cells have a lower affinity for substrate (higher half-saturation coefficient) than

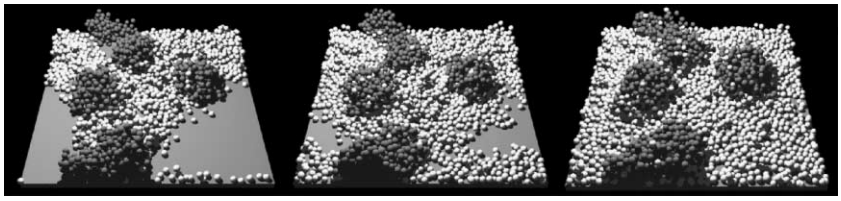


Figure 3 Simulated biofilm structures after 33 hours of development at three migration speeds (5 μm/h left, 10 μm/h middle, 30 μm/h right). PA biofilm simulation is started from a 1:1 mixture of motile (yellow) and immotile (blue) cells. Table 2 (cases 2, 4 and 5) indicates how many motile/immotile cells are present after 33 hours. Animations of the simulation results are available at the website: http://www.biofilms.bt.tudelft.nl/motility_WST/index.html

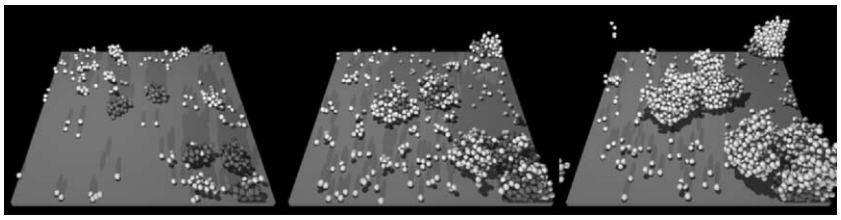


Figure 4 Simulated time development (days 1, 2 and 5, from left to right) of PA biofilm starting from a 1:1 mixture of motile (yellow) and immotile (blue) cells. In the first phase motile cells begin to colonise large surface areas, in the second phase they leave the substratum where the nutrient concentrations become limiting and also reattach on top of the colonies formed by immotile cells. There they start to grow forming the mushroom-like caps

the motile cells. This considerably slows down the growth of immotile cells when the substrate concentration in the bulk liquid drops as a result of biomass accumulation in the biofilm, but still supports growth of motile cells, although at a lower rate.

Qualitatively, at least, this model leads to formation of caps made of motile cells on top of the round colonies built by immotile cells (Figure 4). This happens after the substrate concentration has reached values low enough to trigger the detachment of motile cells. The colonies formed by immotile cells are not allowed to disintegrate by this substrate concentration-induced detachment biofilm. The detachment of motile cells (only if not completely arrested by surrounding cells) is followed by reattachment at a random place on a solid surface (either bare substratum or other biofilm cells). Cells that had the chance to stick to the biofilm top will remain there and form the mushroom-like caps. Whether this proposed mechanism is actually responsible for cap formation in PA biofilms remains to be investigated.

Conclusions

The present motility/growth biofilm model is only intended as a proof-of-principle to explore qualitatively hypothetical mechanisms of "mushroom" formation. Model simulations qualitatively reflect the tendency, observed experimentally, of motile cells to form flat biofilms spreading out on the substratum, in contrast to immotile variants that form only round colonies. Simulations show that motile cells can have a serious ecological advantage by becoming less affected by mass transfer limitations. Motility alone, however, appears to be insufficient to explain formation of mushroom-like capped biofilms. Rather, a substrate limitation-induced detachment of motile cells followed by reattachment could explain this intriguing higher-level biofilm structure formation better.

Several aspects require further study. Firstly, the migration parameters (i.e. migration speed and its relation with the environmental and intracellular processes and parameters) must be quantitatively evaluated. Secondly, the rates of cell attachment and detachment need to be estimated. Thirdly, the contribution of other biological factors such as quorum sensing, chemotaxis, DNA release and social motility to the formation of complex structured biofilms has to be elucidated.

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