

# The sociobiology of biofilms

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## Abstract

Biofilms are densely packed communities of microbial cells that grow on surfaces and surround themselves with secreted polymers. Many bacterial species form biofilms, and their study has revealed them to be complex and diverse. The structural and physiological complexity of biofilms has led to the idea that they are coordinated and cooperative groups, analogous to multicellular organisms. We evaluate this idea by addressing the findings of microbiologists from the perspective of sociobiology, including theories of collective behavior (self-organization) and social evolution. This yields two main conclusions. First, the appearance of organization in biofilms can emerge without active coordination. That is, biofilm properties such as phenotypic differentiation, species stratification and channel formation do not necessarily require that cells communicate with one another using specialized signaling molecules. Second, while local cooperation among bacteria may often occur, the evolution of cooperation among all cells is unlikely for most biofilms. Strong conflict can arise among multiple species and strains in a biofilm, and spontaneous mutation can generate conflict even within biofilms initiated by genetically identical cells. Biofilms will typically result from a balance between competition and cooperation, and we argue that understanding this balance is central to building a complete and predictive model of biofilm formation.

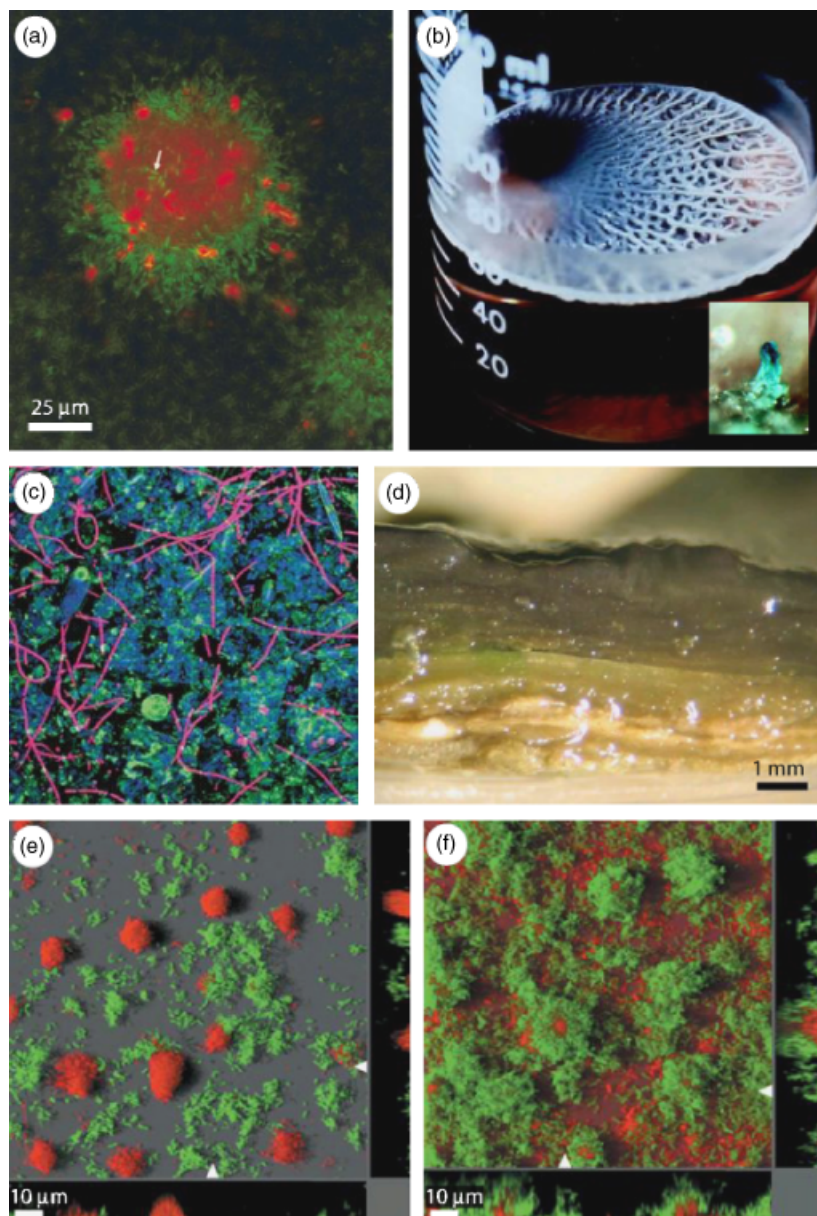
## Introduction

Bacteria are gregarious organisms that commonly form densely populated communities, known as biofilms. These microbial collectives, which are typically encased in secreted polymers, occupy many biotic and abiotic surfaces and frequently contain multiple species (Fig. 1). The ubiquity of biofilms in the natural environment, including plant and animal hosts, suggests that living in groups is critical for bacterial ecology and evolution (Hall-Stoodley *et al.*, 2004). Bacterial aggregates directly impact human lives as well. Biofilm-dwelling cells appear more tolerant to antibiotics than planktonic cells and are responsible for many health threats, including acute and chronic infections and the degradation of implanted prosthetic devices (Watnik & Kolter, 1999; Stewart, 2002; Mah *et al.*, 2003; Heithoff & Mahan, 2004; Costerton *et al.*, 2005; Fux *et al.*, 2005; Lee *et al.*, 2005; Obst *et al.*, 2006; Manos *et al.*, 2007). Bacterial biofilms also contaminate drinking water distribution systems and cause a multitude of industrial problems associated with biofouling (Jass & Walker, 2000).

The significance of biofilms for microbial ecology and human society has motivated an enormous amount of

research on the genetic, biochemical, and physical mechanisms underlying biofilm formation (Hall-Stoodley *et al.*, 2004). This work has shown that the characteristics of biofilms often differ both within and among species as culture conditions change. Diverse bacterial groups form biofilms with different composition and structure (Beloïn & Ghigo, 2005), to the extent that the term biofilm may sometimes be too readily applied (Moxon *et al.*, 2008). However, many biofilms do share common features that set them apart from populations of planktonic cells, and a growing consensus holds that these bacterial communities are functional entities in which cells display specific adaptations to biofilm life (O'Toole *et al.*, 2000a; Hall-Stoodley & Stoodley, 2002; Sauer *et al.*, 2002; Stoodley *et al.*, 2002; Webb *et al.*, 2003a).

Biofilm growth is usually depicted as a series of discrete stages in a life cycle (Stoodley *et al.*, 2002), which begins when planktonic cells contact a surface, either randomly or by taxis toward chemical attractants (Freter & O'Brien, 1981; Pratt & Kolter, 1998; Meibom *et al.*, 2004). Initial attachment is often reversible such that cells can depart from a surface if conditions change. Some bacteria can also position themselves using extracellular pili, which act as miniature



**Fig. 1.** Biofilms often have structural features that appear to suggest coordination or cooperation among bacteria. (a) Monospecies biofilms of *Pseudomonas aeruginosa* with a subpopulation of cells differentiating into diverse phenotypes that lead to localized cell death (Webb *et al.*, 2003b). (b) Floating biofilms of *Bacillus subtilis* form multicellular aerial structures similar to fruiting bodies (inset) in which cells sporulate (Branda *et al.*, 2001). (c) Natural biofilms such as those found on riverbeds can contain many species (cyanobacteria in pink, green algae in blue, EPS in green; courtesy of T. Neu). (d) Different species in a biofilm may separate into discrete layers according to their metabolic properties as the biofilm matures (Roeselers *et al.*, 2008). (e, f) Evolution of species interactions between *Acinetobacter* sp. C6 (red) and *Pseudomonas putida* (green) in a flowcell leads to a more productive biofilm; this occurs not by mutual cooperation but by an increase in the efficiency with which one species exploits the other (Hansen *et al.*, 2007).

grappling hooks and allow cells to move over each other (Klausen *et al.*, 2003a) or along a solid substrate (O'Toole & Kolter, 1998). More stable surface attachment can be achieved via specific adhesin molecules (Hinsa *et al.*, 2003; Latasa *et al.*, 2005). For example, the opportunistic pathogen *Pseudomonas aeruginosa* binds disaccharide units within the mucus lining of the human lung (Wilson *et al.*, 1996), and *Vibrio cholerae* attaches to chitin polymers in the exoskeletons of marine crustaceans (Meibom *et al.*, 2004).

After surface attachment, bacteria grow and divide to form the dense cell groups that characterize biofilms. Microarray studies indicate that diverse model species – including *P. aeruginosa*, *V. cholerae*, *Escherichia coli*, and *Staphylococcus*

*aureus* – differentially express as much as 10% of their genomes when in biofilm vs. planktonic growth conditions (Schoolnik *et al.*, 2001; Whiteley *et al.*, 2001; Schembri *et al.*, 2003; Beenken *et al.*, 2004). As might be expected, these altered transcriptional profiles are associated with phenotypic changes in cell–cell and cell–surface adhesion, metabolism, motility, and extracellular product secretion. However, gene expression studies also illustrate that biofilms of different strains or species may be as different from one another as they are from a planktonic population (Beloin & Ghigo, 2005). Individual biofilms are themselves heterogeneous: genetically identical cells in a biofilm can diverge in a wide variety of traits, including basic metabolic activity

(Xu *et al.*, 1998), antibiotic tolerance (Balaban *et al.*, 2004), spore formation (Branda *et al.*, 2001), and the secretion of extracellular polymers (Vasseur *et al.*, 2005; Vlamakis *et al.*, 2008).

Secreted polymers are a defining feature of biofilms. Cells typically produce and embed themselves in a matrix of extracellular polymeric substances (EPS) composed of polysaccharides and smaller amounts of protein and DNA (Beenken *et al.*, 2004; Hall-Stoodley *et al.*, 2004; Braxton *et al.*, 2005; Flemming *et al.*, 2007). The functions of EPS are not yet clear, but it appears to promote surface attachment and provide structural support; mutants that cannot produce EPS are often deficient in biofilm formation (Davies *et al.*, 1993; Danese *et al.*, 2000; Hammer & Bassler, 2003; Kearns *et al.*, 2005). EPS may also afford protection from external threats, such as antimicrobial compounds and predatory organisms (Mah & O'Toole, 2001; Stewart, 2002; Fux *et al.*, 2005; Matz *et al.*, 2005), or help secreting strains to grow toward nutrient-rich locations (Foster & Xavier, 2007; Xavier & Foster, 2007; Nadell *et al.*, 2008). To disperse from a biofilm, however, bacteria must also be able to escape the binding properties of EPS. Detachment can occur by fluid flow shearing off cells (Telgmann *et al.*, 2004), or by active processes on the part of bacteria. For example, *V. cholerae* cells appear to encourage dispersal by secreting enzymes that break down the surrounding structural matrix (Hammer & Bassler, 2003; Zhu & Mekalanos, 2003; Heithoff & Mahan, 2004; Liu *et al.*, 2007).

An enduring question is how bacteria regulate the changes in gene expression underlying the changes in cell behavior that characterize biofilms. Although no single mechanism is responsible (Hall-Stoodley *et al.*, 2004), many species use quorum sensing to modulate surface attachment (Dunne, 2002), motility (Schuster & Greenberg, 2006), EPS production (Davies *et al.*, 1998; Hammer & Bassler, 2003; Sakuragi & Kolter, 2007), and dispersal (Allison *et al.*, 1999; Dow *et al.*, 2003). Quorum sensing entails the secretion and detection of small diffusible molecules, often known as autoinducers. Bacteria are thought to use autoinducer concentrations as a proxy for population density and thereby tune their behavior according to the local abundance of other cells (Miller & Bassler, 2001; Waters & Bassler, 2005; Bassler & Losick, 2006). It is also possible that bacteria use quorum sensing to monitor the extent of diffusion and fluid flow in their local micro-environment (Redfield, 2002; Hense *et al.*, 2007). Most species upregulate biofilm-associated behaviors at high cell density, and some mutant strains that can no longer detect autoinducers are unable to form biofilms (Parsek & Greenberg, 2005). There are interesting exceptions to this pattern, however: *V. cholerae*, the pathogenic agent responsible for pandemic cholera among humans, downregulates biofilm-associated behavior at high cell density. Mutants of this species that cannot detect autoinducers form exceptionally large biofilms

(Hammer & Bassler, 2003). Importantly, the impact of quorum-sensing regulation on biofilm formation can depend strongly on environmental conditions. While *P. aeruginosa* is known to regulate behaviors such as EPS secretion via quorum sensing (Davies *et al.*, 1998; Sakuragi & Kolter, 2007), for example, there are some culture conditions in which quorum-sensing null mutants and wild-type bacteria produce indistinguishable biofilms (Purevdorj *et al.*, 2002).

The use of quorum-sensing and environmental cues to regulate transitions between seemingly discrete biofilm life-stages has fostered the idea that biofilms emerge from a bacterial developmental program, analogous to those of multicellular organisms (O'Toole *et al.*, 2000b; Danese *et al.*, 2001; Hall-Stoodley & Stoodley, 2002; Stoodley *et al.*, 2002; Webb *et al.*, 2003a). Some have further hypothesized that whole-group coordination – energetically costly intercellular signaling over spatial scales much larger than that of a single cell (Keller & Surette, 2006) – is required for the production of the heterogeneous and apparently complex biofilm structures observed in many species (Stoodley *et al.*, 2002). Further, a prevailing rhetoric within the literature tends, both explicitly and implicitly, to cast biofilms as aggregates in which individual cells cooperate with each other. It is presumed that biofilm-dwelling bacteria display altruistic behavior, decreasing their own fitness – that is, their reproductive output – in order to increase the fitness of other cells and thereby benefit the biofilm community as a whole (Table 1).

Because of their strength in numbers, bacteria residing at high density in biofilms can achieve protection from external threats and impact their biotic and abiotic environments in ways that are simply impossible for individual cells (Stewart, 2003b). Does this mean that biofilms are multicellular in analogy to a metazoan organism, or could it be that biofilms are merely multiorganismal, similar in principle to a

**Table 1.** The four types of social behavior, defined by their effect on the direct fitness (lifetime reproduction) of the actor and recipient

	Effect on recipient	
	+	–
Effect on actor		
	+	–
+	Mutualism	Selfishness
–	Altruism (Coordination*)	Spite
	Cooperation	Competition

Based on Hamilton (1964). A common proxy for direct fitness for many microorganisms is their rate of cell division. For example, consider a microorganism that secretes a costly product that benefits all cells in its neighborhood, including itself. This is an example of cooperation, and if secreting such a product causes a net decrease in the focal cell's growth rate, it is also an example of altruism.

\*In our review, we define coordination as processes that rely on cooperative signaling among cells, which is likely to involve the altruistic production of one or more signaling molecules.

flock of birds, a school of fish, or a swarm of insects? One can evaluate this question by examining the proximate mechanisms that regulate biofilm formation, which is the subject of another upcoming review (Monds & O'Toole, 2008). One may alternatively dissect the ultimate causes of biofilm formation from the perspective of evolutionary biology, which is the purpose of our present paper. Specifically, our goal is to critically evaluate the idea that physical and biological complexity in biofilms is a product of coordination and cooperation among the bacteria residing within them.

Why is it important to understand whether group-living bacteria truly cooperate with each other? We will argue that understanding the degree to which biofilms rely on cooperation among their constituent cells is critical to any predictive model of biofilm formation and to the design of effective strategies for combating biofilm infections. While coordination and cooperation among biofilm-dwelling bacteria are conceivable, two branches of theory warn against assuming that biofilm complexity is evidence of a harmonious social group. First, the theory of collective behavior shows that apparently coordinated aggregates can emerge from the uncoordinated activity of many individuals following simple behavioral rules (Box 1; see section 'Biofilm complexity without coordination'). Second, evolutionary theory predicts that cooperation can only evolve under special circumstances (Box 2; see section 'Competition and cooperation in biofilms'), and that competition will play a strong role governing the behavior of most organisms living in close proximity (Alexander, 1974). In particular, many biofilms contain multiple strains and species (Kolenbrander, 2000; Burns *et al.*, 2001), among which competitive interactions are likely to be common (Hansen *et al.*, 2007).

## Biofilm complexity without coordination

Biofilms are not merely a collection of cells that all behave identically; on the contrary, bacterial groups are heterogeneous by most measures currently available to describe them (Stewart & Franklin, 2008). Fluorescence microscopy reveals that mono- and multispecies biofilms contain segregated subpopulations with different phenotypic and physiological properties. Furthermore, biofilm surfaces are often rugged and uneven, containing large multicellular towers interwoven by fluid-filled channels (De Beer *et al.*, 1994a).

Heterogeneity in physiological state and spatial structure appears to be a fundamental feature of biofilms that is associated with their increased antibiotic tolerance and pathogenicity (Stewart & Costerton, 2001; Stewart, 2002; Fux *et al.*, 2005). It has been suggested that this heterogeneity results from active intercellular signaling that coordinates the biofilm to maximize its productivity (Table 1) (O'Toole *et al.*, 2000b; Danese *et al.*, 2001; Hall-Stoodley & Stoodley, 2002; Stoodley *et al.*, 2002; Webb *et al.*, 2003a). The emergence of biofilm heterogeneity on its own, however, does not necessarily require cooperative, coordinated bacterial behavior. In this section, we review evidence that biofilm composition and structure can also arise as a consequence of bacteria simply adjusting their behavior to the immediate surroundings and competing for limited resources. This perspective is drawn from the study of collective behavior in animal groups (Box 1), and the application of computational biofilm models that capture the interaction between bacterial growth and solute gradients (Wimpenny & Colasanti, 1997; Picioreanu *et al.*, 1998a; Kreft *et al.*, 2001; Xavier *et al.*, 2005, 2007).

### Box 1. Collective behavior: complex patterns from simple rules

Spatially explicit models of biofilm formation have shown that simple cellular responses to the local environment are sufficient to produce complex structures in bacterial groups (Wimpenny & Colasanti, 1997; Picioreanu *et al.*, 1998a, 2004; Noguera *et al.*, 1999; Dockery & Klapper, 2001; Kreft *et al.*, 2001; Xavier *et al.*, 2005). These findings are mirrored in the study of animal behavior. A casual glimpse into the natural world reveals that bacteria are not alone in their proclivity for group living. Organisms from every corner of the animal kingdom form aggregates, with particularly conspicuous cases including insect swarms, fish schools, bird flocks, and mammalian herds (Sumpter, 2006). Many of these animal collectives have a physical coherence that at first appears to require long-range signaling among their individual constituents. Fish schools, for example, exhibit a wide range of structures,

including stationary swarms and cylindrical vortices that rapidly change form in response to predators or prey items.

Theoretical and experimental studies have shown that the integrity and collective behavior of animal groups does not always entail active communication and coordination among constituent members (Sumpter, 2006; Couzin, 2007). Couzin *et al.* (2002) explored the mechanisms underlying group structure using a model that implemented idealized swarming organisms following three simple rules: (1) move away from neighbors that are too close, (2) align with neighbors of intermediate distance, and (3) avoid separation from the group. As the size of the alignment zone increases, the group transitions from a stationary swarm, to a doughnut-shaped group rotating around its collective center of mass, to an aligned group in which all individuals are traveling in the same direction. Couzin *et al.* (2005) later used another model to show that groups of moving organisms can collectively choose the

better of two resource patches even when no single individual has information about both feeding opportunities.

Examples of apparently complex group phenomena can thus be explained by simple behavioral rules on the part of group members. Furthermore, individual animals often join and participate in groups because membership therein provides direct benefits, such as increased foraging efficiency and predator vigilance, an example of mutualism rather than altruism (Table 1) (Krause & Ruxton, 2002). The properties of animal aggregates can even emerge from strong conflicts of interest among group members. For example, the collective behavior of desert locust nymphs appears to be driven by cannibalism: the group proceeds en masse because each individual attempts to eat those in front and avoid being eaten by those behind. This process, combined with high population density, yields an enormous march of insects that culminates in the notorious locust plagues of North Africa and the Middle East (Bazazi *et al.*, 2008).

While group structure and behavior do not strictly require them, coordination via dedicated signaling systems

does play a critical role in the collective activity of some species. For example, the organization of social insect colonies emerges from a mixture of individual behavioral decisions combined with many forms of communication that coordinate colony activity (Bonabeau *et al.*, 1999). Prominent examples include the famous honeybee waggle dance whereby experienced foragers convey the location of flowers to naïve foragers, pheromone trails that lead ants to food patches, and alarm signals that allow wasp colonies to mount a coordinated defense against predators (Ratnieks & Reeve, 1992). These instances of collective behavior require that individuals act in the interests of the colony. In turn, in the absence of feedback benefits, the evolution of individually costly, group-beneficial behavior is only expected among close relatives (Table 1, Appendix B). In general, then, the spatially and temporally complex properties of organismal groups can be deceptive: while whole-group coordination and cooperation do occur, many examples of collective behavior emerge in their absence. Care must be taken to determine which mechanistic and evolutionary processes are relevant to each particular system of interest.

### Diffusion limitation and chemical heterogeneity

Bacteria are often tightly packed in biofilms (Stewart, 2003a). High cell density tends to reduce the bulk flow of any surrounding liquid, and diffusion governs most transport of solutes between the biofilm and its environment. Diffusion is slow compared with cellular metabolism (Characklis, 1990), and as a result, the chemical environment within a biofilm often differs greatly at different depths (Stewart, 2003a). For example, a nutrient in the liquid surrounding a biofilm may be consumed by cells in the outermost layers such that its concentration decreases deeper in the biofilm. Meanwhile, a product secreted by the cells in the biofilm will often accumulate to its highest concentration within the biofilm's interior. Given that many bacteria use multiple growth substrates and release numerous extracellular products, diffusion limitation can produce complex vertical and transverse chemical gradients, particularly when the secretions of some cells are consumed by others (Stewart & Franklin, 2008). These gradients, in turn, generate numerous microniches within the biofilm that can induce pronounced physiological heterogeneity simply because bacteria alter their behavior according to local conditions.

A classical example of chemical and consequent physiological heterogeneity is the commonly observed pattern of oxygen depletion with increasing biofilm depth, which can occur even in small biofilm clusters 40  $\mu\text{m}$  deep (Kuhl *et al.*, 2007). Within large aerobic biofilms, oxygen consumption by bacteria in the outermost 100–200- $\mu\text{m}$  cell layer is

sufficient to create anoxic environments at greater depths (De Beer *et al.*, 1994b). As a result, cells near the biofilm surface utilize aerobic metabolism, while cells within the biofilm must switch to anaerobic metabolism or cease growing (Xu *et al.*, 1998).

### Biofilm growth and species segregation

Spatial heterogeneity is also common in multispecies biofilms, which are often stratified such that species with different metabolic activity occupy discrete layers (Fig. 1d). This stratification can manifest as the separation of aerobic and anaerobic species between the oxygenated and anoxic regions of a biofilm, or more generally as segregation of species due to their use of different electron acceptors for metabolism, of which oxygen is only one (van Loosdrecht *et al.*, 2002). For example, species stratification is common in the biofilms of wastewater treatment reactors. Oxygen is supplied to the reactors at low rates so that it is fully consumed by bacteria residing at the outer surface of biofilms. These aerobes are split into several layers: species in the outermost layer use oxygen to convert ammonium into nitrite, and immediately below them reside other species that aerobically oxidize nitrite to nitrate (Okabe *et al.*, 1999; Lydmark *et al.*, 2006). Finally, species that anaerobically reduce nitrate to elementary nitrogen reside deeper in the biofilm, where oxygen is no longer available. Similar stratification occurs in sulfate-reducing multispecies

biofilms, in which bacteria reducing sulfate are found exclusively in the anaerobic interior (Ramsing *et al.*, 1993).

Species segregation according to metabolic properties might suggest that microbial consortia organize themselves to maximize the productivity of each strain (Stoodley *et al.*, 2002). However, cooperation among species is only expected under restricted conditions (Box 2), and in fact, competitive interactions appear to drive biofilm stratification. The metabolic separation of ammonium oxidation and nitrite oxidation between different species probably occurs because ammonium-oxidizing bacteria grow faster than would a hypothetical species carrying out both steps of nitrification within the same cell (Broda, 1977). By increasing their growth rate in this way, ammonium-oxidizing bacteria decrease the amount of ATP produced for each ammonia molecule oxidized, which reduces their metabolic efficiency. Therefore, the coexistence of ammonium oxidizers and nitrite oxidizers indicates that these bacteria are selected to maximize their own reproductive success rather than the efficiency of the group in which they are living (Costa *et al.*, 2006).

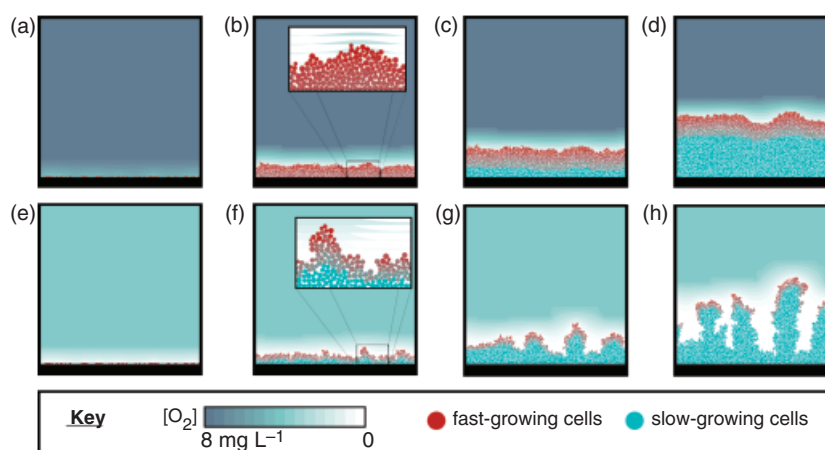
How then can one explain the organization of different metabolic types into discrete layers? A simulation by Picioreanu *et al.* (2004) showed that the differential ability of different species to grow at different depths from the biofilm surface is sufficient to explain their stratification. Species residing deep in sludge granule biofilms grow relatively slowly because they use nitrate as a primary electron acceptor, which yields less energy than aerobic metabolism. Nevertheless, they survive and dominate the inner parts of the biofilm because the species at the surface are obligate aerobes that cannot grow where oxygen has been

depleted (de Kreuk & van Loosdrecht, 2004; Isaka *et al.*, 2006). That is, the segregation of metabolic types within biofilms is consistent with the classic ecological principle of competitive exclusion, whereby competition pushes each species, or cluster of similar species, into a discrete niche (Scheffer & van Nes, 2006).

### Biofilm surface topology

Early microscopy studies revealed that biofilm surfaces vary from smooth and confluent to rough and uneven with tall cell clusters interweaved by fluid-filled channels (Costerton *et al.*, 1994; Klausen *et al.*, 2003b; Wijeyekoon *et al.*, 2004). While one might imagine that the formation of fluid-filled channels reflects coordinated activity within the biofilm to improve waste removal and nutrient uptake, complex cell-cell communication and cooperation are not necessary to explain variability in biofilm surface structure. Mathematical and computational models of biofilm formation have been developed by engineers to identify the environmental factors that promote and inhibit biofilm formation. These models, and empirical tests, have shown that simple differences in environmental nutrient availability can account for a variety of biofilm surface structures (Wimpenny & Colasanti, 1997; Picioreanu *et al.*, 1998b; Dockery & Klapper, 2001; Wijeyekoon *et al.*, 2004).

Nutrient availability is critically important for biofilm surface structure because the outermost cells in a biofilm consume substrates diffusing in from the environment and may thereby prevent nutrients from reaching cells residing at greater depths (Fig. 2). The result is an outer layer of cells



**Fig. 2.** Computational simulations reveal that complex biofilm structure can be explained by simple diffusion limitation of a growth-limiting nutrient. (a–d) When environmental nutrient (here, oxygen) concentration is high, nutrients penetrate well into the biofilm before being depleted, creating a thick layer of actively growing cells (details in b). Biofilm surface irregularities are not amplified, and the biofilm remains smooth. (e–h) When environmental nutrient concentration is low, nutrients are quickly depleted, creating a thin layer of actively growing cells (details in f). Random irregularities in the biofilm surface are amplified because cells residing in the peaks of irregularities continue to grow, while cells residing in the troughs of irregularities cease to grow. This positive feedback process generates towers of cell clusters separated by empty channels in the absence of intercellular communication or cooperation (h).



that typically grows much faster than the rest of the biofilm population. The thickness of this active cell layer depends upon environmental nutrient concentrations, how quickly nutrients diffuse into the biofilm, and how quickly bacteria consume them. When nutrients are abundant, they penetrate deeper into the biofilm before being depleted, and the active cell layer is thick; the biofilm surface remains relatively uniform and homogeneous because a large population of cells at the surface is growing (Fig. 2a–d). When nutrients are sparse, in contrast, they are more quickly depleted, and the actively growing biofilm layer is relatively thin. As a result, irregularities in the surface of the biofilm can become greatly amplified. Specifically, if the depth of surface irregularities is greater than the thickness of the actively growing cell layer, then bacteria residing in the troughs of irregularities will grow poorly, while cells at peaks of surface irregularities retain access to nutrients and grow well. In the absence of any sophisticated intercellular signaling, this positive feedback process gives rise to cell towers interdigitated by empty fluid channels (Fig. 2e–h).

Detachment from biofilms also plays an important role in determining their surface structure. Surface erosion, caused by predator grazing and fluid flow, tends to remove surface irregularities from exposed biofilm regions (Characklis *et al.*, 1990), particularly in long-lived biofilms that have reached a steady state through a balance between new growth and detachment (Kwok *et al.*, 1998). If environmental forces also cause occasional large disturbances, however, biofilms may fail to reach a steady state and instead cycle through periods of growth, sloughing, and regrowth (Lewandowski *et al.*, 2004; Xavier *et al.*, 2005a, b). Detachment may also involve active bacterial behaviors that cause regions of the biofilm to collapse or disperse, yielding heterogeneous surface structure. For example, the soil bacterium *Shewanella oneidensis* detaches from its biofilms in response to oxygen limitation (Thormann *et al.*, 2005, 2006). Some *P. aeruginosa* strains undergo lysis, which liquefies portions of mature biofilms and releases other bacteria into the environment. Partial biofilm lysis is either prophage-mediated (Webb *et al.*, 2003b, 2004) or regulated by quorum sensing (D'Argenio *et al.*, 2002; Allesen-Holm *et al.*, 2006).

This phenomenon may be a coordinated strategy that evolved to allow the biofilm as a whole to release cells for colonizing new resource patches. However, the importance of competition among *P. aeruginosa* strains and conflict between lyso-genic phage and their bacterial hosts remains to be explored for this system.

The studies discussed thus far illustrate that, under the influence of simple physical forces, heterogeneity in biofilm physiology and spatial structure can emerge from the uncoordinated behavior of individual cells. However, the absence of whole-biofilm coordination does not necessarily imply the complete absence of cooperation among group-living bacteria (Box 1), which may take the form of other behaviors such as restrained growth or the secretion of shared products. Furthermore, some biofilm-associated behaviors do appear to entail local coordination. For example, *V. cholerae* uses quorum sensing to terminate EPS secretion and upregulate secretion of extracellular enzymes that promote dispersal en masse at high cell density (Hammer & Bassler, 2003). Are such behaviors cooperative, and is there evidence for whole-biofilm coordination via signaling over large spatial scales? These questions lead us to the following section in which we evaluate the conditions under which coordination and cooperation can evolve among bacteria.

## Competition and cooperation in biofilms

Biofilm-dwelling bacteria exhibit a range of behaviors that influence the reproduction, and thereby evolutionary fitness, of neighboring cells. Each of these social behaviors can be categorized according to its fitness effects on the acting individual and on the recipient individuals (Table 1). As we will see, focusing on fitness effects allows one to identify when natural selection will favor cooperation among bacteria and perhaps coordination on the scale of whole biofilms. Recent reviews have discussed the principles of social evolution in microorganisms (Crespi, 2001; West *et al.*, 2006, 2007; Diggle *et al.*, 2007b; Foster *et al.*, 2007), and here we apply this perspective to the unique problems posed by bacterial biofilms (for another discussion, see Kreft, 2004a).

### Box 2. The evolution of cooperation and altruism: kinship, coercion, and constraint

'If it could be proved that any part of the structure of any one species had been formed for the exclusive good of another species, it would annihilate my theory, for such could not have been produced through natural selection' Darwin (1859).

The evolutionary biologist's fascination with cooperative and altruistic behaviors has a rich history that can be

traced back to Darwin, who discussed examples like the sterile and seemingly selfless workers in social insect colonies and the mutualism that occurs between a nectar-producing orchid and its insect pollinators. Specifically, Darwin realized that natural selection should favor traits that increase personal reproduction, and any behavior that decreases one's own reproduction to increase that of another organism requires additional explanation. Over a hundred years after *On the Origin of Species* was published, William Hamilton – then only a graduate student –

developed the first full theoretical explanation for how cooperative behavior can evolve (Table 1). Since then, a huge literature has developed around the problem (Lehmann & Keller, 2006; Gardner & Foster, 2008), but it can be whittled down to a few central concepts:

*Genetic relatedness* (indirect fitness): the crux of Hamilton's argument was that natural selection can favor altruistic actions (Table 1) that harm personal reproduction when they benefit a family member or other individual that shares alleles at variable loci with the actor. This principle has far-reaching consequences for evolutionary theory. In particular, it predicts that cooperation and altruism will evolve more readily in social groups containing a high proportion of genetic relatives than it will in groups of nonkin. Accordingly, we expect cooperation to evolve more easily in biofilms that always contain a single strain than in biofilms containing multiple strains and species, which are fraught with potential conflicts. While the general effects of changing genetic relatedness in a social group are intuitive, measuring relatedness is not. From an evolutionary perspective, relatedness is not a single value that exists between individuals, but rather a conceptual tool whose calculation depends on the problem at hand. Positive relatedness occurs when two individuals share identical alleles at a *variable* locus: natural selection can only operate when there are competing alleles in the population. Critically, relatedness should be measured at the locus or loci that control the social behavior in question because it is the change in frequency of alleles at these loci that govern the evolution of each social trait. Counterintuitively, bacterial strains that differ at a single nucleotide position may be unrelated in the eyes of social evolution. For example, a null mutation that eliminates pyoverdine secretion in *P. aeruginosa* can produce a cheating strain that exploits any bacteria that continue to altruistically secrete pyoverdine. This consideration is particularly important for biofilms, which endure for many bacterial generations such that groups of initially clonal cells may be subject to exploitation by spontaneous mutants that no longer share the evolutionary interests of others in the biofilm.

*Personal reproductive benefits* (direct fitness): as captured in Darwin's quote, altruism in the strict sense – which lowers an individual's personal reproductive fitness – cannot be favored by natural selection if it occurs between unrelated individuals or different species. How-

ever, cooperation for mutual benefit, which is distinct from altruism, can evolve among unrelated individuals (Table 1). Many explanations for the evolution of cooperative behavior in social groups, therefore, identify conditions under which cooperating with others carries personal benefits that render it mutualistic. Most simply, cooperation can emerge as a byproduct of a selfish action, as may occur when the waste product of one bacterial species can be metabolized by another. Such byproduct effects may turn out to be common among some bacteria within biofilms (Williams & Lenton, 2008). However, the benefits of byproduct mutualism are probably not sufficient to ameliorate all conflicts among all strains and species living together in a group. Species living in tight association within a biofilm will often compete for limited resources – be it nutrients or space – such that the optimum frequency of one species for the other will be different for each (Foster & Wenseleers, 2006). This may mean that one species evolves to become a strong parasite on the other, as occurs in mixed-species biofilms of *Acinetobacter* sp. C6 and *Pseudomonas putida* (Hansen *et al.*, 2007).

Even when species engage in mutualism, one may expect conflicts whose resolution will, in turn, depend on the ability of each species to coerce the other to its advantage. Equally important are constraints, physiological and otherwise, that prevent attempts of coercion from generating an ongoing arms race that destabilizes the mutualism. Stable mutualisms may often involve species with pleiotropic constraints that link genes for cooperation to personal benefits, such that inactivating a gene for social behavior carries a net fitness cost (Foster *et al.*, 2004, 2007). One example is the mutualism in which *Vibrio fischeri* bioluminesces within the bobtail squid *Euprymna scolopes*. The bacteria gain a comfortable living environment in exchange for their light production, which may render it more difficult for other species to pick out the host squid against a reflective ocean surface background. Because light production carries an energetic cost, it is predicted that *V. fischeri* strains with null mutations in the *lux* genes controlling bioluminescence should outgrow light-producing cells within their squid hosts. However, the squid's specialized light-organ that houses its bacterial symbionts is maintained such that bioluminescence null mutants tend to fair poorly in competition with light-producing cells (Visick *et al.*, 2000).

## Growth rate

Like all other organisms, bacteria can affect each other's reproduction simply by using shared and limiting resources.

By dividing rapidly, a cell line can obtain a larger share of such resources and reduce nutrient availability for other members of the population. Rapid growers deplete nutrient pools not only because they grow more quickly but also because they tend to grow inefficiently, yielding less biomass



per unit nutrient consumed. Somewhat paradoxically, then, fast growth may decrease a biofilm's total productivity due to a trade-off between growing quickly (benefiting oneself) and growing efficiently (benefiting the group).

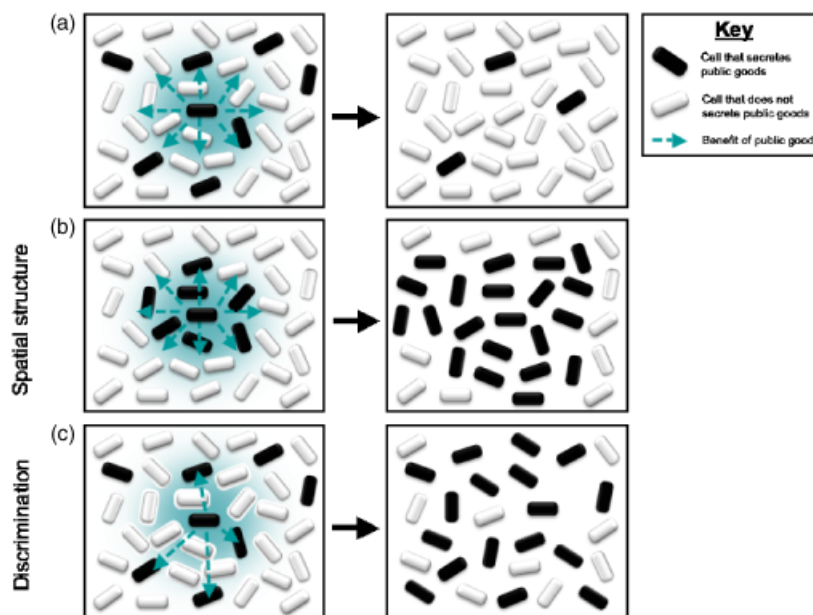
The switch between aerobic and anaerobic metabolism is an illustrative example of the growth rate vs. yield trade-off. Fermentation is a form of anaerobic metabolism that allows cells to grow quickly but at low efficiency (Pfeiffer, 2001; Pfeiffer & Schuster, 2005). In mixed competition between fermentative cells and aerobic cells, evolutionary theory predicts that fermentation may win out simply because it permits rapid growth while decreasing nutrient availability for all other cells in the population. To our knowledge, this prediction has not been directly tested in bacterial biofilms, but an experiment with the budding yeast *Saccharomyces cerevisiae* confirmed that a fermenting strain could out-compete aerobic strains under competition for limited nutrients (MacLean & Gudelj, 2006).

Thus, the frequent use of anaerobic respiration in biofilms may be a signature not only of anoxic conditions but also of conflict between bacteria. Kreft studied this question with an individual-based biofilm simulation and examined competition between a strain that grows rapidly but wastefully and a second strain that grows slowly but efficiently (Kreft, 2004b). The model predicted that the slow-growing high-yield strain could outcompete the fast-growing low-yield strain, but only when biofilms were seeded by a single cell. In the vernacular of social evolution theory, slow-growing high-yield cells are altruists that cooperate with their neighbors by using resources conservatively, increasing nutrient availability for other cells, and allowing the biofilm as a whole to achieve

greater biomass production (Table 1). Biofilms composed solely of slow-growing high-yield cells produce more biomass than biofilms composed solely of fast-growing low-yield cells, but fast-growing cells fare better in direct competition with slow-growing cells within a single biofilm.

Kreft's study illustrates a classic result in sociobiology: altruistic behavior can be successful, but only when it is preferentially directed toward other altruists. Otherwise, exploitative individuals can reap the benefits afforded by altruistic individuals and outgrow them (Box 2, Fig. 3). If altruism is directed toward clones or close relatives, which are more likely than average to share the genes driving the altruistic phenotype, then such genes can increase in frequency in a population (Hamilton, 1964). Natural selection is blind to which individual cells pass on a given set of genes, so long as those genes are replicated and their information content preserved over time. Under some circumstances, a cell's best strategy with respect to its genetic heritage may be to sacrifice itself while benefiting other cells that are genetically identical. Thus, the probability that altruists benefit others with which they share genes is a critical variable controlling the evolution of social behavior. This probability is typically measured as genetic relatedness, where high relatedness more strongly favors the evolution of cooperation and altruism. In biofilms seeded by multiple strains and species, most cells in the biofilm will be genetically different, and slow-growing high-yield strains are open to exploitation by fast-growing low-yield strains. On the other hand, when biofilms are seeded by a single cell, relatedness among cells will be maximal, and altruistic strategies that maximize biofilm productivity are more likely to evolve (Wilson, 1975; Foster, 2004).

**Fig. 3.** The evolution of selfishness or altruism in mixed populations. (a) In mixed populations, individuals that pay a cost to secrete a diffusible public good can be exploited by neighbors that do not secrete public goods and thereby grow faster. (b) *Spatial structure*: even if altruistic cells direct public goods indiscriminately to nearest neighbors, the benefits to clonemates can be high if for any reason a population is structured such that cells of the same genotype tend to be close to each other. (c) *Discrimination*: altruism can evolve in well-mixed populations when cells produce public goods that can only be utilized by other cells of the same genotype.



## Diffusible secreted products

Biofilms comprise not only bacterial cells but also a myriad of compounds that the cells release into their surrounding environment. Many of these compounds are diffusible substances, including digestive enzymes and chelating compounds that aid nutrient acquisition. When these secreted products are costly to synthesize but benefit other cells in the population, they are termed 'public goods' (West *et al.*, 2006). As for restrained growth, evolutionary theory predicts that public good production will be favored when its benefits can be directed toward relatives or clonemates (Fig. 3). In mixed populations, therefore, cells that exploit public goods without investing in them may be able to outcompete public good-producing cells. Griffin *et al.* (2004) confirmed this simple prediction using competition experiments with *P. aeruginosa*, which can grow in iron-limited environments by secreting pyoverdine, an iron-scavenging siderophore. Pyoverdine synthesis is costly, and mutants that do not produce it grow faster than wild type in rich media. In iron-limited media, such mutants dominate mixed cultures by exploiting the pyoverdine secreted by wild-type cells. When growing alone in iron-limited media, however, wild type is far superior to the pyoverdine mutant.

The release of autoinducers by quorum-sensing bacteria may represent another example of bacterial altruism that provides information for the good of a group, while imposing direct costs on cells that secrete autoinducers (Table 1). However, some quorum-sensing systems may instead be driven by the release of cellular waste products rather than dedicated signaling molecules (Miller & Bassler, 2001; Waters & Bassler, 2005; Bassler & Losick, 2006). For example, many species, both Gram-positive and Gram-negative, possess protein machinery required for responding to autoinducer 2 (AI-2); this autoinducer molecule is a waste product that results from the degradation of a key metabolite, S-adenosylhomocysteine (Vendeville *et al.*, 2005). In this case, autoinducer secretion may be tantamount to excretion, and cells are probably not releasing AI-2 to communicate and coordinate their activity with other members of the population (Keller & Surette, 2006). A convincing case for an active coordination system in a biofilm, therefore, requires evidence that cells secrete an autoinducer specifically to elicit a cooperative response from other members of the population, rather than merely to unburden themselves of waste material (Keller & Surette, 2006; Diggle *et al.*, 2007b). In our experience, many find it unnecessary to distinguish between communication by active signaling vs. eavesdropping on a waste product, at least on first thought. However, the difference is critical for understanding whether a bacterial aggregate is behaving as a coordinated group, like a multicellular organism, as opposed to a collection of independent individuals. If a

given quorum-sensing system evolved to benefit a group of bacteria, it would suggest that biofilms formed by the species in question are highly cooperative at the spatial scale over which signaling occurs and, accordingly, that this intercellular communication may involve dedicated regulatory systems that are good targets for biofilm control strategies.

How can one recognize when autoinducer production has evolved, at least in part, to elicit responses from other cells and perhaps to coordinate group activity? One potential indicator is an energetic cost accompanying autoinducer production and secretion. Unless waste product secretions serendipitously possess optimal properties for intercellular communication, natural selection for communication among cells is expected to generate new signal production pathways – or modify existing waste secretion pathways – such that energetic investment is required on the part of secreting cells. Moreover, such signaling costs imply altruism in a group of many cells because little or none of the benefit of releasing a signaling molecule may return to the secreting cell (Table 1).

Identifying any costs of quorum sensing is likely to be challenging when intercellular signaling has evolved via modification of a waste product excretion pathway, as may have occurred for acyl homoserine lactone autoinducers. Nevertheless, a cost to autoinducer production was recently demonstrated in *P. aeruginosa*; wild-type cells that express the autoinducer synthase *lasI* are outcompeted in mixed culture by *lasI* null mutants that exploit the autoinducer molecules released by others (Diggle *et al.*, 2007a). In this example, responding to autoinducer concentrations also entails a cost: *lasR* mutants, which do not possess the receptor required for responding to the *lasI* autoinducer product, outcompete wild type in mixed planktonic culture (D'Argenio *et al.*, 2007; Sandoz *et al.*, 2007). Wild-type *P. aeruginosa* upregulates many costly social behaviors, including the production of siderophores and several extracellular digestive enzymes, in response to high autoinducer concentration. Like the pyoverdine-exploiting strain discussed above, *lasR* mutants fail to invest in the production of extracellular public goods, including proteases that are required for growth in media supplied only with protein substrates. In mixed culture conditions, *lasR* mutants can exploit the extracellular enzymes produced by wild-type cells and outgrow them, which may explain why mutants with *lasR* null mutations often arise spontaneously in the lung infections suffered by cystic fibrosis patients (D'Argenio *et al.*, 2007).

Although some microbial social evolution experiments focusing on public good production were conducted in standing cultures that allow limited biofilm growth on the sides of culture vessels (Griffin *et al.*, 2004; Diggle *et al.*, 2007a), the majority of cells analyzed were likely in the planktonic phase (S. Diggle, pers. commun.). Critically, cells

in shaken or standing liquid media are more mobile and therefore more likely to experience identical conditions than cells in a biofilm environment, which move less and experience different microhabitats. This difference between growth conditions in planktonic vs. biofilm culture may have important consequences for the outcome of social evolution; nevertheless, the study of planktonic cells illustrates that simple changes in strain mixture can strongly favor (clonal culture) or disfavor (mixed culture) the evolution and maintenance of public good secretion.

These results may have implications for bacterial pathogenesis because many extracellular public goods, including extracellular proteases and iron-chelating siderophores, are virulence factors used by pathogens to exploit their host environment and establish infections (Arvidson, 2000; Visca *et al.*, 2007). Indeed, understanding whether biofilms and their associated pathogenesis are a product of competition or cooperation among their constituent cells is central to predicting how strain diversity in a biofilm will affect its virulence. When virulence is the outcome of competition among multiple strains and species within a host, minimizing the diversity of microorganisms contributing to infection will more effectively mitigate their effect on host organisms. However, when virulence is the outcome of cooperation among bacteria, promoting competition among strains and species – for example, by the introduction of probiotics – will tend to decrease the severity of infections (Brown *et al.*, 2002).

### Extracellular polymeric substances

EPS production is a common bacterial behavior that lends biofilms their sticky or slimy nature. EPS may sometimes be a shared beneficial substance that protects biofilms from external threats, such as antibiotic compounds (Mah & O'Toole, 2001), predator grazing (Matz *et al.*, 2005), and cells of host immune systems (Vuong *et al.*, 2004). In many cases, EPS is also important for the structural integrity of biofilms, and EPS null mutants tend to be deficient at biofilm formation (Danese *et al.*, 2000; Hammer & Bassler, 2003; Rainey & Rainey, 2003; Kearns *et al.*, 2005). Specifically, bacteria that do not secrete EPS often cannot bind together efficiently and fail to attach to hard surfaces or to aggregate on liquid–air interfaces (Friedman & Kolter, 2004). When grown in beakers partly filled with liquid medium, EPS-secreting cells of *Pseudomonas fluorescens* cooperate to form a mat at the liquid–air interface, which affords better access to oxygen relative to the planktonic phase below. However, null EPS synthase mutants can exploit the collective support of wild-type cells without paying the cost of EPS secretion, which eventually compromises biofilm structure and sends the whole mat into the anoxic liquid phase below (Rainey & Rainey, 2003).

While EPS secretion may sometimes reflect cooperation among biofilm-dwelling bacteria, indirect evidence suggests that EPS can also mediate competition in biofilms. *Vibrio cholerae* mutants with high constitutive EPS expression rapidly dominate solid-surface biofilms in which they are coinoculated with a strain that produces little or no EPS (Hammer & Bassler, 2003), and hypermucoid *P. aeruginosa* mutants that produce copious EPS consistently evolve and invade chronic biofilm lung infections suffered by cystic fibrosis patients (Martin *et al.*, 1993; Govan & Deretic, 1996). Might EPS production provide a competitive edge in biofilms?

A recent individual-based simulation predicted that EPS secretion allows a cell lineage to push its descendants up to the oxygen-rich biofilm surface while simultaneously suffocating any neighboring cells that do not secrete EPS (Xavier & Foster, 2007). This putative advantage of secreting EPS, however, is only obtained when different strains of bacteria compete within the same biofilm. When biofilms are clonal and natural selection favors behaviors that maximize whole-biofilm productivity, excessive EPS secretion may be disfavored because it decreases the yield of biomass per unit nutrient consumed (Xavier & Foster, 2007) and may generate other negative effects (e.g. overproduction of EPS predisposes biofilms to sloughing and lysis in *Vibrio vulnificus*, McDougald *et al.*, 2006). For EPS-secreting bacterial lineages to outcompete other strains, the biofilms in which they reside together must persist for several bacterial generations: daughter cells of EPS-secreting strains can then benefit from favorable spatial locations and increased nutrient availability afforded by the EPS secretion of parent cells. Because the benefits of EPS secretion are delayed by multiple generations, natural selection may also favor the use of quorum sensing to downregulate EPS secretion at high cell density and redirect resources from EPS secretion into growth before detachment (Pesci *et al.*, 1997; Hammer & Bassler, 2003; Nadell *et al.*, 2008). *Vibrio cholerae* appears to use this strategy to disperse from its host's intestinal environment (Hammer & Bassler, 2003; Zhu & Mekalanos, 2003; Liu *et al.*, 2007).

### Genetic exchange in biofilms

The closely packed environment in biofilms makes them ideal candidates for genetic exchange among cells (Sørensen *et al.*, 2005), and in fact some conjugative plasmids appear to carry genes that promote biofilm formation (Ghigo, 2001). *Vibrio cholerae* becomes naturally competent in biofilms growing on chitin (Meibom *et al.*, 2005), and the gene encoding cholera toxin, a key virulence factor in cholera infections, is transferred among cells by phage (Waldor & Mekalanos, 1996). Under low-nutrient conditions, the spore-forming bacterium *Bacillus subtilis* also

becomes naturally competent via a set of processes that pull DNA into the cell (Chen *et al.*, 2006) and activate genes responsible for homologous recombination (Dubnau *et al.*, 1973). *Bacillus subtilis* may have evolved this competence response to fish for DNA released from other cells; whether the goal is to gain new adaptive traits, to repair damaged genes, to acquire nutrients, or to perform another unknown function remains to be seen.

DNA release and the activation of natural competence within biofilms suggest that genetic exchange may be an important facet of bacterial group living. How could DNA swapping influence the evolution of cooperation among microorganisms? In some cases, genetic exchange may promote cooperation among cells. For example, when genes for cooperative secreted products are carried by mobile genetic elements, such as plasmids or lysogenic viruses (Waldor & Mekalanos, 1996), DNA exchange between bacteria via conjugation or transduction can increase the frequency of cooperative cells in a population (Smith, 2001). However, gene transfer may also involve conflict. Natural competence in *Streptococcus* species, for example, occurs alongside the activation of multiple toxin–antitoxin systems that selectively lyse surrounding cells that are not competent (Claverys & Håvarstein, 2007; Claverys *et al.*, 2007). Indeed, conflict can occur at an even lower level of organization if mobile genetic elements replicate at the expense of cells in which they reside (Paulsson, 2002). Recent evidence suggests that some transposons may replicate so quickly relative to their hosts that they can drive whole bacterial lineages extinct (Wagner, 2006).

### Differentiation and development in biofilms

We have discussed evidence that bacteria slow their growth to use resources efficiently and secrete molecules that benefit other cells in their surroundings. These cooperative behaviors are often regulated by costly quorum-sensing systems that may reflect communication and coordination, at least on the spatial scale of a few cell lengths. Although impressive for organisms that were once considered largely solitary, these examples still do not approach the sophistication of a multicellular animal or plant, in which many types of differentiated cells communicate across the whole organism to produce a highly integrated functional unit. Do bacterial aggregates ever reach comparable sophistication? Coordinated phenotypic diversification may carry advantages for bacterial groups, including insurance against uncertainty of future environmental conditions (Kussell & Leibler, 2005), or a division of labor in which cells specialize on single tasks to increase the entire group's productivity (Michod & Roze, 2001). Is there evidence that bacterial differentiation represents an adaptation for the benefit of whole biofilms?

One example of bacterial differentiation that may represent both a division of labor and insurance against environ-

mental uncertainty is the formation of dormant persister cells. Subpopulations of persister cells have been observed in planktonic cultures and within the biofilms of pathogenic bacteria (Brooun *et al.*, 2000; Spoering & Lewis, 2001). Persister cells are immune to many antibiotics and are thought to protect a cell lineage from large-scale disturbances that induce mortality in the growing population (Lewis, 2007). Theoretical work suggests that persister formation requires a degree of global cooperation because rapidly dividing strains that do not differentiate into persisters can readily replace strains that curtail growth in favor of stress tolerance (Gardner, 2007). A related finding from *P. aeruginosa* is the observation that several phenotypically distinct lineages often arise from a single clone during biofilm formation (Boles *et al.*, 2004). These diverse phenotypes are heritable, and their appearance depends on the expression of RecA, a DNA repair enzyme that catalyzes recombination. In addition, the *P. aeruginosa* variants that emerge during biofilm growth appear to be functionally specialized for either dispersal or accelerated biofilm formation. Importantly, some variants are more resistant to oxidative stress and can thus protect a cell lineage from large-scale disturbances that induce mortality in the growing population.

A second example of cell differentiation found within *P. aeruginosa* biofilms may also represent a division of labor (Klausen *et al.*, 2003a). Under some conditions, surface-attached cells migrate by twitching motility to form clusters and then mushroom-shaped microcolonies. These mushrooms appear to be structurally reinforced by extracellular DNA, which is putatively released by quorum-sensing-mediated autolysis of a subpopulation of cells (Allesen-Holm *et al.*, 2006). Meanwhile, another subpopulation in the cap of the mushroom expresses the *pmr* operon in response to some antimicrobials, which increases their stress tolerance via cell wall modifications (Haagensen *et al.*, 2007). Of course, this apparent division of labor occurs in the presence of steep chemical gradients that may induce cells to activate different metabolic pathways (see section 'Biofilm complexity without coordination', Pamp *et al.*, 2008). More work is required to determine whether differentiation by *P. aeruginosa* is an example of global coordination or another instance of cells simply optimizing their behavior to local conditions.

Among bacteria observed thus far, arguably the most complex social behaviors occur in two spore-forming species: *B. subtilis* and *Myxococcus xanthus*. Aggregation by these species comes closest to multicellular development: sporulation in both results from a series of temporally separated checkpoints that produce a predictable, directional sequence of differentiation events (Monds & O'Toole, 2008). *Bacillus subtilis* also provides a dramatic example of bacterial differentiation; populations of a single strain

display bimodal distributions for multiple phenotypes, including competence, chaining (Losick & Desplan, 2008), motility (Kearns *et al.*, 2004), EPS production (Vlamakis *et al.*, 2008), and spore formation (Branda *et al.*, 2001). A subpopulation of cells in wild *B. subtilis* biofilms produces spores in small fruiting body structures that develop at the tips of biofilm surface irregularities. Larger groups of spores are formed by *M. xanthus*, in which starving cells aggregate to form a fruiting body whose cell density approaches that of many biofilms (Julien *et al.*, 2000). It is also striking that in both species spore-destined cells kill other members of the population during fruiting body formation (Wireman & Dworkin, 1977; Gonzalez-Pastor *et al.*, 2003). In *B. subtilis*, cell death releases nutrients that increase the viability of the remaining population, allowing it to delay commitment to sporulation (Gonzalez-Pastor *et al.*, 2003). This violent ending might therefore represent altruistic sacrifice by cells that lyse, similar to programmed cell death within a developing multicellular organism. Alternatively, cell death in *B. subtilis* and *M. xanthus* biofilms may be the signature of strong evolutionary conflict in which different strains compete to become spores. Consistent with this latter interpretation, different environmental *M. xanthus* strains can exploit and poison one another (Fiegna & Velicer, 2005), and toxin secretion is a well-known strategy that bacteria use to compete with other strains and species (Durrett & Levin, 1997; Cascales *et al.*, 2007).

## Future work

Our perspective highlights four major questions that will help us to better understand and predict the behavior of bacteria in biofilms.

### Function: what are the constituent genes and phenotypes that define the biofilm?

Before studying biofilm-associated behaviors, one must obviously identify them. Our understanding of biofilms will continue to rely on microbial genetics, which dissects biofilms into discrete behaviors and the genes that control them (Stoodley *et al.*, 2002; Hall-Stoodley *et al.*, 2004; Beloin & Ghigo, 2005). Genetic manipulation is critical for identifying traits associated with biofilm formation, such as the synthesis of adhesion factors and extracellular products, and for uncovering their relationship with the virulence and antibiotic tolerance of pathogenic bacteria. Bacterial genetics also reveals the mechanisms by which biofilm-associated behaviors are regulated, which partially determine whether it is appropriate to draw comparisons between biofilm growth and multicellular development. For example, the discovery of hierarchically ordered gene expression circuits that direct a series of irreversible biofilm growth steps might reflect a system of cooperative and coordinated compo-

nents, comparable to a metazoan zygote (Monds & O'Toole, 2008). Finally, only by finding the genes that drive biofilm formation can one assess the evolutionary forces acting upon biofilms through the comparison of sequence variation within and between species (Smith *et al.*, 2005).

### Coordination: are biofilms orchestrated communities?

We have seen that complex biofilm structures may arise from the uncoordinated behavior of many cells responding to variable microenvironments. This observation, in turn, suggests that any explanation of biofilm structure that assumes whole-biofilm coordination must be tested against the more parsimonious hypothesis that biofilm structure does not strictly depend on intercellular communication. Comparing alternative models of biofilm formation will benefit from a close interaction between laboratory experiments and theoretical approaches that allow rapid *in silico* manipulation of bacterial behaviors and environmental conditions. Such an interaction between theory and experiment may be helped by recent microfluidic chamber techniques, with which nutrient gradients and the density and position of bacteria can be manipulated at a high spatial resolution (Keymer *et al.*, 2006). How can these techniques be used to identify instances of coordination among bacteria? A promising direction is to further explore examples, such as *B. subtilis* competence, in which differentiation into multiple cell types occurs in the absence of nutrient gradients but *does* require quorum-sensing systems and high population density (Dubau & Losick, 2006). Another enduring question concerns how biofilms achieve stability in the face of disturbances, such as physical trauma or antimicrobial treatment. Do the robustness and adaptability of biofilms rest upon coordination and cooperation among bacteria, or can they arise among cells that are merely competing to grow as quickly as possible (Bonabeau *et al.*, 1999)?

### Cooperation: what are the costs and benefits of biofilm-associated behaviors?

Understanding the evolution of biofilm-associated behaviors requires that we identify which cells are affected by each behavior. The two key questions are (1) what are the costs and benefits of a behavior to the cell that expresses it and (2) what are the costs and benefits of a behavior to cells other than the individual that expresses it? Answering these questions will require biofilm competition experiments with isogenic strains that differ only at the loci controlling a social behavior of interest. Focusing on one or a few social behaviors at a time – and on all parties affected by each social behavior – is required to determine whether such behaviors truly represent the evolution of cooperation

(Table 1; Box 2). On the other hand, mixed-species biofilm observations can be difficult to interpret. Showing that a two-species biofilm is more than twice as productive as either of the corresponding one-species biofilms, for example, does not prove mutual cooperation; it may instead reflect exploitation of one species by the other (Hansen *et al.*, 2007). Assessing the costs and benefits of quorum-sensing and biofilm-associated behaviors will also inform our distinction between uncoordinated and coordinated bacterial aggregates, for which the difference between eavesdropping and altruistic signaling is so important. Understanding the fitness effects of biofilm-associated behaviors may also reveal the Achilles heel of pathogenic species, which often rely on secreted enzymes and nutrient-sequestering compounds for their virulence. Theory predicts that altruistic traits will be the most unstable to social manipulations, such as the introduction of probiotic bacterial species in the digestive tract (Saxelin *et al.*, 2005).

### Ecology: which strains and species are in the biofilm, and how are they arranged?

A better understanding of the natural ecology of biofilms is required to assess how the costs and benefits of bacterial behaviors measured in the laboratory translate into fitness effects. A first step is to identify all the species and strains within natural biofilms, for which culture-independent techniques such as phylochips (DeSantis *et al.*, 2007) and high-throughput sequencing show promise. Such coarse-grained diversity measurements provide a useful first proxy of the potential for cooperation within bacterial groups: the higher the proportion of a single strain within a biofilm, the more likely cooperative behavior can evolve and remain stable against exploitative mutants. Assaying diversity across time and space is also important, however, because strong initial competition may whittle down an initially diverse biofilm to a few species that then cooperate (Cascales *et al.*, 2007). In addition, cell lineages may partially segregate within biofilms simply because cell division and limited movement tend to produce clonal clusters (Kreft, 2004b; Xavier & Foster, 2007). Under such conditions, the evolution of public good secretion may occur more easily and remain more stable than in mixed culture, where cooperative cells are readily susceptible to exploitation (Griffin *et al.*, 2004; Diggle *et al.*, 2007a; Sandoz *et al.*, 2007).

### Conclusion

The ecological success of biofilms is underlined by their resilience in the face of numerous challenges, be they the shear forces of a river current or the roaming macrophages of a host's immune system. It is not yet clear whether biofilms' structural properties and capacity for perseverance rely on organization and harmony among the cells that

reside in them. While some biofilms may contain differentiated cells that work in concert, many are likely to be dominated by bacteria that simply compete to gather limited resources and to divide as rapidly as possible. Understanding the extent of coordination and cooperation in biofilms is paramount to building a complete picture of how biofilms form and how to manipulate them. While biofilms are a highly visible hallmark of bacteria sociality, the application of concepts from sociobiology to biofilm research is only now beginning.

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### Authors' contribution

C.D.N. and J.B.X. contributed equally to this work.

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