The evolution of groups of cooperating bacteria and the growth rate versus yield trade-off

Micro-organisms are ever more widely recognized as social. Many researchers, whose primary interest is the evolution of cooperation, have turned to microbes as the organisms of choice to test fundamental theories on the evolution of cooperative behaviour (e.g. Crespi, 2001; Brown & Johnstone, 2001; Ferriere *et al.*, 2002; Turner & Chao, 2003; Velicer, 2003; Travisano & Velicer, 2004; Greig & Travisano, 2004; Griffin *et al.*, 2004). The key problem for the evolution of cooperation is the fitness cost for the cooperating individual: cooperation between individual members of a group produces a public good that may benefit all group members, whether they cooperate or not, but only the cooperators pay the costs of producing the public good. Those who do not cooperate are called defectors, and those that gain an advantage from defection are called cheaters. Altruism is a behaviour that decreases the fitness of the altruistic individual while benefiting others. Cooperative behaviour in the presence of cheaters constitutes altruism towards the cheaters. The problem, therefore, is cheaters. Investment in cooperation reduces the fitness of the cooperating individual relative to the cheater, while the fitness of the group is increased relative to a group with a lower level of cooperation. In other words, we have a conflict of interest between individuals and the group. For recent more in-depth treatments, see Velicer (2003) and Travisano & Velicer (2004).

Mathematical models allow us to study the consequences of a coherent set of assumptions about the characteristics and composition of the system under study even when this system has complex dynamics and spatial structure. Using models, we can ask, for example, what are the minimum requirements for the evolution of cooperation? For several decades, models have shed light on the evolution of cooperation; for example, they have revealed the importance of repeated interactions and of spatial structure; see Sigmund (1994) for an introduction.

In biofilms or other complex dynamic microbial assemblages, bacteria live in crowded environments, where they interact with many neighbours in multiple positive and negative ways, much like our life in cities (Watnick & Kolter, 2000). Given that biofilms are multicellular, whether as a *multicellular community* or a *multicellular organism*, they are often seen as models for the development and evolution of multicellular organisms.

A report by Pfeiffer *et al.* (2001) was the first to identify a trade-off between growth yield and growth rate in heterotrophic organisms. They realized that trading an increase in growth yield for a decrease in growth rate is cooperative behaviour where the cooperation is 'passive' and indirect, consisting in restraint from competition over the use of limiting, shared (external) resources. Furthermore, this study also highlighted a connection between the evolution of cooperation in resource use and the evolution of multicellularity (see the section on strategies below).

As an extension of this study, Pfeiffer & Bonhoeffer published an individual-based model of variants of the above cooperative strategy in 2003, while another individual-based model of the evolution of cooperation in biofilms had been submitted (Kreft, 2004), raising questions of comparability of the two models, which will appear similar to the non-specialist reader, yet differ in a number of basic assumptions.

Individual-based models are mathematical models of population dynamics without any specifications for population behaviour; rather, the characteristics of the higher level of organization, the population, result from the dynamics on the lower level of organization, the individual organisms: population characteristics emerge from the actions and interactions of the individuals with each other and the environment. While this is true for all bottom-up models, individual-based models are those bottom-up models that explicitly allow variability among individuals (DeAngelis & Gross, 1992). For example, an individual-based model of biofilms would not contain a single line of programming code describing biofilm structure or function. Rather it describes the properties of the bacteria (such as metabolism, growth, motility and quorum sensing), the system geometry (such as liquid flowing over a flat, inert substratum) and mass transport processes (such as diffusion and convection) and studies the consequences of these properties on biofilm formation.

The report by Kreft (2004) looks at the above yield versus rate trade-off from a more microbiological perspective, examining the evolution of altruism in biofilms, and the consequences this entails for biofilm structure and characteristics, pointing out the importance of purification steps for clusters of cooperating cells – see the 'purification step' section below.

The aim of this Comment is threefold. Firstly, we compare the above two models of the evolution of cooperation and cooperating groups, on the one hand the studies of Pfeiffer *et al.* (Pfeiffer *et al.*, 2001; Pfeiffer & Bonhoeffer, 2002, 2003), and on the other the study of Kreft (2004). Secondly, the Comment wishes to emphasize the importance of conflicts of interest between the individual and higher levels of organization in biofilms and why this requires a cluster purification step. Thirdly, we hope that the Comment spawns a debate of the

question of why biofilms have not evolved into multicellular organisms.

Comparison of models

Strategies: muscle cells versus *Holophaga*. Both models provide the setting for the competition of two alternative survival strategies, which are based on a trade-off between growth rate and growth yield. One strategy is to grow fast at a low yield (rate strategy), the other is to grow slow at a high yield (yield strategy).

The trade-off, in turn, is based on irreversible thermodynamics which states that the rate of a process, such as microbial growth, is proportional to the thermodynamic driving force if the process is not too far from thermodynamic equilibrium. Consider the diffusion of a nutrient from a transient point source as a simple example. The rate of this process, the flux of nutrient, is proportional to the concentration gradient, which is the driving force in this case. Over time, the flux will decrease with decreasing force until equilibrium is reached. Other 'driving' forces are temperature gradients for heat flow or gradients of chemical potential for chemical reactions (Westerhoff & van Dam, 1987).

In the studies by Pfeiffer *et al.*, the two strategies reflect the switch from respiration to fermentation plus respiration as found in, for example, muscle cells and yeasts. These studies show that respiration, which results in higher yield but slower substrate turnover and growth rate, is in fact a group-beneficial trait because a high growth yield is equivalent to an economic utilization of the resource, which benefits all those sharing the (limiting) resource. Using fermentation in addition to respiration is a selfish trait, since it results in lower yield but higher substrate turnover and growth rate. They argue further that the evolutionary transition to multicellularity gives the newly formed multicellular organism an immediate advantage when the constituent cells use respiration, because the conflict of interest between the individuals (growth rate advantage) and the group (growth yield advantage) is diminished by aligning the interests of the individuals with the interest of the group. While many of the typical advantages of multicellularity became available only later, when further evolution had led to increasingly more sophisticated division of labour, forcing cells to cooperate in the use of common resources may have been the initial advantage of multicellularity.

The parameters of the rate versus yield trade-off used in the Kreft (2004) report were abstracted from growth data of the anaerobic bacterium *Holophaga foetida* which can double its growth rate at the cost of a halved yield, which is a moderate difference between the parameters of the high rate and the high yield strategy, compared to the difference in ATP yield between respiration (32 ATP per glucose) and fermentation (2 ATP per glucose) and the 100-fold higher maximal substrate consumption rate of fermentation (Pfeiffer & Bonhoeffer, 2003).

System: flatland versus highland. The system domain of the Pfeiffer & Bonhoeffer (2003) model may be pictured as a flat landscape on which individual cells and clusters grow in one layer, while the substrate first 'rains' down (stochastic allocation of substrate into the grid cells) and then diffuses on this plane. The system domain of the Kreft (2004) model in contrast may be pictured as a vertical landscape, tailored for biofilms, which grow from a flat substratum surface upwards towards the bulk liquid, which stays separated from the biofilm by a diffusion boundary layer of constant height (Fig. 1). The main consequence of the different system domains is in the way substrate diffuses. In the biofilm model, substrate diffuses down the concentration gradient that is actively generated by the substrate consumption of the biomass. Therefore, biomass clusters with higher substrate turnover rate act as stronger sinks for the diffusive substrate flow; they will receive a larger share of the substrate flux: those who consume more will get more. In particular, for clusters of the same size and density, the substrate consumption rate of rate strategy clusters is higher than that of yield strategy clusters. Substrate rations received by the grid cells in the flatland system do not depend on the activity or presence of biomass in that grid cell.

Four important consequences of the fact that substrate flux is driven by the cells' activity can be seen in Fig. 1. (1) The top layer of the biofilm receives more substrate than the inside and the sides.

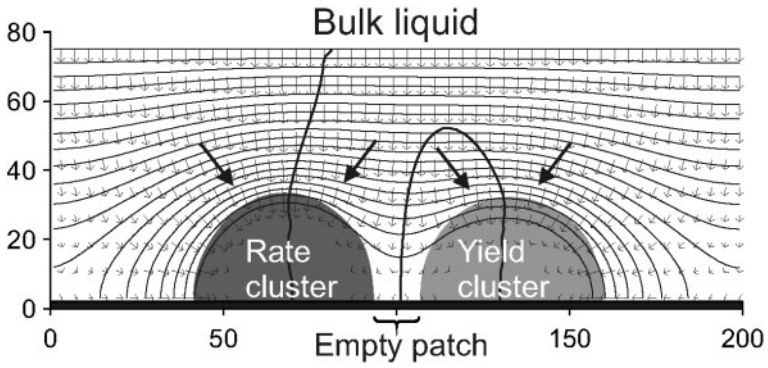

Fig. 1. The system domain (length scale in μm) of the biofilm model is a vertical landscape where substrate diffuses down the substrate gradient: from source to sink, i.e. from the bulk liquid through the concentration boundary layer to the substrate-consuming biomass. Two equally sized biofilm clusters are shown as grey areas: to the left is a cluster of the high rate strategists (darker grey) with higher substrate turnover rate, and to the right is a cluster of the high yield strategist (lighter grey). The 20 contour lines indicate substrate concentrations from 100 % in the bulk liquid at the top, down to 5 % near the cluster surfaces, in steps of 5 %. The arrows indicate the substrate flow and the two thick lines demarcate, at higher resolution, the regions in which the *horizontal component* of the diffusive flow is either to the left or to the right, in order to emphasize point (4) in the text.

(2) A cluster with higher substrate consumption rate will clear a larger area of substrate and receive a larger share of the total substrate flux. (3) Substrate flux may be sideways rather than top down. (4) Empty patches surrounded by active biomass may be empty due to the neighbouring clusters effectively clearing the patch of substrate, thus not allowing any growth in this patch. In the Pfeiffer & Bonhoeffer (2003) model, however, such an empty patch could be colonized from surrounding biomass by motile cells since the allocation of substrate into the spatial grid does not depend on whether the patch is surrounded by biomass or not. This gives motile single cells an advantage because they can spread into this patch, while the immotile clustering strategy cannot, resulting in an intrinsic disadvantage of the clustering strategy (see the section on clustering below for an explanation of this clustering strategy).

Cell division: surface-bound versus throughout. The Pfeiffer & Bonhoeffer (2003) model investigates whether the trade-off between growth rate and growth yield may lead to the evolution of active clustering of cells and may thus be linked to the evolutionary transition from single cells to undifferentiated multicellular organisms. The model uses a grid to represent space: only one individual organism is allowed per grid cell, and cell division is not possible if the four grid cells in the neighbourhood are already occupied. Although this assumption may appear somewhat unrealistic for bacterial communities, it is a worst case scenario as it impairs the evolution of cluster formation. However, as a consequence of this assumption, the cells living in a grid also compete for space, and hence the Pfeiffer & Bonhoeffer (2003) model examines competition for resources and space in combination. The Kreft (2004) report uses the previously described program BacSim (Kreft *et al.*, 1998, 2001), where individual bacteria are represented as spheres living in continuous space. In BacSim, cell division may well occur inside the biofilm, given nutrients are available, growth and division of spheres inside the biofilm simply pushes neighbouring cells away, leading to a flow of biomass towards the biofilm surface

Clustering: active versus passive. Clustering of cooperating cells is a necessary condition for the evolution of cooperation that is based on the rate versus yield trade-off, in other words on the economical utilization of resources, as both models show. However, the models differ in the way in which clustering is achieved. In the Pfeiffer *et al.* (2001) model, clustering is passive and is the consequence of low rates of cell diffusion. In the Pfeiffer & Bonhoeffer (2003) model, clustering is active and is assumed to be the consequence of a mutation leading to incomplete separation of cells after division, thus gluing the cells and their evolutionary interests together. In their model simulations, they compete clustering and non-clustering cells with high rate or high yield strategies to demonstrate that active clustering may evolve as a consequence of resource competition. In the biofilm study (Kreft, 2004), clustering is assumed to be passive, consistent with the hypothesis that this form of altruism is as old as life because it is simply based on the economical use of resources rather than on any direct or specific interactions between individuals. Accordingly, cells were assumed to be immotile since the first cells presumably were immotile. Then, clustering results from the multiplication of immotile cells. Growth and division of cells leading to the expansion of the biofilm may result in a certain degree of mixing (convection), but the method of biomass spreading used in the biofilm model (Kreft, 2004) does not result in extensive mixing, which may occur in models using Cellular Automata rules for biomass spreading – see Fig. 7 of Kreft *et al.* (2001) and further discussion in Picioreanu *et al.* (2004).

Cell death: uniform probability versus none. While death is assumed to occur with fixed probability rather than to depend on substrate availability in Pfeiffer & Bonhoeffer (2003), death was disabled in the BacSim model used in Kreft (2004) for the sake of simplicity.

Conclusions and debate

Robustness. The models differ with respect to substrate flux, cell division and the way in which clustering is achieved. Moreover the difference between the growth parameters of the two strategies is more pronounced in the studies of Pfeiffer *et al.*, where respiration and fermentation of glucose is contrasted. Nevertheless, both simulation studies obtain qualitatively the same results, indicating that the results are robust with regard to model and parameter choice.

Purification step. Clusters of individuals cooperating in resource use clearly have an advantage in the long term, as both models show, but the conflict of interest between higher growth rate, advantageous for the individual and higher yield, advantageous for the cluster (and indirectly advantageous for the individual members of the cluster) remains. This will be seen whenever a mutant or immigrant with a higher growth rate and lower yield appears in the cluster since the offspring of this faster growing cell will increase its proportion in the cluster, deriving benefits from the economical use of resources of its neighbours while not paying the cost of a decreased growth rate. In order for the cooperating individuals to survive in the long term, the spread of such cheaters, whose appearance is unavoidable, must be limited. In the biofilm study (Kreft, 2004), it was argued that the simplest way of limiting the spread of cheaters in clusters of cooperating cells is the occasional break-up of clusters into single cells that colonize other patches, effectively refounding the clonal clusters. Such a purification step is essential, and in the case of clusters arising simply from the multiplication of immotile cells, shear or other mechanical forces of detachment or disruption of the biofilm structure may be sufficient. Active detachment of cells from the cluster may have evolved later to provide additional purification events if advantageous. For the survival strategy of active clustering by incomplete cell division (Pfeiffer & Bonhoeffer, 2003), the simultaneous evolution of a purification mechanism is not addressed in the report. However, it is conceivable that following the evolution of active clustering, mechanisms evolve that result, for example, in the budding of individual cells from the cluster.

Evolutionary transition to multicellularity. Bacteria living in biofilms have, by and large, not evolved into multicellular

organisms whilst showing aspects of functional multicellularity. Rather, biofilms are transient and *ad hoc* assembled communities of many species; the phases of biofilm formation ('biofilm growth') correspond to *adaptation* and *ecological succession* rather than the life cycle or *developmental programme* of a multicellular organism (cf. O'Toole *et al.*, 2000): from colonization by pioneers ('attachment'), via population growth (while colonization continues, the environment changes, and the bacteria adapt, move, quorum sense, etc. in this 'maturation' phase), to actively regulated (biological) as well as shear forced (physical) detachment.

Despite the involvement of genetic programmes and the observation of repeatable patterns (O'Toole *et al.*, 2000; Webb *et al.*, 2003a, b), biofilm succession is unlike development of a multicellular organism because a multicellular organism can control or enforce its own developmental programme even in the face of challenges. These may be physical disruption or biological interference from other species or strains. Typically, the cells of a multicellular organism form a tissue, a clone of cells all derived from a single stem cell. Note that going through a single cell stage as part of the life cycle constitutes a purification step as discussed above. The cells in this tissue do not mix with cells of other species; rather, the boundary of a multicellular organism is defined and defended. Its formation is robust. Microcolonies in biofilms do not have such a defined boundary between self and non-self. Some multicellular organisms do not follow this scheme of development and form by aggregation of cells into a fruiting body, such as *Myxococcus xanthus* and *Dictyostelium discoideum* (Travisano & Velicer, 2004). Here, *cell sorting* by coordinated cell movement is crucial; in the case of *D. discoideum*, the cell sorting is achieved by differential chemotactic cell movement (Vasiev & Weijer, 1999). For this aggregation type of development to work in natural habitats, differential cell sorting of one type of cells from a mix of cells of various species must be possible. To our knowledge, differential cell sorting in multi-species biofilms has not been demonstrated.

However, the evolution of multicellularity in bacteria is possible, since multicellularity has independently arisen among bacteria several times in actinomycetes, cyanobacteria and myxobacteria (Bonner, 2001). Moreover, experimental microbial evolution demonstrates that cooperative behaviour can readily evolve in bacteria (Rainey & Rainey, 2003; Velicer & Yu, 2003). The work of Pfeiffer *et al.* argues that the combination of multicellular organization and economical use of resources (e.g. respiration) could represent a major fitness advantage that does not require cell differentiation and thus benefits the simplest multicellular organism derived from respiring cells as soon as it arises. Why, then, have biofilm bacteria not evolved into multicellular organisms?

One possible reason that could impair the evolution of multicellularity in bacteria is that multicellularity may create a '*feeding problem*'. The solution of the feeding problem typical for eukaryotes is 'eating': the ingestion and exclusive digestion of large food items. While the structure of eukaryotic cells with a cytoskeleton-driven phagocytosis mechanism may have facilitated the evolution of eating, the bacterial cell structure with a rigid mesh wall and lack of a phagocytosis mechanism could have constrained evolution, not allowing the feeding problem to be solved efficiently. The best solution possible for bacteria is the 'wolf-pack feeding' of myxobacteria (Bonner, 2001); arguably, such external digestion by a cooperatively produced cocktail of exoenzymes is intrinsically less efficient due to the diffusive loss of enzymes and degradation products. (Note that such a feeding problem does not exist for phototrophic organisms, and cyanobacteria are indeed one of the three groups of multicellular prokaryotes.) Another reason is that the trade-off in heterotrophic resource use is most pronounced when comparing the yield of fermentation versus respiration. Thus, the benefit of cooperative resource use may have been sufficiently large only after oxygen levels reached a sufficient level, which could explain why the evolutionary transition to multicellularity in heterotrophic eukaryotes appears to have taken so long. Identifying the ecological and evolutionary factors that promote the evolution of multicellularity rather than complex microbial ecosystems such as biofilms remains a challenging problem for future research on competition in microbial communities.

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