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Biofilms promote altruism

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The origin of altruism is a fundamental problem in evolution, and the maintenance of biodiversity is a fundamental problem in ecology. These two problems combine with the fundamental microbiological question of whether it is always advantageous for a unicellular organism to grow as fast as possible. The common basis for these three themes is a trade-off between growth rate and growth yield, which in turn is based on irreversible thermodynamics. The trade-off creates an evolutionary alternative between two strategies: high growth yield at low growth rate versus high growth rate at low growth yield. High growth yield at low growth rate is a case of an altruistic strategy because it increases the fitness of the group by using resources economically at the cost of decreased fitness, or growth rate, of the individual. The group-beneficial behaviour is advantageous in the long term, whereas the high growth rate strategy is advantageous in the short term. Coexistence of species requires differences between their niches, and niche space is typically divided into four 'axes' (time, space, resources, predators). This neglects survival strategies based on cooperation, which extend the possibilities of coexistence, arguing for the inclusion of cooperation as the fifth 'axis'. Here, individual-based model simulations show that spatial structure, as in, for example, biofilms, is necessary for the origin and maintenance of this 'primitive' altruistic strategy and that the common belief that growth rate but not yield decides the outcome of competition is based on chemostat models and experiments. This evolutionary perspective on life in biofilms can explain long-known biofilm characteristics, such as the structural organization into microcolonies, the often-observed lack of mixing among microcolonies, and the shedding of single cells, as promoting the origin and maintenance of the altruistic strategy. Whereas biofilms enrich altruists, enrichment cultures, microbiology's paradigm for isolating bacteria into pure culture, select for highest growth rate.

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INTRODUCTION

In the context of evolutionary theory, altruism is defined as behaviour that benefits others while costing self. In the context of human behaviour, altruism implies a concern for the welfare of others as the motivation for that behaviour, and this is how the term altruism is used in everyday language. However, for evolutionary altruism, the motivation, if any, for altruistic behaviour is not considered. Since benefits and costs are relative as well as local, altruistic behaviour may be defined more precisely, from the perspective of multi-level selection theory, as behaviour that increases the fitness of the group relative to other groups while it decreases the fitness of the altruist relative to others within the group (Sober & Wilson, 1998). The economical use of limiting resources is such a case of altruism because of a trade-off between specific growth rate (rate of biomass increase per time and biomass) and growth yield (biomass formed per amount of resource used) (Pfeiffer *et al.*, 2001). High growth yield, in other words, the economical use of common resources, increases group fitness. However, this can only be attained at the cost of a decreased growth rate, which decreases individual fitness. This form of altruism does not require memory of past interactions, recognition of individuals, sophisticated interactions or behavioural repertoires, or direct interactions between individuals. It is therefore the simplest form of altruism.

Spatial structure is well known to facilitate the evolution of cooperation and altruism (see e.g. Nowak & May, 1992; Sigmund, 1994; Hofbauer & Sigmund, 1998; Hauert *et al.*, 2002). For the altruistic strategy studied here, spatial structure is absolutely necessary since recognition of other individuals and their behaviour, i.e. social structure that could substitute for spatial structure, is beyond the means of bacteria. However, this requirement is easily met, because the spatial structure is automatically generated simply by the asexual division of immotile cells, which leads to clonal clusters. This self-organized structure merely needs to be maintained. Since the earliest forms of life were presumably immotile, asexual cells unable to recognize the individuals

Abbreviations: YS, yield strategy – *high* growth *yield* at the cost of low growth rate; RS, rate strategy – *high* growth *rate* at the cost of low growth yield.

with which they interact, our simplest form of altruism could have evolved already at the beginning of life.

An example of the rate versus yield trade-off is the branched catabolism of the anaerobic bacterium *Holophaga foetida* (Kreft & Schink, 1993). It can double its maximum specific growth rate at the cost of a halved growth yield (Kappler *et al.*, 1997) by switching catabolism from higher to lower ATP yield. From this example, two growth strategies, high growth rate versus high growth yield, have been abstracted and their fitnesses compared in this study. In *H. foetida*, the two strategies are followed by one and the same organism, depending on physiological conditions (Kappler *et al.*, 1997), but for the purpose of comparing and studying these strategies, they have been 'extracted' from measurements with *H. foetida* (see Table 1) and assigned to separate virtual organisms each following one heritable, immutable, and pure strategy exclusively. Like *H. foetida*, the aerobic bacterium *Acetobacter methanolicus* (Müller & Babel, 1993) and the yeast *Saccharomyces kluyveri* (Møller *et al.*, 2002) can boost their growth rates by shifting catabolic substrate flow into less energy-conserving branches, resulting in lowered biomass yields.

Examples of the trade-off can also be found among different bacteria using alternative catabolic pathways. The fermentation of 3 lactate to 2 propionate and 1 acetate by *Clostridium homopropionicum* using the acrylyl-CoA pathway yields only 1 ATP whereas the methylmalonlyl-CoA pathway of *Propionibacterium freudenreichii* yields 2·3 ATP (Seeliger *et al.*, 2002). This leads to a 2·75-fold higher growth rate and a 0·43-fold lower growth yield of *C. homopropionicum* (Seeliger *et al.*, 2002).

Non-equilibrium thermodynamics predicts a linear relationship between the rate of a reaction and its driving force (the Gibbs free energy change) near equilibrium (Westerhoff & van Dam, 1987). This prediction agrees reasonably well with measurements. For *H. foetida* (Kappler *et al.*, 1997), a doubled growth rate is coupled with a doubled Gibbs free energy dissipation per C-mol biomass formed (Heijnen & van Dijken, 1992) ($-\Delta G_D^{0\prime}$). For the lactate fermenters, the 2·75-fold higher growth rate is coupled with a 3·5-fold higher Gibbs free energy dissipation per C-mol biomass formed, calculated from Seeliger *et al.* (2002). These results argue for a thermodynamic necessity of the trade-off. Thermodynamic necessity combined with the simplicity of the altruistic strategy implies that the evolutionary choice, and conflict, between these selfish and altruistic strategies is as old as life. Microbiologists have tried to understand 'what sets and limits the specific growth rate [of bacteria]' (Koch, 1985); see also Marr (1991). Due to the rate versus yield trade-off, the question for a bacterium is not so much how fast could it grow as how fast should it grow.

Micro-organisms will colonize and grow on almost any available surface, thus forming a biofilm where cells are embedding themselves in a slimy matrix while their metabolism creates substrate and product gradients entailing a very heterogeneously structured microenvironment to which the cells in turn will adapt (Costerton *et al.*, 1995). If this surface happens to be a part of or associated with the human body, such biofilms are of particular concern due to their enhanced resistance to antimicrobials (Costerton *et al.*, 1999; Gilbert *et al.*, 2002). Biofilms are teeming with a diversity of bacteria, and life in biofilms has been likened to our human life in cities (Watnick & Kolter, 2000). The high cell density means that most cells have many neighbours close by. Bacteria may stay in a neighbourhood for prolonged periods of time, punctuated by sudden events such as emigration, dispersal, sloughing, etc.

Under such conditions of high cell density, bacteria could benefit from division of labour, collective actions, and other forms of altruistic behaviour or cooperation with their neighbours, and many examples of such cooperations are known (Bradshaw *et al.*, 1994; Caldwell *et al.*, 1997; Turner & Chao, 1999; Velicer *et al.*, 2000, 2003; Strassmann *et al.*, 2000; Crespi, 2001; Palmer *et al.*, 2001; Gilbert *et al.*, 2002). However, the benefits of cooperation can be exploited by selfish individuals or groups not contributing to the costs

Table 1. Growth parameters for the high yield (YS) and the high rate (RS) strategies (relative values)

The initial slope was assumed to be the same for both strategies; therefore, the ratio for K_s must equal the ratio for μ_{max}. All other ratios were slightly rounded from measurements of *H. foetida*, which was using alternative pathways of its branched catabolism, depending on growth conditions (Kappler *et al.*, 1997).

Parameter	Symbol	YS	RS
Maximal specific growth rate	μ_{max}	1	2
Growth yield coefficient	Y_s	1	½
Monod half-saturation constant	K_s	1	2
Initial slope	μ_{max}/K_s	1	1
Maximal substrate consumption rate	$q_{max} = \mu_{max}/Y_s$	1	4
Gibbs free energy dissipation per C-mol biomass	$-\Delta G_D^{0\prime}$	1	2

of cooperation, for example, exoenzyme production or signal production for quorum sensing (Brown & Johnstone, 2001). Therefore, conflicts of interest arise between cells in a cluster or clusters in different parts of the biofilm, even in single-species biofilms, and a case of general protection against invaders by rhamnolipid surfactant production has been reported (Davey *et al.*, 2003). Conflicts of interest between individuals and groups over the use of resources exist not only for bacteria but also for humans, where this conflict is known as the 'tragedy of the commons' (Hardin, 1968).

In this study, I will show how the clustered growth and substrate gradients in biofilms promote altruism, and make the following predictions on biofilm characteristics: (1) biofilms formed by altruists have a higher surface area coverage, (2) biofilms are predominated by altruists, (3) such altruists have to grow and stay in clusters and this is why clusters are the main unit of biofilm architecture, and (4) such clusters have to propagate, from time to time, by breaking up into single cells rather than staying as a unit. The study ends with conclusions on the mechanisms maintaining biodiversity as well as the selection of fast-growing microbes by enrichment cultures and how this has biased mainstream microbiological research, and finally asks why biofilms have not evolved into multicellular organisms.

THE BIOFILM MODEL

The biofilm simulations furnish the setting in which bacteria with different survival strategies compete. The RS bacteria have a high growth *rate* at a low yield *strategy* and the YS bacteria have a high *yield* at a low growth rate *strategy*. The YS bacteria use resources economically and are therefore altruists. The RS bacteria follow an egoistic strategy of resource use. The ratios of maximal specific growth rate and yield are given in Table 1. The dependence of specific growth rate μ on substrate concentration s was assumed to follow Monod kinetics:

$$\mu = \mu_{max}[s/(K_s + s)]$$

for both RS and YS (Fig. 1). The initial slopes (μ_{max}/K_s) of the Monod curves were assumed to be identical since the aim of this study was the comparison of strategies based on the rate versus yield trade-off, rather than the distinct trade-off between growth rate at low versus high substrate concentrations, *r*- versus *K*-strategy (Velicer *et al.*, 1999; Velicer & Lenski, 1999), which would result in a cross-over of the two Monod curves. Initial slopes have to my knowledge only been measured for one example of the rate versus yield trade-off: The initial slope of *C. homopropionicum* (RS strategist) can be roughly estimated from the data in Seeliger *et al.* (2002) to be about 0·7-fold that of *P. freudenreichii* (YS strategist).

Biofilm simulations were carried out using the individual-based model BacSim (Kreft *et al.*, 2001). This program simulates bacterial cells as spheres in continuous space and diffusion-reaction of substrates and products on a lattice. The cells grow (Monod kinetics), divide when the cell volume has reached a critical value, and push other cells away if they overlap. The substrate is transported by diffusion from the bulk liquid (source) through a diffusion boundary layer (concentration boundary layer) into the biofilm (sink) (Fig. 2). Since the active biomass acts as a sink, it drains substrate from the surroundings, from the top *and the sides*; this is the most important difference from a

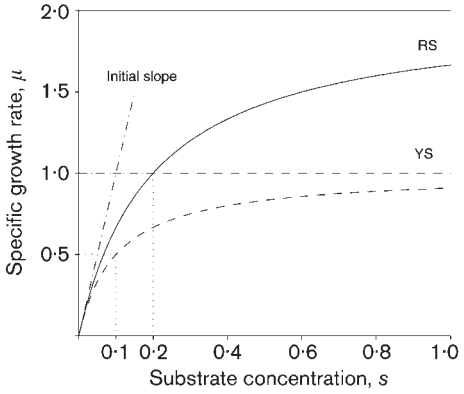

Fig. 1. Dependence of specific growth rate μ on substrate concentration s according to Monod kinetics: $\mu = \mu_{max}[s/(K_s + s)]$. High rate strategist RS (—): $\mu_{max} = 2$, $K_s = 0{\cdot}2$; high yield strategist YS (– –): $\mu_{max} = 1$, $K_s = 0{\cdot}1$. The initial slope (– · –), μ_{max}/K_s, of the curves was assumed to be the same for both strategies.

modelling study of how the high energy yield of respiration may have facilitated the evolutionary transition to multicellularity (Pfeiffer *et al.*, 2001; Pfeiffer & Bonhoeffer, 2003). The Pfeiffer & Bonhoeffer (2003) model considers a flat landscape on which clusters grow in one layer; the substrate is first allocated stochastically into the grid cells, and then diffuses on this flat landscape. Here, a biofilm that grows up towards the substrate loaded bulk liquid is considered, and substrate is not allocated but transported by diffusion, which is driven by substrate consumption. Therefore, clusters with higher substrate demand (RS strategy) will also receive more.

Further important differences are that in the Pfeiffer & Bonhoeffer (2003) model, only one cell is allowed per grid cell, and cell division is only possible as long as there are free grid cells in the neighbourhood. This makes the cells also compete for space, and gives clusters an intrinsic disadvantage by assuming that growth within clusters is impossible. In addition, clustering of cells in the Pfeiffer & Bonhoeffer (2003) model is assumed to be forced by a mutation that prevents motile cells from separation upon division, whereas in this study, clustering is considered to be passive, resulting from the division of immotile, attached cells; therefore clustering does not prevent the passive motion (convection) of cells with the expanding biofilm – see the fan-shaped structures in Figs 4(b_{10}, e_{10}) and 5(c_{10}). The flow of biomass is most obvious in movies of the simulations available from the author's website (http://www.theobio.uni-bonn.de/people/jan_kreft/).

Since substrate diffuses down through the boundary layer into the biofilm, cells at the top will receive more substrate than cells further down or inside the biofilm (Fig. 2) and therefore grow faster, producing more offspring. The offspring in turn will be even higher, like the new leaves of a tree already above the canopy. This positive feedback loop is the cause of finger formation in biofilms and fractal colony edges (Picioreanu *et al.*, 1998). This can also be seen as lateral inhibition because those cells which are higher than their neighbours divert substrate flux away from these neighbours, thereby inhibiting their growth. Once a certain 'finger' is higher than the neighbours (see the YS clusters which are higher than their neighbouring RS clusters in Fig. 4c_3), it will win the competition due to this lateral inhibition. As soon as lateral inhibition suppresses the growth rate of the neighbouring clusters below that of the topmost or leading cluster, the neighbouring clusters no longer have any chance to overgrow the leading cluster, and the outcome of competition is decided. The later

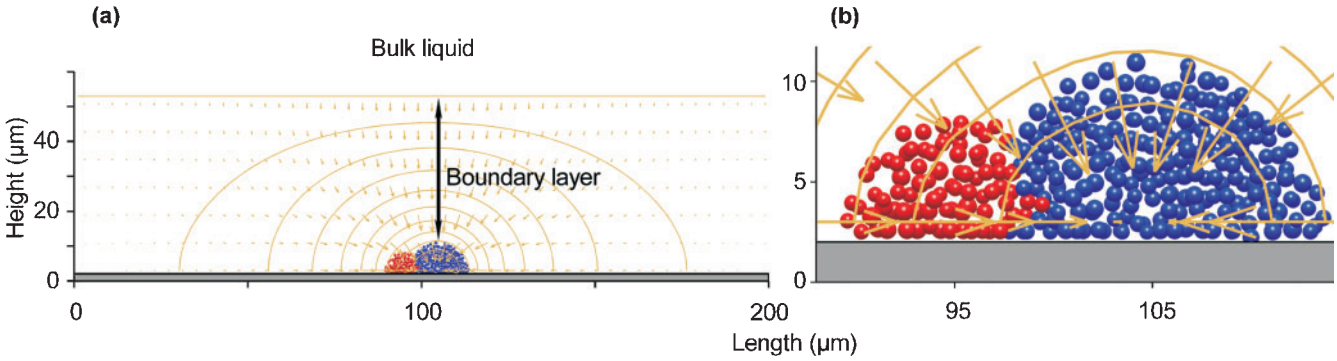

Fig. 2. Simulated biofilm setup. Substrate is transported by diffusion (2D), indicated by arrows, from the well-mixed bulk liquid (constant substrate concentration), beginning at 40 μm above the biofilm top, through the stagnant boundary layer, into the biofilm, which is growing attached to an inert substratum. The contours indicate normalized substrate concentration, from 1·0 in the bulk liquid down to 0·1 inside the biofilm in steps of 0·1. The biofilm in this example consists of one microcolony each of RS (blue) and YS (red) cells. The bacterial cells are modelled as spheres in continuous space (a very thin 3D slice). All boundaries are periodic, except in the vertical dimension (constant concentration boundary condition at the top, zero flux into the substratum boundary condition at the bottom). In this example, the higher specific substrate turnover rate of the RS cluster, in combination with a higher biomass, creates a sink that is strong enough to drain substrate flux away from the YS cluster. (a) Whole domain. (b) Enlarged view of the biofilm.

this 'decision time', the more the long-term advantage of YS will pay off. This is a case of truncation selection (Sober & Wilson, 1998), as also found in the desert leaf-cutting ant *Acromyrmex versicolor*, where the first colony to produce workers as offspring will win the competition because these workers will first raid the neighbouring colonies (Sober & Wilson, 1998).

An experimental study of a mutant of *Pseudomonas fluorescens*, which overproduces a sticky polymer, thereby creating clusters of cooperating cells, differs from this biofilm study in that it does not show aspects of lateral inhibition (Rainey & Rainey, 2003): the mutant forms a mat at the air/liquid interface, and rather than outcompeting the ancestral strain in the same habitat, it colonizes a self-made and spatially separated niche.

To obtain quantitative results, the generic, abstract strategies RS and YS were applied to the concrete example of an ammonia-oxidizing bacterium. This matters insofar as ammonia oxidizers grow slowly and their small substrates oxygen and ammonia diffuse rapidly. For the sake of simplicity, only one substrate (oxygen) was assumed to be limiting growth, substrate inhibition by ammonia was assumed to be absent, and the maintenance rate was set to zero. Model parameters were as described by Kreft *et al.* (2001). Most importantly, the oxygen concentration was 1 mg l^{-1}, the simulated domain was $200 \times 200 \times 2$ μm wide, high and deep, respectively, and the height of the concentration boundary layer was 40 μm. The growth parameters for YS were those given for the ammonia oxidizer (Kreft *et al.*, 2001), and the growth parameters of RS were chosen relative to the values for YS (Table 1). The source code and Java program of the model are available from my web site (http://www.theobio.uni-bonn.de/people/jan_kreft).

RESULTS AND DISCUSSION

Competition in chemostats

In chemostats (well-mixed, continuous systems), the RS strategist will always outcompete the YS strategist, because RS strategists grow faster at every substrate concentration above zero (Fig. 1). Although hardly any natural system can be regarded as a chemostat, empirical and theoretical studies of microbial competition have focused on chemostats since their introduction about 50 years ago. Even in chemostat studies, wall growth (biofilms growing on the walls of the chemostat) often occurs but it is rarely taken into account. Further, as shown by Christensen *et al.* (2002) and here, competition experiments in chemostats cannot be used to predict results for biofilm competition because the yield differences that are completely irrelevant in chemostats have a strong impact in biofilms and other natural systems.

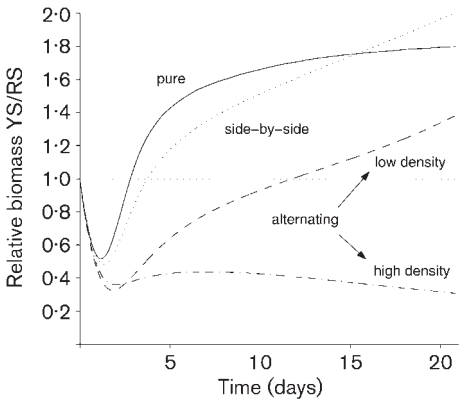

Fig. 3. Growth of YS relative to RS in biofilms grown from various initial line-ups of RS and YS cells. —, Pure biofilms grown from 20 cells. · · · ·, One continuous stretch of 10 RS cells placed side-by-side (see Fig. 4f_3) with a second stretch of 10 YS cells. – –, – · –, Alternating arrangement of RS and YS (see Fig. 4c–e), at lower (5 cells each, – –) and higher (10 cells each, – · –) densities.

However, even in chemostats, yield differences become important when growth is limited by more than one substrate.

Competition in biofilms

In biofilms, cells grow in clusters (microcolonies); this creates a substrate gradient into and within the clusters. Such clustered growth, plus its inevitable combination with substrate gradients, is necessary but not sufficient to change the outcome of competition in favour of YS (Figs 3 and 4). Comparing pure biofilms of RS and YS strategists, it is obvious that the latter's higher yield allows more biomass to accumulate in the long run despite an initial advantage of RS (Fig. 3). In direct side-by-side competition (Figs 3 and 4), qualitatively the same picture emerges, independent of the initial density (1–50 cells each, data shown for 10 cells each). When the arrangement of clusters is alternating, RS will win the competition only if the density of clusters is above a

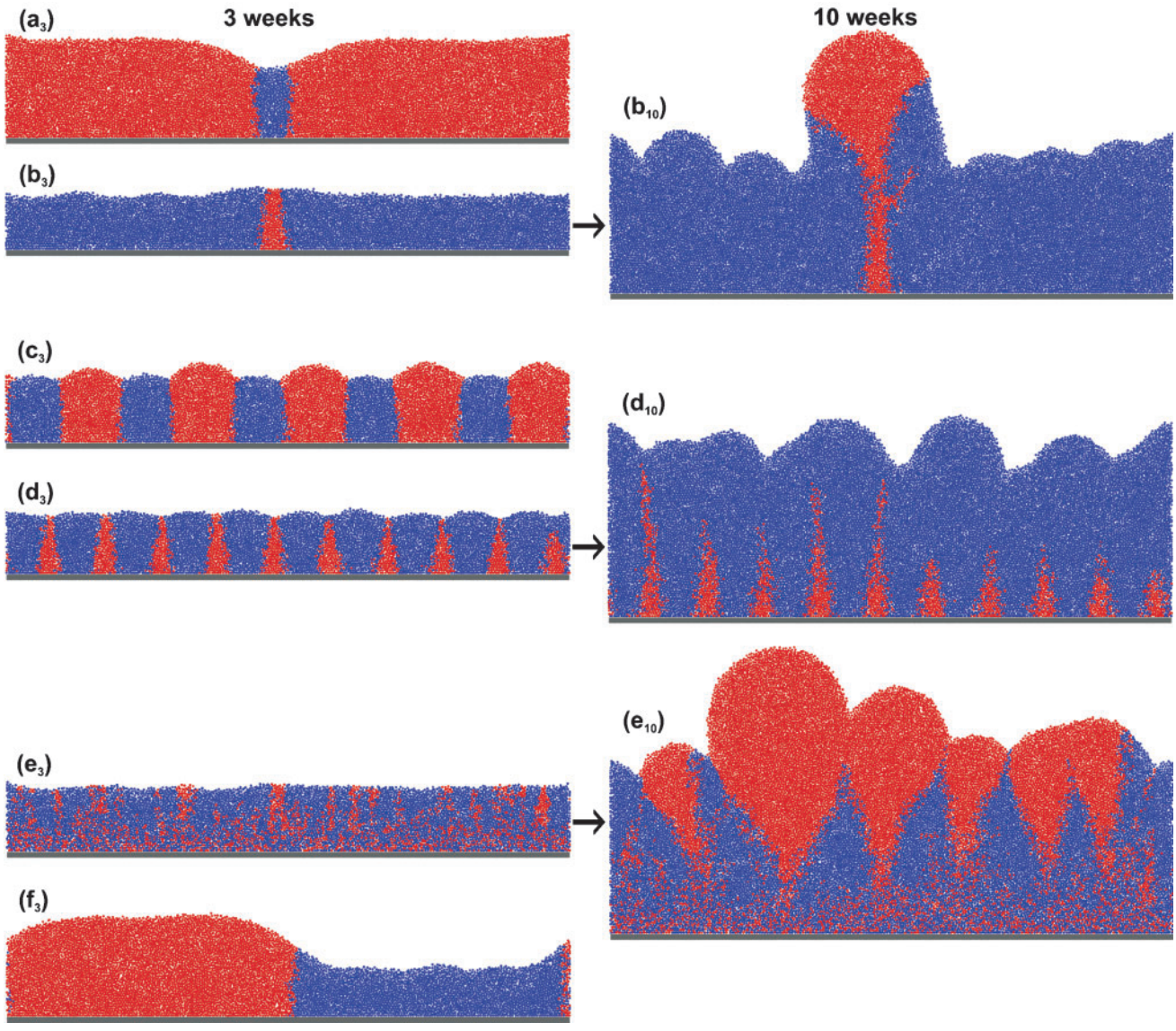

Fig. 4. Competition among RS (blue) and YS (red) clusters in biofilms grown from various initial line-ups of RS and YS cells. The length of the domain was 200 μm. The left panels (a_3–f_3) show 3-week-old biofilms and the right panels (b_{10}, d_{10}, e_{10}) show 10-week-old biofilms only in those cases where the 'time of decision' had not passed and further development was not obvious. (a_3) One RS cell in a pure YS neighbourhood (seeded by 19 cells). (b_3, b_{10}) Vice versa, one YS cell in a pure RS neighbourhood (seeded by 19 cells). (c–e) Alternating arrangement of seeds at increasing density: (c_3) 5 cells each; (d_3, d_{10}) 10 cells each; (e_3, e_{10}) 50 cells each. (f_3) Side-by-side arrangement of 10 cells each; here, other densities lead to qualitatively similar results (not shown). Movies of all these simulations can be found on the author's webpage (http://www.theobio.uni-bonn.de/people/jan_kreft/).

threshold that depends on growth parameters and environmental conditions such as substrate transport rate (Figs 3 and 4). However, at even higher densities, the self-organization into clusters (*clustering effect*) that results from the cell divisions of immotile cells gives YS the chance to spread out of a dominantly RS biofilm in the shape of wedges or fans (Figs $4b_{10}$, $4e_{10}$ and $5c_{10}$; Movie 1, included as supplementary data with the online version of this paper at http://mic.sgmjournals.org). Wedge-like growth patterns have long been known in dental plaque (Lai *et al.*, 1975; Listgarten, 2000). Clustering has also been reported in *P. aeruginosa* biofilms (Davey *et al.*, 2003). Clustering can also result from polymer production, which effectively binds cells and their evolutionary interests together (Rainey & Rainey, 2003; Velicer & Yu, 2003).

Ironically, YS rather than RS clusters in the neighbourhood may help RS clusters to win, because competition among RS clusters is stronger (the higher substrate turnover by RS leads to steeper gradients of the resource and more severe substrate limitation) than competition between RS and YS clusters. This is why a single YS cluster wins (Fig. $4b_{10}$) whereas RS wins (Fig. $4d_{10}$) when RS and YS clusters are alternating at the same density (compare Fig. 4 panels b_3 and b_{10} with d_3 and d_{10}). A single RS cluster (Fig. $4a_3$), in contrast to a single YS cluster (Fig. $4b_3$), will not win.

In principle, the same results as above were obtained at higher substrate concentrations or thinner boundary layers (data not shown), where the short-term initial advantage of RS becomes more pronounced. Therefore, for the alternating arrangement, the switch from YS winning to RS winning occurs at a lower density.

These results demonstrate how both strategies can originate in an environment dominated by the other strategy, albeit in different ways. The YS strategy can arise in a spatially structured environment dominated by RS because of (a) more intense competition among RS clusters themselves, (b) the clustering effect, or (c) the overall density of cells being low enough due to a scarcity of resources. The RS strategy can arise in well-mixed environments such as the planktonic phase, and in patchy environments if the short-term advantage of RS (see Fig. 3) dominates over the long-term advantage of YS. In summary, YS wins the competition in the long run if it has not been overgrown by RS during the initial phase of biofilm growth.

Invasibility by individuals and groups

For a given strategy to survive in the long term, at least some conditions under which the strategy can arise *de novo* must first of all exist, and the preceding section has demonstrated that suitable conditions for the origin of both strategies can easily be found. Secondly, once a strategy has emerged, it must be able to be maintained against competing strategies, and this is studied in this section, employing the usual invasibility criterion. A strategy is called an evolutionarily stable strategy (ESS) if a population of individuals using that strategy cannot be invaded by a rare mutant adopting a different strategy (Maynard Smith, 1982). If group-level selection plays a dominant role, the invasibility criterion should be applied on the individual and group levels of selection separately.

To test for invasibility by individuals, a single cell at the top or in the middle of a developing, fairly even and uniform biofilm was 'mutated' into one with an alternative strategy (data not shown). As expected from the Monod kinetics of the two strategies (Fig. 1), initially the RS strategist always had a higher growth rate than the one a YS strategist would have had in the same place. In real biofilms, this 'mutant' is more likely to be an immigrant or arise from phase variation (phenotypic switching; Drenkard & Ausubel, 2002).

To test for invasibility by groups, vertical strips of cells (5–100 μm) were changed into cells of the other strategy. RS clusters could not invade YS biofilms, but YS clusters could invade RS biofilms (Fig. 5). The growth of a strip of YS strategists invading an RS biofilm, relative to a same-sized strip of RS strategists invading a YS biofilm, showed a trend of increasing advantage for the YS clusters with increasing cluster size (data not shown). Comparing the growth of pure RS and YS biofilms with a randomly mixed biofilm (same number of cells for RS and YS to begin with) initially shows the expected results (data not shown): pure YS biofilms grow much better than pure RS biofilms, and the mixed biofilm is in between, with RS strategists growing slightly better than the YS strategists in the mixed biofilm. However, YS strategists overtake in the long run because of the clustering effect (Fig. $5c_3$, c_{10}; Movie 2, included as supplementary data with the online version of this paper at http://mic.sgmjournals.org).

Individual RS strategists can always invade groups of YS strategists due to their higher relative growth rate under the same conditions (see Monod curves in Fig. 1), but clusters of RS strategists above a critical size cannot, since such clusters will deplete resources more quickly in their own microenvironment. Conversely, yet for the same reasons, individual YS strategists can never invade but sufficiently large clusters can. Therefore, YS and RS strategies are both evolutionarily stable and unstable – on different levels of selection. In summary, whenever group-level selection is strong enough, the YS strategy can both arise and be maintained.

Within each cluster (neighbourhood), the proportion of RS strategists will tend to increase because of their unconditional growth rate advantage (Fig. 1), giving the RS strategy a higher relative fitness in individual-level selection. But clusters will grow better and produce more offspring the higher the fraction of YS strategists is, under the same conditions, giving the YS strategy a higher relative fitness in group-level selection. This may lead to a global increase of the number of YS strategists although the proportion of YS strategists decreases in all clusters, a counter-intuitive result that is known as Simpson's Paradox (Sober & Wilson, 1998).

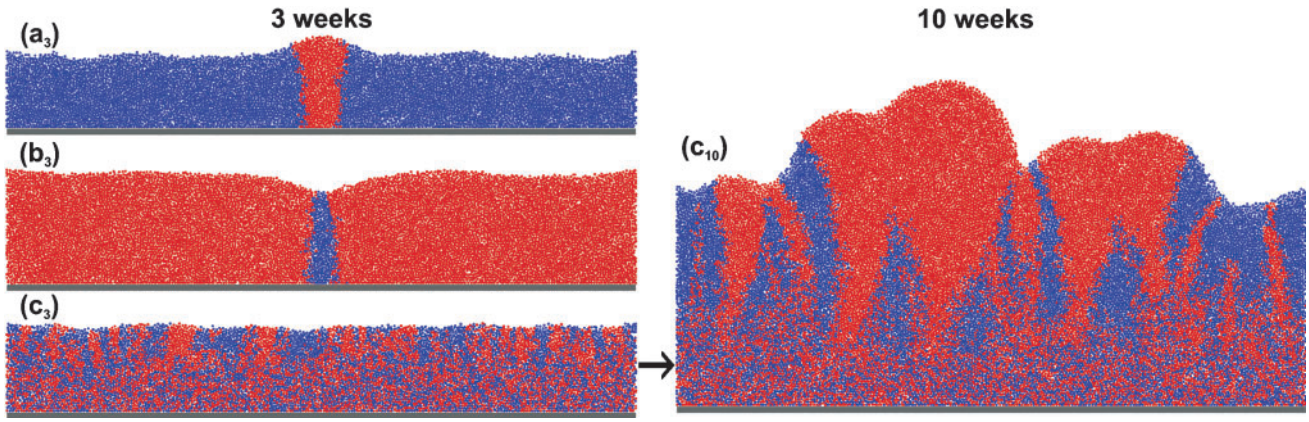

Fig. 5. Invasion of a pure biofilm by a group of cells of opposite strategy. An essentially flat biofilm aged 1 week was used as the template to set up all runs. Blue, RS cells; red, YS cells. The length of the domain was 200 μm. Biofilms aged 3 weeks on the left (a_3–c_3) or 10 weeks on the right (c_{10}). (a_3) Vertical strip of YS (10 μm) invades an RS biofilm or (b_3) vice versa. (c_3, c_{10}) Mixed: half of the cells of a 1-week-old YS biofilm were randomly replaced by RS cells. After 2 more weeks of growth, a re-formation of clusters is discernible (clustering effect), but who wins the competition becomes obvious only later (c_{10}). Movies of all these simulations can be found on the author's webpage (http://www.theobio.uni-bonn.de/people/jan_kreft/).

Despite major differences with respect to substrate transport, cell growth, cell motility, and biomass spreading, the results of this study are qualitatively similar to those of Bonhoeffer and coworkers (Pfeiffer *et al.*, 2001; Pfeiffer & Bonhoeffer, 2003), suggesting that these results are very robust.

Predictions on biofilm characteristics

Based on these findings, a set of predictions on the characteristics of biofilm life follows, which are then compared with the empirical evidence when available.

(1) Higher substratum coverage of YS biofilms. YS strategists should form biofilms with higher surface area coverage than RS strategists, due to the weaker competition between neighbouring clusters.

(2) Dominance of YS in biofilms. Altruism can evolve in natural biofilm communities and biofilms should be dominated by YS strategists. In contrast, planktonic bacteria that are primarily growing as single cells should be RS strategists. Planktonic cells of *P. aeruginosa* grow twice as fast as *P. aeruginosa* cells after attaching to a surface (Rice *et al.*, 2000), suggesting that they switch strategy from RS to YS upon surface attachment.

(3) Clustering of YS. YS strategists should have a tendency to literally stick to themselves and thereby facilitate and maintain clustering. RS strategists should try to avoid self and mix, e.g. by surface-bound twitching motility. Bacteria from natural river biofilms show either a tendency to avoid self, called 'spreading, rolling, or shedding maneuvers', or to cluster, called 'packing maneuver' (Lawrence & Caldwell, 1987). Biofilms as a whole should resemble an ensemble of distinct, clonal and identifiable microcolonies with a very limited amount of mixing between these clusters. The most direct studies of mixing within biofilms involve cells of the same strain tagged with different colours. Movement of *Pseudomonas putida* cells between and within microcolonies was frequently observed apart from flagellum-less mutants; however, the extent of mixing was too limited to change the overall composition or structure of the microcolonies (Tolker-Nielsen *et al.*, 2000). Recently, *P. aeruginosa* biofilm structure was shown to depend on the carbon source, and an initial phase of clonal growth was followed by a phase of twitching-motility-driven mixing (Klausen *et al.*, 2003). Similar studies of dual-species biofilms show the same trend: mixing is surprisingly limited even for commensalistic associations where satellite microcolonies are formed, growing on the products of a big cluster in the centre (Nielsen *et al.*, 2000; Tolker-Nielsen & Molin, 2000; Christensen *et al.*, 2002). Formation of *P. aeruginosa* microcolonies has been proposed to occur by aggregation (O'Toole *et al.*, 2000), but the evidence is conflicting among various laboratories (Chiang & Burrows, 2003; Klausen *et al.*, 2003). The typically limited mixing explains why microcolonies are the basic structural unit of biofilms (Costerton *et al.*, 1995; Tolker-Nielsen *et al.*, 2000).

(4) Biofilms are not 'units of proliferation'. Maintenance of YS requires clusters to disperse. Even an originally pure YS cluster will not stay pure forever, due to mutation, phase variation and immigration. In a mixed cluster, RS strategists will grow faster than their cluster neighbours, thereby increasing the fraction of RS strategists within the cluster. A 'purification' step is required for YS strategists to survive: clusters must at least occasionally be broken up into single cells that leave the cluster to colonize another surface. Supporting this view,

biofilms shed not only clusters but also single cells in proportion to the total biofilm mass (Stoodley *et al.*, 2001). If the biofilm community were the 'unit of proliferation' (Caldwell *et al.*, 1997), the YS strategists would become extinct.

Conclusions

Two conditions are necessary and sufficient for the origin and maintenance of simple altruistic strategies without direct and recognition-based interactions: (a) spatial structure (clustering), and (b) dissociation of clusters into individuals before the RS strategists have taken over the cluster. (A third condition, resource limitation, is a necessary consequence of clustering.) These conditions are clearly met in biofilms.

Economy of resource use has probably been overlooked as a form of altruism in biofilms (Caldwell *et al.*, 1997) because it does not involve the specific, direct interactions that would catch one's attention, yet here I argue that it is the earliest form of altruism, widespread since life began and having a profound impact on biofilm structure and function ever since.

The range of possible strategies from selfish to altruistic, whether pure or mixed, obligate or facultative, opens up a neglected dimension for the diversification of life. There are a plethora of mechanisms and effects that help maintain biodiversity (Chesson, 2000). While resources, predators, time and space are commonly viewed as axes of niche space (Chesson, 2000), cooperation should be added as the fifth major axis.

The study of fast-growing bacteria in liquid culture has formed the mainstream of microbiological research since Beijerinck and Winogradsky established enrichment cultures about a century ago (Brock, 1998). Unfortunately, enrichment cultures tend to select RS strategists, thereby ensuring that most laboratory studies have been carried out with RS strategists: growth of bacteria was found, using mosaic non-equilibrium thermodynamics, to be optimized for maximization of growth rate while keeping efficiency as high as possible (Westerhoff & van Dam, 1987). Also, the organization of the *Escherichia coli* metabolic network was found to be optimized to maximize growth (Edwards *et al.*, 2001). However, these results may not hold for YS strategists, which probably predominate in nature. Isolating bacteria by dilution culture (dilution of the inoculum prior to isolation) rather than enrichment culture will pick the most abundant species able to grow under the given conditions. Using dilution culture of samples from spatially structured habitats should favour isolation of YS strategists. It may be expected that the more frequent use of dilution cultures will allow a fraction of so-called unculturables to become cultured. A case in point is the isolation of *H. foetida* (Bak *et al.*, 1992) from which the growth parameters of this study were derived and other members of the class *Acidobacteria* (Joseph *et al.*, 2003).

Given that biofilms promote the evolution of clusters with group-beneficial behaviour, why did biofilms not develop into multicellular organisms? After all, multicellular organisms have evolved mechanisms to counter selfish defection of individual cells from altruistic behaviour (cancer). Yet bacteria have, by and large, not evolved into multicellular organisms over more than 3 billion years, despite the ease with which cooperating groups of bacteria evolve (Rainey & Rainey, 2003; Velicer & Yu, 2003). Two main reasons for this can be envisaged. (1) The architecture of prokaryotic cells with haploid genomes constrains evolution. (2) The main ecological advantage of bacteria would be lost upon transition to obligate multicellularity. There are many links in the carbon, nitrogen and sulphur cycles provided by the unique metabolic capabilities of bacteria, whereas eukaryotes are metabolically far less diverse. If metabolic versatility is the foundation for the evolutionary success of bacteria, it seems likely that the flexibility in time and space of the metabolic capacities of bacterial communities requires a modular construction which would be lost if the single bacterial cells (the modules) were to integrate into defined and permanent larger units. The relationship between community-level metabolic flexibility and performance on the one hand and modularity of organization on the other is the topic of a future study.

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REFERENCES

Bak, F., Finster, K. & Rothfuß, F. (1992). Formation of dimethylsulfide and methanethiol from methoxylated aromatic compounds and inorganic sulfide by newly isolated anaerobic bacteria. *Arch Microbiol* **157**, 529–534.

Bradshaw, D. J., Homer, K. A., Marsh, P. D. & Beighton, D. (1994). Metabolic cooperation in oral microbial communities during growth on mucin. *Microbiology* **140**, 3407–3412.

Brock, T. D. (1998). *Milestones in Microbiology: 1546 to 1940.* Washington, DC: American Society for Microbiology.

Brown, S. P. & Johnstone, R. A. (2001). Cooperation in the dark: signalling and collective action in quorum-sensing bacteria. *Proc R Soc Lond B Biol Sci* **268**, 961–965.

Caldwell, D. E., Wolfaardt, G. M., Korber, D. R. & Lawrence, J. R. (1997). Do bacterial communities transcend darwinism? *Adv Microb Ecol* **15**, 105–191.

Chesson, P. (2000). Mechanisms of maintenance of species diversity. *Annu Rev Ecol Syst* **31**, 343–366.

Chiang, P. & Burrows, L. L. (2003). Biofilm formation by hyperpiliated mutants of *Pseudomonas aeruginosa*. *J Bacteriol* **185**, 2374–2378.

Christensen, B. B., Haagensen, J. A. J., Heydorn, A. & Molin, S. (2002). Metabolic commensalism and competition in a two-species microbial consortium. *Appl Environ Microbiol* **68**, 2495–2502.

Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R. & Lappin-Scott, H. M. (1995). Microbial biofilms. *Annu Rev Microbiol* **49**, 711–745.

Costerton, J. W., Stewart, P. S. & Greenberg, E. P. (1999). Bacterial biofilms: a common cause of persistent infections. *Science* **284**, 1318–1322.

Crespi, B. J. (2001). The evolution of social behavior in microorganisms. *Trends Ecol Evol* **16**, 178–183.

Davey, M. E., Caiazza, N. C. & O'Toole, G. A. (2003). Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **185**, 1027–1036.

Drenkard, E. & Ausubel, F. M. (2002). *Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature* **416**, 740–743.

Edwards, J. S., Ibarra, R. U. & Palsson, B. O. (2001). In silico predictions of *Escherichia coli* metabolic capabilities are consistent with experimental data. *Nat Biotechnol* **19**, 125–130.

Gilbert, P., Maira-Litran, T., McBain, A. J., Rickard, A. H. & Whyte, F. W. (2002). The physiology and collective recalcitrance of microbial biofilm communities. *Adv Microb Physiol* **46**, 202–256.

Hardin, G. (1968). The tragedy of the commons. *Science* **162**, 1243–1248.

Hauert, C., De Monte, S., Hofbauer, J. & Sigmund, K. (2002). Volunteering as red queen mechanism for cooperation in public goods games. *Science* **296**, 1129–1132.

Heijnen, J. J. & van Dijken, J. P. (1992). In search of a thermodynamic description of biomass yields for the chemotrophic growth of microorganisms. *Biotechnol Bioeng* **39**, 833–858.

Hofbauer, J. & Sigmund, K. (1998). *Evolutionary Games and Population Dynamics*. Cambridge: Cambridge University Press.

Joseph, S. J., Hugenholtz, P., Sangwan, P., Osborne, C. A. & Janssen, P. H. (2003). Laboratory cultivation of widespread and previously uncultured soil bacteria. *Appl Environ Microbiol* **69**, 7210–7215.

Kappler, O., Janssen, P. H., Kreft, J.-U. & Schink, B. (1997). Effects of alternative methyl group acceptors on the growth energetics of the *O*-demethylating anaerobe *Holophaga foetida*. *Microbiology* **143**, 1105–1114.

Klausen, M., Heydorn, A., Ragas, P., Lambertsen, L., Aaes-Jørgensen, A., Molin, S. & Tolker-Nielsen, T. (2003). Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. *Mol Microbiol* **48**, 1511–1524.

Koch, A. L. (1985). The macroeconomics of bacterial growth. In *Bacteria in their Natural Environments*, pp. 1–42. Edited by M. Fletcher & G. D. Floodgate. London: Academic Press.

Kreft, J.-U. & Schink, B. (1993). Demethylation and degradation of phenylmethylethers by the sulfide-methylating homoacetogenic bacterium strain TMBS4. *Arch Microbiol* **159**, 308–315.

Kreft, J.-U., Picioreanu, C., Wimpenny, J. W. T. & van Loosdrecht, M. C. M. (2001). Individual-based modelling of biofilms. *Microbiology* **147**, 2897–2912.

Lai, C. H., Listgarten, M. A. & Rosan, B. (1975). Immunoelectron microscopic identification and localization of *Streptococcus sanguis* with peroxidase-labeled antibody: localization of *Streptococcus sanguis* in intact dental plaque. *Infect Immun* **11**, 200–210.

Lawrence, J. R. & Caldwell, D. E. (1987). Behavior of bacterial stream populations within the hydrodynamic boundary layers of surface microenvironments. *Microb Ecol* **14**, 15–27.

Listgarten, M. A. (2000). The structure of dental plaque. *Periodontology* **5**, 52–65.

Marr, A. G. (1991). Growth rate of *Escherichia coli*. *Microbiol Rev* **55**, 316–333.

Maynard Smith, J. (1982). *Evolution and the Theory of Games*. Cambridge: Cambridge University Press.

Møller, K., Christensen, B., Förster, J., Piskur, J., Nielsen, J. & Olsson, L. (2002). Aerobic glucose metabolism of *Saccharomyces kluyveri*: growth, metabolite production, and quantification of metabolic fluxes. *Biotechnol Bioeng* **77**, 186–193.

Müller, R. H. & Babel, W. (1993). Oxidative capacity determines the growth rate with *Acetobacter methanolicus*. *Acta Biotechnol* **13**, 3–11.

Nielsen, A. T., Tolker-Nielsen, T., Barken, K. B. & Molin, S. (2000). Role of commensal relationships on the spatial structure of a surface-attached microbial consortium. *Environ Microbiol* **2**, 59–68.

Nowak, M. A. & May, R. M. (1992). Evolutionary games and spatial chaos. *Nature* **359**, 826–829.

O'Toole, G., Kaplan, H. B. & Kolter, R. (2000). Biofilm formation as microbial development. *Annu Rev Microbiol* **54**, 49–79.

Palmer, R. J., Kazmerzak, K., Hansen, M. C. & Kolenbrander, P. E. (2001). Mutualism versus independence: strategies of mixed-species oral biofilms in vitro using saliva as the sole nutrient source. *Infect Immun* **69**, 5794–5804.

Pfeiffer, T. & Bonhoeffer, S. (2003). An evolutionary scenario for the transition to undifferentiated multicellularity. *Proc Natl Acad Sci U S A* **100**, 1095–1098.

Pfeiffer, T., Schuster, S. & Bonhoeffer, S. (2001). Cooperation and competition in the evolution of ATP-producing pathways. *Science* **292**, 504–507.

Picioreanu, C., van Loosdrecht, M. C. M. & Heijnen, J. J. (1998). Mathematical modeling of biofilm structure with a hybrid differential-discrete cellular automaton approach. *Biotechnol Bioeng* **58**, 101–116.

Rainey, P. B. & Rainey, K. (2003). Evolution of cooperation and conflict in experimental bacterial populations. *Nature* **425**, 72–74.

Rice, A. R., Hamilton, M. A. & Camper, A. K. (2000). Apparent surface associated lag time in growth of primary biofilm cells. *Microb Ecol* **40**, 8–15.

Seeliger, S., Janssen, P. H. & Schink, B. (2002). Energetics and kinetics of lactate fermentation to acetate and propionate via methylmalonyl-CoA or acrylyl-CoA. *FEMS Microbiol Lett* **211**, 65–70.

Sigmund, K. (1994). *Games of Life*. Oxford: Oxford University Press.

Sober, E. & Wilson, S. D. (1998). *Unto Others: the Evolution and Psychology of Unselfish Behaviour*. Cambridge, MA: Harvard University Press.

Stoodley, P., Wilson, S., Hall-Stoodley, L., Boyle, J. D., Lappin-Scott, H. M. & Costerton, J. W. (2001). Growth and detachment of cell clusters from mature mixed-species biofilms. *Appl Environ Microbiol* **67**, 5608–5613.

Strassmann, J. E., Zhu, Y. & Queller, D. C. (2000). Altruism and social cheating in the social amoeba *Dictyostelium discoideum*. *Nature* **408**, 965–967.

Tolker-Nielsen, T. & Molin, S. (2000). Spatial organization of microbial biofilm communities. *Microb Ecol* **40**, 75–84.

Tolker-Nielsen, T., Brinch, U. C., Ragas, P. C., Andersen, J. B., Jacobsen, C. S. & Molin, S. (2000). Development and dynamics of *Pseudomonas* sp. biofilms. *J Bacteriol* **182**, 6482–6489.

Turner, P. E. & Chao, L. (1999). Prisoner's dilemma in an RNA virus. *Nature* **398**, 441–443.

Turner, P. E. & Chao, L. (2003). Escape from Prisoner's Dilemma in RNA phage phi6. *Am Nat* **161**, 497–505.

Velicer, G. J. (2003). Social strife in the microbial world. *Trends Microbiol* **11**, 330–337.

Velicer, G. J. & Lenski, R. E. (1999). Evolutionary trade-offs under conditions of resource abundance and scarcity: experiments with bacteria. *Ecology* **80**, 1168–1179.

Velicer, G. J. & Yu, Y. T. (2003). Evolution of novel cooperative swarming in the bacterium *Myxococcus xanthus*. *Nature* **425**, 75–78.

Velicer, G. J., Schmidt, T. M. & Lenski, R. E. (1999). Application of traditional and phylogenetically based comparative methods to test for a trade-off in bacterial growth rate at low versus high substrate concentration. *Microb Ecol* **38**, 191–200.

Velicer, G. J., Kroos, L. & Lenski, R. E. (2000). Developmental cheating in the social bacterium *Myxococcus xanthus*. *Nature* **404**, 598–601.

Watnick, P. & Kolter, R. (2000). Biofilm, city of microbes. *J Bacteriol* **182**, 2675–2679.

Westerhoff, H. V. & van Dam, K. (1987). *Thermodynamics and Control of Biological Free-Energy Transduction*. Amsterdam: Elsevier.