Spatial Structure, Cooperation, and Competition in Biofilms

Carey D. Nadell[1], Knut Drescher[1], Kevin R. Foster[2]

[1] Max Planck Institute for Terrestrial Microbiology, Marburg D-35043, Germany
[2] Department of Zoology, University of Oxford, Oxford OX1 3PS, UK

Correspondence to: kevin.foster@zoo.ox.ac.uk; carey.nadell@mpi-marburg.mpg.de

Abstract

Biofilm formation, in which cells form matrix-enclosed communities, is a major mode of microbial life. The study of biofilms has revealed vast complexity both in terms of their resident species composition and phenotypic diversity. Despite this complexity, theoretical and experimental work in the past decade has identified common principles for understanding microbial biofilms. In this Review, we discuss how the spatial arrangement of genotypes within a community influences the cooperative and competitive cell-cell interactions that define biofilm form and function. Furthermore, we argue that a perspective rooted in ecology and evolution is fundamental to progress in microbiology.

Introduction

Microorganisms frequently live in dense and diverse communities, termed biofilms, which can be surface-bound or free-floating and are usually encased in a secreted polymer matrix[1,2]. Biofilms are indispensible to global biogeochemical cycling[3,4] and to the normally functioning microbiota of multicellular organisms[5]; more troublingly, they cause devastating antibiotic-tolerant infections[6] and destroy surfaces and flow systems in medical and industrial settings[7-9].

Biofilm-dwelling cells interact intimately and influence each other's evolutionary fitness via a wide range of social phenotypes (Box 1)[10,11]. Many of these behaviors are simple forms of cooperation that benefit neighboring cells, such as secreted nutrient chelators[12,13], digestive enzymes[14], surface adhesins[15], wetting agents[16], structural polymers[17], and signaling molecules[18-20]. For example, the pathogen *Vibrio cholerae* forms biofilms on environmental particles of the structural polymer chitin, which it digests via communally beneficial chitinases[21,22]. Diverse biofilm-dwelling bacteria also produce siderophores, which bind and solubilize otherwise inaccessible iron, a frequently limiting nutrient in the abiotic environment and within host organisms[12,23]. Biofilms achieve much more through cooperative action than single cells can alone, including increased resilience against external threats and efficiency in digesting complex nutrient sources[22,24-27]. Microorganisms are thus fundamentally social organisms, and their cooperative behaviors are pivotal to how they affect the world around them.

However, social interactions can also be competitive, and cells within a microbial community should not be assumed to work together harmoniously[11]. Competition for limited space and resources is pervasive[28-30], and many social phenotypes serve as weapons that harm other strains and species. Antibiotic secretion, direct injection of toxins into adjacent cells, and mechanisms for displacing or suffocating neighbors[31-34] all target competitors for elimination and can substantially alter biofilm composition[35,36]. *P. aeruginosa*, for instance, engages in bouts of Type 6 secretion system (T6SS) attack specifically in response to T6SS-mediated antagonism from other bacteria[35,37]. *V. cholerae* and *P. fluorescens* produce extracellular matrix materials that give secreting cells a positional advantage over competitors, which are physically displaced[33,38] or cut off from nutrient access[39].

The spatial arrangement of different strains and species within biofilms strongly influences the relative benefits of cooperative and competitive phenotypes. Furthermore, by altering the reproductive rates of neighboring cells, social phenotypes can cause compositional and structural changes in microbial communities, which shape their overall

function and – in the case of pathogens – their virulence[40-42]. In order to understand microbial communities, therefore, we must consider the balance of cooperation and competition within biofilms, and how this balance influences their macroscopic properties. This goal poses significant difficulties. Biofilms are complex, often heterogeneous systems that emerge from an interplay of many physical forces and local interactions among cells[43,44]. Nevertheless, a growing body of theoretical and experimental literature has begun to dissect the intricacy of biofilms and to identify general rules of cell-cell interaction within them. In this Review, we discuss these recent findings, focusing on the central importance of spatial structure for understanding and predicting microbial social behaviors.

How spatial structure affects microbial social interaction

Microbial communities can contain hundreds of strains and species, and we are only beginning to understand how and why different genotypes arrange themselves in space. Patterns of immigration can establish structure in nascent biofilms, as can spatial heterogeneity in environmental stress, predation, nutrient availability, and suitable attachments sites (reviewed in Ref. [45]). As surface-adhered cells grow, divide, and interact with each other, the structure of their emerging community may change, sometimes quite dramatically. An initially disordered mixture of strains and species, for instance, can become highly structured such that the final community contains large single-genotype patches spanning many cell lengths (**Figure 1**).

In general terms, a social phenotype will be favored or disfavored by natural selection depending on its costs, its effects on other cells, and the genotypes of the affected cells (**Box 1**). In biofilms, the last factor – namely the genotypes of cells that are most strongly affected by a social phenotype – is strongly determined by spatial structure, because microbial social behaviors typically have the greatest influence on immediate neighbors[10,46]. How cells are arranged in space is therefore critical to whether competitive or cooperative interactions are advantageous in a given environmental context[47,48]. Understanding the spatial structure of biofilms and how it affects the evolution of social phenotypes (and *vice versa*) often requires specialized computational models (Box 2)**.** To summarize this literature with an intuitive guide, we consider three key scenarios of spatial structure within biofilms and their relationship to patterns of competitive and cooperative behavior (**Figure 1**).

First, cells may be dispersed at low density on a surface, such that they are essentially solitary (**Figure 1A**). While important as an early phase of biofilm growth, this scenario generally disfavors the expression of social phenotypes, many of which are likely to have

evolved to influence nearest neighbors (but see Ref. [49] for a recent example of long-range interactions). Cooperative and antagonistic phenotypes can have the strongest impact on evolutionary dynamics when population density is high enough for cells to affect each other, either through direct contact or the release of diffusible substances. This Review focuses on such high-density conditions, where cell lineages (i.e., different mutants, strains, and species) may be segregated (such that cells primarily interact with their own genotype, **Figure 1B**) or mixed (such that multiple genotypes interact with each other, **Figure 1C**). We first address how shifts between these regimes of spatial structure are expected to impact the evolution of cooperative and antagonistic phenotypes. We then examine how spatial structure influences the regulation of these social phenotypes and how microbial social interactions can, in turn, feed back onto and alter population spatial structure.

Spatial segregation: benefits within genotypes

Due to the constraint on movement that is common in biofilms, clonal clusters can be generated passively – that is, without active adhesion or aggregation among clonemates – as cells grow and divide. This phenomenon has been observed *in silico*, *in vitro*, and *in vivo*[20,38,50,51] and causes clonal patchiness as a function of surface colonization, birth, death, and dispersal rates[52]. Even when different strains or species are initially well-mixed, populations that grow toward a limited nutrient pool often experience strong spatial bottlenecks as some cell lineages are cut off from access to the actively growing front[53,54] This process, referred to as gene surfing or spatial genetic drift, induces population subdivision into monoclonal sectors[55,56] and has been documented in agar colonies of *Escherichia coli*[55], *Bacillus subtilis*[57], *P. aeruginosa*[54], *Saccharomyces cerevisiae*[55,58,59], and *Dictyostelium discoideum*[60].

Spatial structure can be critical to cooperation within species and the evolution of simple public goods, which many microorganisms require in order to take advantage of nutrient reservoirs in their natural habitats. For example, pathogens and saprophytes harvest tissues that are composed of large polymers, which must be digested into soluble components by secreted enzymes before they can be imported and catabolized[61]. *Clostridium difficile* uses secreted enzymes to digest host connective tissue[62], and numerous bacteria[63,64] and fungi[65] produce exoenzymes to digest cellulose, the ubiquitous plant structural compound. The nutrients released by extracellular enzyme activity are potentially available for uptake by nearby cells, a principle that extends to other secreted compounds, including nutrient-chelators and communal adhesins[12,23,40,66,67]. These behaviors result in public good dilemmas:

the public good-producing behaviors can be eliminated or reduced in frequency by cheating mutants that no longer invest in the group-beneficial trait but nevertheless reap the rewards of others' investment[18,68] (**Box 1**). Public good production and exploitation have been most heavily explored in laboratory settings, but recent work suggests they are important in clinical[69,70] and natural environments[23] as well. The latter study used a bioinformatic and phylogenetic analysis of wild *Vibrio* populations and observed frequent loss of genes for production of iron-chelating siderophores, but not the loss of the corresponding cognate receptors, consistent with a producer-cheater dynamic for siderophore secretion[23].

When costly to produce, the evolutionary fate of secreted cooperative compounds depends on the ability to benefit clonemates rather than competing strains and species [10,67]. This will depend on how far the secreted public good travels, which is affected by its production, uptake, decay, and transport[10,46,71,72]. However, when the spatial scale of public good sharing is similar to the spatial scale on which clonal clustering occurs , public goods dilemmas can be resolved[14,46,71,73,74]. . Clonal clustering thus tends to promote the evolution of public good production[10], so long as the public good in question does not rapidly diffuse throughout the system[20,22]. The logic of this prediction dates back to the birth of social evolution as a field and was originally conceived with animal behavior in mind[48], but it is also upheld for microbial systems with cooperative phenotypes[75]. For instance, extracellular digestive enzyme production is more strongly favored as clonal cluster size increases in biofilms of *Vibrio cholerae* on chitin particles[22], which this organism digests using secreted chitinases. Similarly, competition experiments performed on agar plates have demonstrated that siderophore secretion by *P. aeruginosa* is more strongly favored as agar concentration is increased, which decreases public good diffusivity and limits the receipt of cooperative help to neighboring clonemates[74,76]. When *P. aeruginosa* is grown on glass, siderophore exchange becomes limited to direct neighbors, which in combination with local clonal clustering virtually prevents exploitation by cheating mutants[77]. Finally, recent work using colonies of *Bacillus subtilis* showed that stronger cell lineage segregation favors the secretion of extracellular polymer that cooperatively aids cells in spreading along agar surfaces[57,78].

Computational simulations of biofilm growth (**Box 2**) predict that the lineage segregation that occurs in expanding populations can dramatically favor neighbor-benefiting behaviors by generating clonal clusters on large scales relative to diffusion of cooperative secreted compounds[46,53,60,79] (**Figure 2A**). This prediction has been upheld by experiments using different cooperative phenotypes and model organisms, including yeast[58] and bacteria[57,80-83] (**Figure 2B**). By contrast, the clonal clustering that emerges spontaneously due

156 to spatial genetic drift can destabilize cooperation between different strains or species by
157 separating the mutually beneficial partners from each other (see below)[75].
158 Many complementary studies to date show that spatial segregation of cell lineages in
159 biofilms increases the frequency of interactions between cells of the same genotype;
160 generally speaking, these conditions favor investment into cooperative behaviors that
161 heighten the ecological productivity of clonal patches and, as a result, the biofilm as a whole.
162
163 ***Spatial mixture: conflict between genotypes.***
164 Although clonal clustering occurs readily in biofilms due to limited movement, it is not
165 universal. Cell lineages may become spatially mixed for many reasons, including frequent
166 dispersal and recolonization, diffusive cell motility, and homogeneous nutrient abundance[53].
167 When multiple strains and species encounter each other often, the default expectation is that
168 competitive phenotypes will predominate, as the primary action of natural selection is to
169 favor genetic lineages that benefit themselves over others[48,84-86]. Such competition has led to
170 the evolution of diverse competitive strategies, which range from rapid growth and resource
171 acquisition[87] to the use of adhesion and matrix production to reach the best nutrient-rich
172 locations within biofilms (see below)[33,88]. Perhaps the clearest incarnation of competitive
173 strategies, however, is the secretion of broad- and narrow-spectrum toxins, coupled with
174 privatized anti-toxins that prevent self-poisoning[89].
175 A common example of such competitive strategies is the production of antibiotics and
176 bacteriocins, which is widely documented in microorganisms[32] and has been studied for some
177 time in the theoretical ecology literature[90]. While it has been suggested that antibiotics can
178 function as cooperative signals at sub-inhibitory concentrations[91], the evolutionary basis for
179 this idea is unclear, and parsimony suggests that their primary role is to kill competitors[92,93].
180 Most simply, antibiotics – and other secreted toxins – benefit the lineages that possess toxin
181 resistance by eliminating cells that do not. Lysed neighbors may also be directly harvested
182 for raw materials, including their genetic content[94]. Theory predicts that microbial poison-
183 secretion strategies will be most strongly favored when competition for resources is localized
184 and competing cell lineages are moderately well mixed in space[41,95,96]. When community
185 mixture is too high, each toxin-secreting strain's density may be too low to launch an
186 effective attack. By contrast, when communities are clonally segregated there may be no cells
187 of other genotypes in the vicinity for toxin-secretors to target. Indeed, simulations and
188 experiments show that when cell lineages are segregated, toxin-sensitive species readily
189 coexist or even outcompete toxin-secretors within the same biofilm[90,96-99] (**Figure 2C-D**).

Though classical antibiotics and bacteriocins are secreted into the extracellular space[32], other toxins are directly placed into or onto neighboring cells via Type 5 secretion systems (T5SSs; responsible for contact-dependent inhibition) or Type 6 secretion systems (T6SSs, which are derived from contractile phage tails[36,100]). *Bacteroides fragilis*, a common symbiont of the gutmicrobiome, uses T6SSs to compete and persist in the mammalian intestine, in a manner predicted to be dependent on spatial genotype mixing[101]. The opportunistic pathogen *Proteus mirabilis*, on the other hand, also expresses a T6SS along with the motility machinery needed for collective movement on agar surfaces[102]. When isolates with incompatible T6SSs encounter each other, the mutual killing generates clearance zones (Dienes lines[103]) on the border of their adjacent swarming colonies. In this manner, *P. mirabilis* appears to deploy a preemptive attack against susceptible competitors as it prepares to migrate. This behavior has the notable effect of maintaining the clonal structure of a growing cell cluster; many social phenotypes, in fact, have a strong reciprocal influence on spatial structure, which we discuss in the last section of this review.

Spatial mixing: benefits between genotypes.

While antagonism between strains and species is common[48,84-86], spatial population mixing also allows cells to receive benefits from other strains or species[75] (**Figure 1C, Figure 2E-H**). In the simplest cases, such benefits are unidirectional: cells of one genotype release a factor that benefits another genotype, receiving nothing in return. *Bacteroides* spp., for example, digest host-ingested polysaccharides and can secrete acetate as a metabolic waste product. This is used as a carbon source by other members of the microbiome that do not, as known at present, produce anything useful in return[104]. When the released factor is costly to produce (i.e., is not simply a waste product, Box 1), the recipient of such unidirectional benefits is essentially a cheating strain, as discussed in the previous section. A recently introduced idea, qualitatively similar to cross-species cheating, is that of black queen evolution, in which one species survives the loss of a catabolic capacity because another species in the vicinity leaks complementary metabolites into their shared environment[105]. This process is thought to have occurred for the marine bacterial group *Pelagibacter ubique*, which depends on reduced sulfur released by co-habiting plankton[106]. Cheating and black queen effects both rest on sufficiently high cell density to generate usable concentrations of the exchanged compound, and on sufficiently mixed community structure in which recipient cells can access the compounds released by producers[107,108].

Spatial mixing of cell lineages can also allow for reciprocal benefits and the evolution of cooperation between species[75,108,109] (**Figure 2E-H**). A potential evolutionary trajectory to such mutualisms is through syntrophic relationships[110], in which a waste product of a first species renders a core metabolic reaction thermodynamically unfavorable. If this waste product also serves as a nutrient source for a second species, the latter species can, by absorbing the waste product, help the first species to grow[108]. This kind of interaction occurs within oil-degrading microbial communities; the recently-sequenced *Desulfatibacillum alkenivoransi* can metabolize alkanes when paired with *Methnospirillim hungatei* JF-1, which absorbs the hydrogen and formate released by *D. alkenivorans*[111]. This form of exchanged benefit might emerge whenever two species with complementary pre-evolved metabolic profiles are in close proximity, and it is particularly evolutionarily stable because it does not require either species to pay a cost for the sake of the other (**Box 1**).

In principle, between-species cooperation that requires costly investment from each party may also arise, including cross-feeding partnerships where metabolites released by one species mitigate the auxotrophy of another, and *vice versa*[112]. Several groups have synthetically constructed obligate mutualisms of this kind, including a pair *E. coli* amino acid auxotrophs that complement each other in co-culture[113]. Recent work has also found evidence for evolved cooperation between *Bacteroides* species in the human gut[114], but the wider prevalence of cooperation between species remains to be determined. Importantly, both cross-feeding and syntrophy can also represent commensalism, or even mutual exploitation, depending on byproduct consumption rates and the extent of interspecific competition among interacting partners[107,115]. Theory and experiments with synthetic systems agree that some mixing of cell lineages is essential for mutualisms to evolve[75,108,116] (**Figure 2F-I**). On the other hand, overly homogeneous mixing can undermine mutualistic interactions by exposing them to cheating genotypes, or to passive genotypes that neither benefit from nor contribute to the mutualism but "socially insulate" mutually beneficial partners from interacting with each other [59,75,107].

In sum, spatial mixing of genotypes can favor strong antagonism, as is widely seen in antibiotic warfare. However, lineage mixing also enables dependencies to evolve between strains whereby one uses the beneficial products of another. Under specific conditions, these dependencies may further evolve into mutualistic cooperation. However, too much spatial genetic mixing can compromise between-genotype cooperation due to cheating and social insulation.

Spatial structure and the regulation of microbial social behavior

We have so far discussed cooperative and competitive traits within biofilm communities as though they are expressed constitutively. In reality, social phenotypes are often strictly regulated in response to biotic and abiotic inputs. The evolution of these regulatory strategies ultimately depends on how the costs and benefits of a particular trait change as a function of a cell's chemical and biological environment.

Cutting the cost of social phenotypes is among the broadest principles underlying their regulation. *P. aeruginosa*, for example, controls the synthesis of the iron-scavenging molecule pyoverdin according to iron availability in a manner that minimizes its marginal production cost[117,118]. Pyoverdin is durable over multiple bacterial generations, and *P. aeruginosa* reduces its investment into pyoverdin secretion as the compound accumulates locally, again reducing its trans-generational expense and rendering it difficult to exploit by non-producers in realistic settings[118,119]. *P. aeruginosa* also secretes copious rhamnolipid surfactants, which are thought to aid both motility and resource acquisition at the edge of expanding colonies. Even though rhamnolipid production involves substantial resource allocation, its mode of regulation results in little negative impact on cell division rate: rhamnolipids are only synthesized by cells with access to more carbon than they need for growth[16]. This strategy of metabolic prudence appears to operate for many secretion phenotypes, which can prevent the evolutionary invasion of non-producing mutants[16,120].

In addition to reducing their cost burden, the regulation of social traits can also increase the likelihood that their associated fitness effects are delivered to the appropriate target cells. As discussed above, the evolutionary fitness consequences of a particular secretion phenotype depend heavily on whether there are other cells nearby, and their genetic identity. Consequently, natural selection can be expected to favor regulatory networks that predict both the density and identity of cells in the vicinity[121], i.e., that differentiate the population structure scenarios described in Figure 1. Two of the most common avenues of information cells use to distinguish biofilm spatial structures include molecules that correlate with cell density and environmental stressors.

Many cooperative secretion phenotypes fall under quorum-sensing control, a regulatory mechanism involving the secretion, detection, and response to diffusible molecules termed autoinducers[19,122-124]. Quorum sensing has been conceived as a means of assessing local cell population density and of monitoring fluid transport processes in the immediate environment[125]. Theoretical and experimental work shows that these two interpretations are not mutually exclusive[126-128]. Biofilm modeling and experiments with *V.*

291 *cholerae* in microfluidic devices indicate that quorum sensing could also be used to tune the
292 timing of extracellular matrix secretion, which confers an advantage in competition for
293 limited space but reduces dispersal ability[38,129]. Simulations also show that quorum sensing
294 can be used to predict when clonal patches will occur along cell group fronts; this predictive
295 ability can then improve the targeting of public goods to clonemates[130]. However, quorum
296 sensing and the phenotypes it regulates within biofilms are themselves susceptible to
297 exploitation by mutants that either do not produce or do not respond to autoinducers, as has
298 been observed *in vivo* for *P. aeruginosa*[40].
299 Quorum sensing has also been found to regulate competitive traits including
300 bacteriocin production by *Streptococcus* spp.[131,132] and *Lactobacillus* spp.[133]. This is
301 consistent with the logic that toxin-secreting strains can only mount effective attacks at
302 sufficiently high density or restricted fluid transport conditions[121]. Regardless of their
303 population density, toxin-secretors cannot gain a net benefit from their antagonistic behavior
304 without the presence of victim cells to target. This information can be gleaned from many
305 other diffusible cues that are not canonical quorum sensing autoinducers but still correlate
306 with the density of a target cell population[121,134]. For example, *P. aeruginosa* releases the
307 toxin pyocyanin in response to *N*-acetylglucosamine shed from the cell walls of gram-
308 positive bacteria[135].
309 Another mechanism for detecting the presence of competitors is *via* the stresses they
310 induce when they are in close proximity. Such "competition sensing" can manifest as a
311 response to nutrient limitation or, perhaps more reliably, to cell damage[121]. Indeed, anti-
312 bacterial toxin secretion is commonly up-regulated after recognition of stresses associated
313 with competitors (e.g., starvation, cell wall degradation), but not stresses that are strictly
314 abiotic in origin (e.g., heat or osmotic shock). The *P. aeruginosa* T6SS, for example, is
315 activated in retaliation to heterologous T6SS attack from *V. cholerae* and *Acinetobacter*
316 *baylyi*[37]. Similarly, *E. coli* was recently shown to induce the production of reactive oxygen
317 species after exposure to T6SS-mediated aggression or antibiotic attack[136]. Mounting
318 evidence suggests that biofilm production itself confers a competitive advantage to matrix-
319 secreting strains[33,38,39,88], and wild isolates of *P. aeruginosa* up-regulate biofilm production
320 upon encountering bacteriocins secreted by competing cells[92]. A recent study suggested a
321 related mechanism of competitor detection: *P. aeruginosa* up-regulates extracellular matrix
322 secretion and its T6SS after detecting the solutes released by lysed clonemates[137]. This result
323 implies the intriguing idea that bacteria can indirectly sense competitive pressure via the
324 harm that has been done to nearby clonemates, and respond accordingly[134].

Collectively, these studies demonstrate that bacteria experience highly variable chemical and social environments, and their regulatory networks have evolved to make sense of this complexity. Decisions to up-regulate social traits are a function of their costs, their benefits, and whether there are cells in the environment, be they friend or foe, that can be effectively targeted.

How microbial social interaction affects spatial structure

The spatial arrangement of different genotypes within microbial communities is central to the evolution of cooperative and antagonistic phenotypes and their regulatory patterns. Importantly, these phenotypes also feed back heavily onto biofilm structure, creating a mutual dependence between social behavior and biofilm spatiotemporal composition. These feedbacks fall into two general categories. First, any of the cooperative or competitive phenotypes discussed in the previous section can modify neighbors' fitness, indirectly changing population structure by increasing, or decreasing, the local abundance of different strains (**Figure 3**). Second, many microorganisms can modify their interaction neighborhoods via adhesion-driven spatial assortment or the secretion of matrix components that organize biofilm architecture.

Population structuring via neighbor fitness modification. The fields of ecology and evolution have recognized for many years that social interactions influence population structure by locally altering reproductive rate[138-140], and the same principle has been clearly demonstrated in theoretical and experimental work with microorganisms (**Box 2**). Public good secretion, for example, can combine with restricted movement and nutrient limitation to generate patches of a single genotype[53,54,75,141,142]. This effect is partially an amplification of the effects of limited dispersal, but the full picture can be subtler. As discussed above, biofilm growth is often limited to individuals on an advancing front, such that fitness can depend strongly on presence in the front[141]. Public good secretion can allow a cooperative genotype to bloom locally, expand, and propelling itself into the cell group front[55,143,144]. This effect can completely choke off non-cooperating cell lineages from further access to growth substrate, preventing them from replicating for the duration of biofilm growth[10,53] (**Figures 2A-B, Figure 3C**). A recent study that co-inoculated wild type *S. cerevisiae* and an invertase null mutant on agar surfaces provides direct support for this prediction[58]. Invertase digests sucrose at the cell wall into glucose and fructose, both of which can diffuse away from the cell and act as public goods. When the two strains are mixed and spotted on agar, clonal

clustering occurs spontaneously due to spatial genetic drift, allowing wild type invertase secretors to preferentially benefit their clonemates. As a result, clusters of invertase secretors expand more rapidly than those of cheating mutants and eventually dominate the entire colony front[58] (**Figure 2B**).

In addition to public good secretion, antagonism and mutualistic interactions also strongly impact the distribution of genetic lineages within biofilm communities. For example, bacteriocin production and T6SSs can destroy susceptible competitor cells in the vicinity. Some of the earliest experiments exploring antagonistic interactions among bacteria growing on agar surfaces showed local clearance of susceptible cells by bacteriocin-secreting *E. coli*[145]. As a result, inter-strain antagonism can also increase genetic segregation by locally eliminating all but one cell type[96]. This result has the interesting implication that toxin secretion, by reducing the local abundance of other genotypes, breaks down the well-mixed population structure that favored it in the first place (**Figure 3A**). It is perhaps unsurprising then that bacteriocidal toxin secretion is often tightly regulated based on cues of competitors in close proximity (see above).

Mutualistic and commensal interactions between strains or species can have the opposite effect to toxin secretion; theory predicts that lineage mixture increases specifically among those cell lineages that benefit from each other's presence[75,108,109,116,146]. Mutualistic cell types grow faster in proportion to their proximity with each other and can become entangled as they divide, which can even exclude potential cheating strains that do not contribute to the mutualism[75,109,116,147-149] (**Figure 3B**). This theoretical prediction was first experimentally verified using strains of *S. cerevisiae* engineered to behave as obligate mutualists, including an adenine-secreting lysine auxotroph, a lysine-secreting adenine auxotroph, and a cheating lysine auxotroph that secretes nothing[109,116]. In liquid culture the cheating strain can exploit the two mutualists. On solid surfaces, however, colonies of the two mutualistic strains spontaneously interdigitate, spatially excluding the cheating strain and obtaining a collective competitive advantage (**Figure 2G,H**).

Population structuring by adhesion and matrix secretion. Given the strong links between spatial structuring and the outcome of competitive dynamics for social phenotypes, it is not surprising that microbial species have evolved strategies to directly influence population structure. Such active structuring can serve at least two complementary functions. First, it can allow cells to bias their interaction toward preferred partners of the same or other genotypes.

392 Secondly, it can allow cell lineages to collectively alter their location within biofilms and
393 gain optimal access to limited resources.
394 Many examples of genotypic assortment are now known in microorganisms and
395 appear to evolve rapidly under a wide variety of conditions[150]; we focus here on examples
396 that are most relevant to biofilm-like growth (see ref. [151] for a broader discussion). Different
397 cell lineages of *Neisseria gonorrhoeae* can self-assort from initially mixed populations due to
398 variation in the density and post-translational glycosylation of cell surface pili[152]. The yeast *S.*
399 *cerevisiae* associates with cells of the same genotype by flocculation under physical and
400 chemical stresses[153]. The resulting flocs, like bacterial biofilms, are far more resistant to
401 various environmental assaults than individual cells. Yeast cell aggregation occurs based on
402 expression of FLO1, a surface protein that binds to the cell wall of other cells. Cells lacking
403 FLO1 are predominantly omitted from flocs and killed under stressful conditions. In the
404 vernacular of social evolution, FLO1 is a greenbeard gene that identifies copies of itself in
405 other cells and selectively confers a cooperative benefit to them[154].
406 Cells can also increase the chances of residing next to clonemates simply by
407 remaining attached to their progenitors following cell division. Such mother-daughter cell
408 adhesion is pronounced in a number of facultatively unicellular prokaryotes and eukaryotes,
409 and it is widely thought to be a primary driver of evolutionary transitions to
410 multicellularity[155-159]. Natural strains of *S. cerevisiae* form small multicellular clonal clumps,
411 and lab strains that lost this phenotype during domestication can re-evolve it rapidly[160-162].
412 Moreover, clusters of yeast cells are better able to use cooperative digestive enzymes than
413 single cells, which lose the majority of digestion products to the environment[162,163]. Bacteria,
414 too, control their population structure using adhesion strategies and even their cell shape.
415 Numerous species – such as *Anabaena* spp. and *Streptomyces* spp.[159] – perform incomplete
416 cell division to produce multicellular filaments or clusters that confer protection against
417 environmental stresses, especially predation by protists and the phagocytosing cells of host
418 immune systems[164,165]. The lake-dwelling bacterium *Caulobacter crescentus* exploits a
419 surface-adhesive polar holdfast and its curved shape to increase the likelihood that daughter
420 cells are deposited onto substrata directly adjacent to mother cells under fluid flow. This
421 behavior creates a foundation on which clonal microcolonies are subsequently built[34].
422 The secreted matrix, a ubiquitous and defining feature of biofilms, plays a central role
423 in organizing local and global architecture[166] as well as cell lineage spatial arrangement
424 [2,17,167]. Shortly after initiating biofilm growth, *V. cholerae* secretes the matrix protein RbmA
425 to enforce tight binding of mother cells and daughters cells to each other and to the

surrounding polysaccharide matrix[168-171]. Moreover, cell clusters bound by RbmA are guarded from invasion by cells in the surrounding planktonic phase, protecting local genetic similarity within the biofilm[172,173]. In addition to genotypic assortment, a second parallel function of matrix-driven population structuring is to achieve favorable spatial positions within a biofilm community relative to competitors. . Individual based-modeling (**Box 2**) has identified at least two ways by which cell lineages can improve their spatial position in such contexts (**Figure 4**).

Secreting extracellular matrix can expand cell lineage volume more rapidly than cell division alone, placing these cells at the edge of advancing fronts in a manner analogous to plants competing for access to light[174]. This result has been observed experimentally in agar colony biofilms of *P. fluorescens* [39], in which mutants arise that hyper-secrete matrix and position themselves on the outer surface of colonies. Another mechanism for improving spatial position with biofilms is simply through strong adhesion to substrata[33]. This result was observed experimentally within *V. cholerae* biofilms, in which matrix-secreting strains physically displace non-secreting strains from biofilms through increased cell-cell and cell-substratum adhesion[33,38].

The feedback between microbial social behavior and biofilm spatial structure is strongly reciprocal. Phenotypes that help or hurt neighbors can dramatically alter biofilm structure via their effects on local population dynamics. Microorganisms have also evolved to use specialized adhesins and the extracellular matrix to alter biofilms structure directly and thereby tip the balance of social engagements in their favor.

Outlook

The ubiquity of biofilms has dramatically shifted our understanding of microbial natural history[175]. Despite the complexity of biofilm communities, the application of ecological and evolutionary thinking has identified core principles underling many of their key properties and phenotypes. Central amongst these principles is the importance of spatial structuring for how cells interact and shape biofilms.

However, significant challenges remain. Studies of spatial organization in microbial communities have mostly relied on laboratory assays that do not closely replicate natural environments[176]. Advances in microfluidics and microscopy, including single-cell imaging of biofilm-dwelling bacteria[166], have greatly improved our ability to study complex biofilm microhabitats[44,177]. Yet we know relatively little about the spatial details of cell-cell interactions in ecologically realistic settings (see references [45,50,178-180] for new strides on this

front). Several important questions thus remain to be answered. For example, how common are competitive versus cooperative phenotypes in nature? What are the typical spatial structures of different strains and species within biofilms in soil or on host epithelia? Studies of microbial consortia in natural settings have been revolutionized by metagenomics, but this approach (by necessity) largely ignores the small spatial and temporal scales on which microorganisms interact with each other. We need new theoretical and experimental tools to determine how the ecological and evolutionary dynamics that occur within biofilms relate to the compositional changes in community structure revealed by metagenomics.

Biofilm communities affect many aspects of our lives. They can be devastating as agents of infection and industrial contamination, but highly beneficial in their contribution to healthy microbiota, biogeochemical cycling, and bioremediation. Understanding how to disrupt, or promote, the function of microbial communities is a priority for modern microbiology. What, then, is the key to making or breaking a productive biofilm? We predict that clonal patchiness will often increase ecological productivity by stabilizing local cooperative traits and limiting the damage from antibiotic warfare. Conversely, spatial mixing of genotypes will shift the system towards antagonism by placing competing strains next to one another. The exception is when cooperation between species is essential for biofilm community functioning, in which case spatial genotype mixing can promote productivity. Highly connected networks of mutualism may be unstable, however, because the loss of a small number of species can compromise the whole community[181]. How to shape microbial cooperation in order to control both productivity and stability is a fundamental question for the future.

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Text boxes

Box 1: Social Evolution: what is a "social" phenotype?

The goal of social evolution theory is to explain phenotypes that evolved to exert fitness effects on individuals other than the actor. The field first developed in the context of animal behavior, seeking to explain now-famous examples of behavioral interaction, such as self-sacrificial cooperation within honeybee societies and intense male-male competition among polar bears. However, it is now clear that social phenotypes are important in all living systems, including microbial communities. The key determinants of social evolution are the fitness costs of a phenotype to the actor, its fitness effects on recipients, and the genetic identity of recipients[48,73,182-184]. The third factor is often phrased in terms of a "relatedness", which refers broadly to the genetic similarity of an actor and recipient, relative to the population average[48,73,185,186]. In asexual microorganisms, relatedness and the balance of social evolution within a species can often be reduced to a simple genotypic view of microbial interactions: *if cells have the same genotype at the locus defining a social trait, then cooperative interaction is favored, but if they are of a different genotype, then competition is usually favored*. Cooperation can evolve between genotypes, particularly between species that do not compete for resources, but the conditions are much more restrictive than for cells of one genotype[107,187].

An important limitation to the social evolution approach is that it can, by design, only explain and predict phenotypes that evolve *because* of their social effects on recipients. It is not always easy to empirically resolve the distinction between social phenotypes that have evolved to influence other individuals, as opposed to asocial phenotypes that incidentally affect other individuals. However, there are common signatures of social phenotypes: they are often energetically costly to a cell because investment in a social trait can direct resources away from other functions[11]. Linked to this, many social phenotypes are regulated in response to the density and the composition of the local population (see main text). Social phenotypes' fitness benefits also depend on the presence of suitable target cells. A bacteriocin-secreting strain, for instance, suffers a net fitness loss from bacteriocin secretion if there are no susceptible target cells in the vicinity. On the other hand, in all communities, individuals coincidentally influence each other's fitness due to asocial traits that evolved without regard to their effects on con- and hetero-specifics. In microbial groups, this phenomenon can manifest as one strain producing metabolic waste products that may benefit (e.g., by providing a new nutrient source) or harm (e.g., by changing environmental pH) other strains. The secreting cell benefits from releasing its waste product regardless of whether other cells are affected. While such accidental effects can be important for understanding a given community, they are not formal examples of cooperation or antagonism. Social evolutionists like to compare plants and pollinators with elephants and dung beetles. Plants evolved to make nectar cooperatively *because* of the return benefits from pollinators, but elephants did not evolve to make dung for beetles.

Box 2: Individual-based modeling of biofilms

Biofilms arise from many interacting processes, including cell-surface and cell-cell adhesion, physical shoving among cells as they grow and divide, solute diffusion, bulk fluid transport, shear forces exerted by local flow, cells' secretion of various compounds into the extracellular space, and biofilm matrix rheology[44]. Consequently, developing predictive theory for biofilm behavior and community dynamics is difficult, but engineers have answered this challenge for the past two decades using spatially explicit simulation techniques[188-192]. They implement idealized microorganisms as independent agents responding to their local microenvironment, which is continually modified by cells' consumption and secretion of different solutes or extracellular matrix polymers. Environmental heterogeneities are tracked by iteratively solving reaction-diffusion equations that describe solute concentration gradients in relation to bulk transport and consumption or secretion within the community. This approach is powerful for exploring questions about biofilm structure and composition, often achieving excellent consistency with experiments. New techniques for imaging biofilms at single-cell resolution promise to further tighten the interaction of experimental biofilm studies and individual-based simulations[166].

Over the last ten years, spatial biofilm simulations have been coopted for studying evolution in microbial communities. The first study of this kind[193] suggested that spatial structuring in biofilms could promote the evolution of metabolic strategies that maximize biofilm ecological productivity instead of individual growth rate. Other groups have since used related methods to explore questions on the boundary between biofilm microbiology and social evolution. These are summarized in the table below, along with their experimental support where available. The modeling traditions of engineering have been instrumental in bridging gaps between the abstract literature of social evolution theory and the more mechanistic culture of experimental microbiology. This topic is discussed in detail in another recent review[10].

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Theoretical Prediction		Summary	Experimental Support	
Kreft (2004)	194	- Spatial structuring in biofilms favors yield-maximizing metabolic strategies with group-level benefits.	No direct tests	
Xavier & Foster (2007)	88	- Secreted matrix confers cell lineages with greater access to locations with higher nutrient availability.	Nadell and Bassler (2011) Kim et al. (2014)	38 39
Nadell et al. (2008)	129	- Quorum sensing regulation of matrix secretion tunes a tradeoff between biofilm competition and dispersal.	No direct tests	
Nadell et al. (2010) Nadell et al. (2013)	53 10	- Genetic drift in expanding cell groups is proportional to their active layer width. - Spontaneous lineage segregation favors the evolution of diffusible public good compounds as a function of population structure and public good transport.	Buttery et al. (2012) Van Dyken and Desai (2013) Datta et al. (2013) van Gestel et al. (2014) Mitri et al. (2015)	60 58 80 57 54
Bucci et al. (2011) Weber et al. (2014) Borenstein et al. (2015)	96 98 99	- Bacteriocin secretion is favored when lineages are mixed and nutrient competition is local. - Toxin-sensitive strains can coexist with or outcompete secretors under cell lineage segregation conditions.	Tait and Sutherland (2002) Weber et al. (2014) Borenstein et al. (2015)	97 98 99
Mitri et al. (2011)	75	- Competition with other species can socially "insulate" cooperators against cheating. - Lineage mixing favors evolution of mutualistic secretion behaviors, while segregation does not.	Momeni et al. (2013b) Muller et al. (2014)	109 59
Mitri et al. (2011) Momeni et al. (2013a) Estrela & Brown (2013)	75 116 108	- Cross-feeding and detoxification mutualism both induce spatial mixing of mutualists.	Momeni et al. (2013a)	116
Mitri et al. (2011) Momeni et al. (2013b) Pande et al. (2015)	75 109 146	- Self-organized mixing of cross-feeding mutualists can protect them against invasion by cheating strains.	Momeni et al. (2013b) Pande et al. (2015)	109 146
Schluter & Foster (2012)	195	- Hosts supplied nutrients can select strongly for microorganisms at the epithelium.	No direct tests	
Schluter et al. (2015)	33	- Cells with higher cell-surface and cell-cell adhesion properties can physically displace less-adhesive strains from biofilms and outcompete them.	Schluter et al. (2015)	33

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Figure Legends

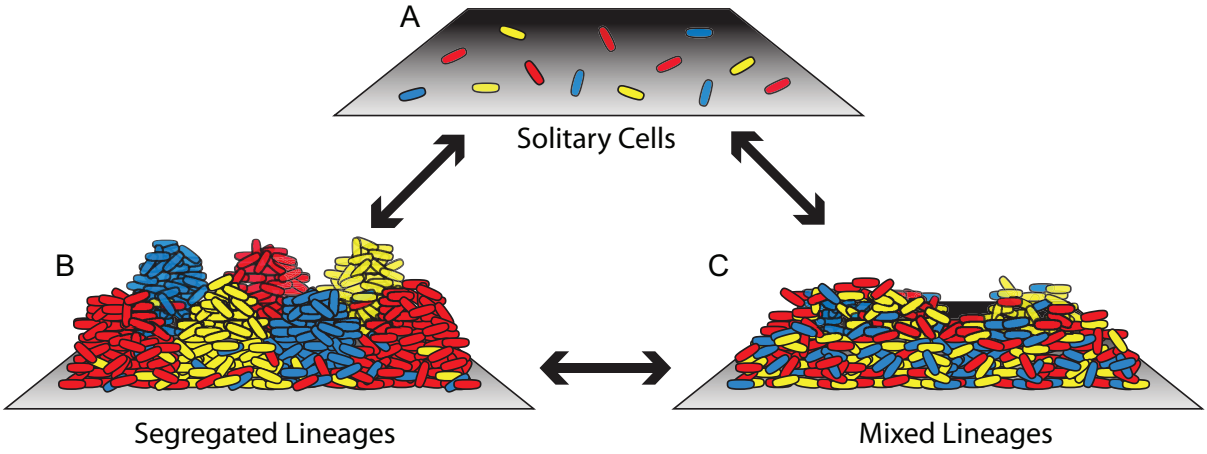
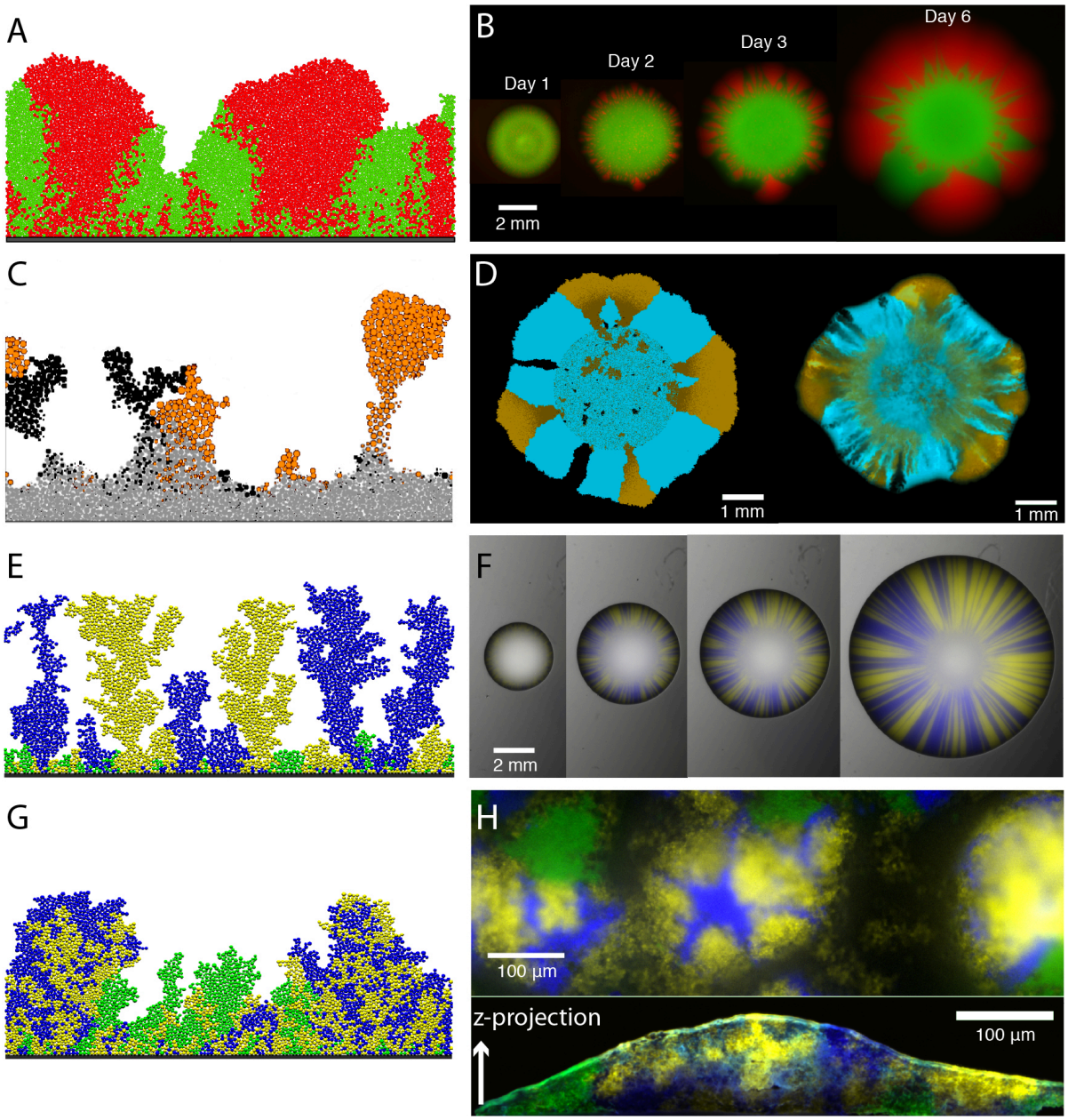


Figure 1. A conceptual guide to spatial structuring in microbial biofilms and its influence on the evolution of social phenotypes. Cells of the same color represent distinct cell lineages, (i.e., different species, or different strains within a species). (A) When cells are solitary on surfaces, their social phenotypes are often down-regulated due to the absence of suitable targets for either cooperative or antagonistic interaction. There are notable exceptions to this pattern, however, including extracellular matrix secretion[168] and aggregative surface motility[196]. (B) When biofilms contain segregated genetic lineages at high population density, cooperative public goods are often favored, because each cell's neighbors (which are often most strongly affected by social phenotypes) are almost exclusively clonemates. (C) When biofilms contain mixed lineages at high density, interactions are expected to be predominantly antagonistic, though inter-strain commensalism or mutualism is also possible. Whether biofilms transition from initial surface colonization to a high density segregated or mixed state depends on numerous factors[53], but lineage segregation can occur by default as cells divide while spatially constrained. Segregation is further strengthened by spatial bottlenecks due to limited growth along an advancing front[55], or by mechanisms supporting mother-daughter cell attachment[168]. Populations can be shifted toward lineage mixture, on the other hand, by physical perturbation, spatially homogeneous growth rates, diffusive cell movement, rapid population turnover due to migration, and mutualistic cross-feeding interactions[75,108,116]. Lastly, high-density biofilms can be reverted to sparse groups of solitary cells by dispersal or disturbance events that remove or destroy most of the population[197].



584
585 **Figure 2**. Individual-based simulations of cooperative microbial social behaviors with
586 experimental support. (A) Simulations by Nadell et al. (2010)[53] predicted that cell lineage
587 segregation on expanding cell group fronts allows public good-secreting cells (red) to
588 preferentially benefit themselves and outcompete non-secreting cells (green). (B) The
589 prediction in panel A has been verified by several studies, including a public goods system
590 using wild type and invertase null mutants of *S. cerevisiae* (producers: red; non-producers:
591 green) developed by van Dyken et al. (2013)[58]. (C) Biofilm simulations by Bucci et al.
592 (2011)[96] and (D, left) Weber et al. (2014)[98] illustrate the potential for coexistence between
593 toxin-secreting cells (black) and susceptible cells (orange) when cell lineages segregate in
594 space. In panel (C), gray cells are quiescent due to lack of nutrients. (D, right) A related study
595 by Weber et al. (2014)[98] also included resistant but non-toxin-secreting cells (teal) and an
596 experimental verification using bacteriocin-secreting, -sensitive, and -resistance cells of *E.*
597 *coli*. (E) Simulations by Mitri et al. (2011)[75], Estrela and Brown (2013)[108], and Momeni et al.
598 (2013a,b)[109,116] predict that mutually beneficial strains on expanding fronts spatially
599 segregate when mutualism is weak relative to competition (yellow and blue cells are
600 mutually beneficial strains; green cells are non-producers). (F) Segregation of strains in the

synthetic yeast mutualism of Müller et al. (2014)[59], when mutualism is negligible relative to competition. (G) When mutualism is strong relative to competition, simulations predict that mutually beneficial strains will spatially assort together and exclude non-producer (cheater) strains[75]. (H) Spatial mixing of beneficial genotypes, and exclusion of non-beneficial genotypes, was demonstrated experimentally by Momeni et al. (2013b)[109]; see also Momeni et al. (2013a)[116] and Müller et al. (2014)[59] for instances of spatial intermixing induced by cross-feeding mutualism. All images are reproduced from their original sources with permission of the authors. In panels A, C, D, E, G and H the color schemes were altered from the original to facilitate comparison.

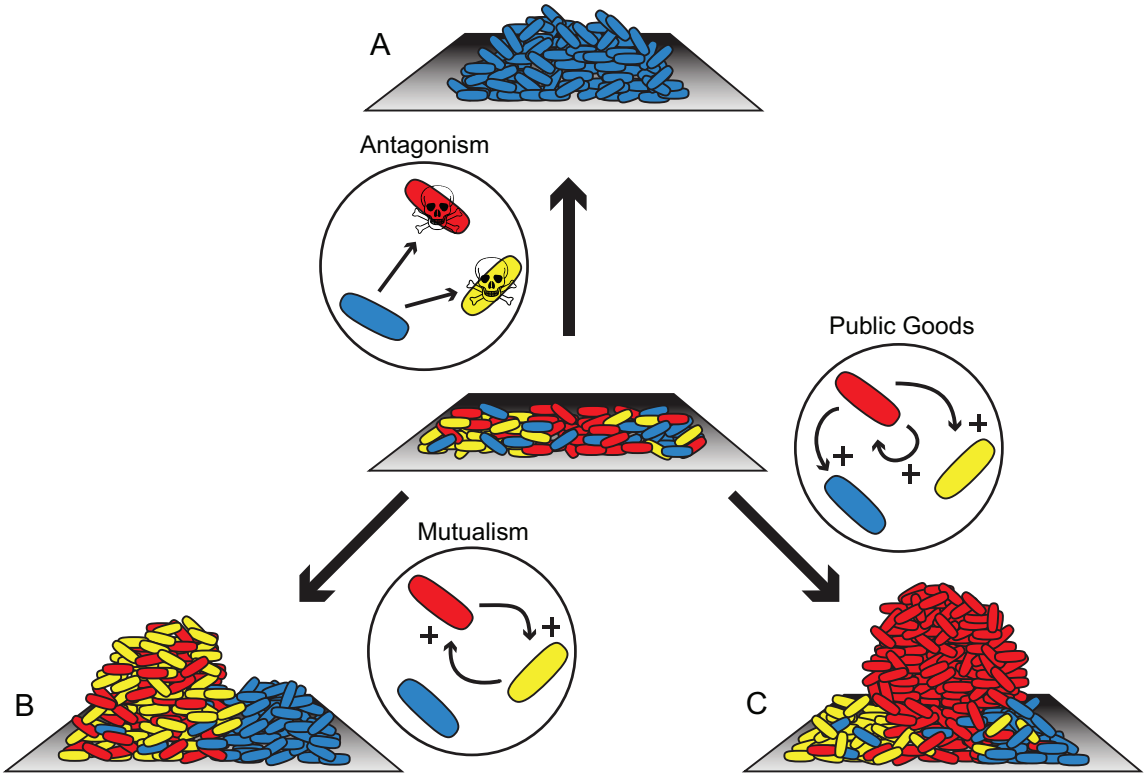


Figure 3. A conceptual guide to the influence of social interaction on the emergent structure of biofilm communities. Cells of the same color represent distinct cell lineages, as in Figure 1. (A) From an initially well-mixed population, antagonistic phenotypes such as secreted toxins or type VI poison delivery systems can eliminate all susceptible cells in the vicinity, culling the population to one genotype[96]. (B) Two cell lineages that mutually benefit one another tend to become entangled, as they grow better in proportion to their spatial proximity with each other. This can result in spatial mixing of the mutualists and exclusion of cheating or non-interacting third parties[59,75,108,109]. (C) If populations contain limited early clonal clustering (e.g., due to spatial constraint or spatial genetic drift), then cells secreting public goods can preferentially benefit clonemates, which proliferate more rapidly than neighboring lineages and cut them off from the actively growing front of a biofilm[53,58].

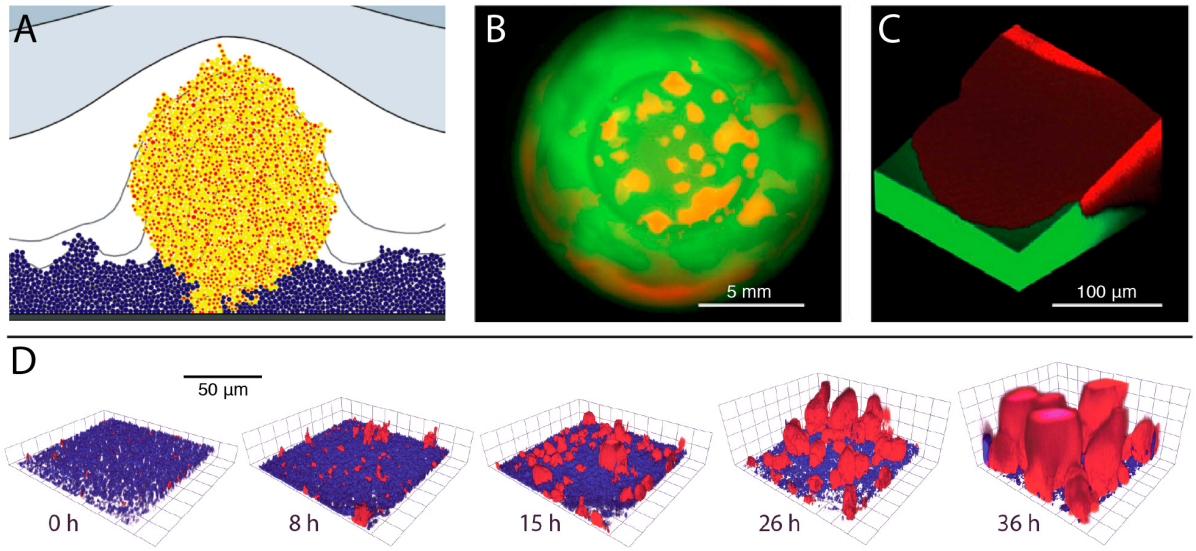


Figure 4. Matrix-secreting cells outcompete non-secreting cells within bacterial biofilms. (A) Xavier and Foster (2007)[88] first predicted that extracellular matrix (yellow), if spatially retained by secreting cells (red), could allow producers to expand in volume more rapidly than non-producing competitors (blue), propelling themselves into regions of higher nutrient availability (denoted by nutrient isoconcentration lines). This prediction was confirmed by Kim et al. (2014)[39] using laboratory evolution experiments with *P. fluorescens*, from which mutants (red) consistently emerge that hyper-secrete matrix relative to wild type (green) when inoculated on agar (B, fluorescence micrograph; C, confocal 3D reconstruction). (D) Nadell and Bassler (2011)[38] illustrated that matrix-secretors (red) also expand in volume and gain a competitive advantage against isogenic non-secretors (blue) in the pathogen *V. cholerae*. By the end of this experiment at 36 h, matrix-secreting clusters reach the ceiling of the flow chambers in which they're growing. This system supports additional predictions made by Schluter et al. (2014)[33], who use simulations to show that cell-cell and cell-surface adhesion can allow matrix-secreting cells to physically displace competitors from biofilms[33].

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Author Biographies

Kevin R. Foster is Professor of Evolutionary Biology at the University of Oxford, UK. His laboratory combines evolutionary and ecological theory with molecular microbiology to study the social lives of bacteria. Foster Lab Homepage. Knut Drescher is Professor of Biophysics and a Max Planck Research Group Leader with the MPI for Terrestrial Microbiology in Marburg, Germany. His laboratory uses methods from physics and molecular biology to study the dynamics of bacterial biofilm formation. Drescher Lab Homepage. Carey D. Nadell is an Alexander von Humboldt Fellow at the MPI in Marburg; he has a background in social evolution, theoretical ecology, and population dynamics within biofilms, with emphasis on the relationship between biofilm matrix structure and community assembly.

Bullet Point Summary

- Bacteria often live in biofilms, which are surface-bound or free-floating cell groups bound together by a secreted polymer matrix. These microbial collectives are important for how bacteria occupy diverse ecological niches, contribute to biogeochemical cycling, and cause disease in multicellular organisms.
- While residing in biofilms, bacteria interact with each other closely via cooperative phenotypes, such as digestive enzyme production, and antagonistic phenotypes, such as Type 5 or Type 6 secretion. The evolutionary dynamics of these social phenotypes depend on their costs, their effects on other cells, and specifically which other cells they tend to affect in a cell group.
- Many bacterial social phenotypes are secreted products, which affect neighbors in a distance-dependent manner. As a result, interaction networks within biofilms are largely determined by their spatial structure, namely the arrangement in space of different clones, strains, and species.
- When biofilms are segregated into clonal clusters, a given cell's neighborhood mostly contains clonemates, and natural selection often favors the secretion of compounds that benefit all recipient cells; i.e. public goods such as digestive enzymes or communal surfactants. When different strains and species are spatially mixed within biofilms, however, cells primarily interact with other genotypes, and antagonistic behavior is often favored. Under certain circumstances however, between-species commensalism or mutualism can also evolve and remain stable against cheating.
- Cooperative and antagonistic phenotypes fall under the control of sophisticated sensory mechanisms, such as competition sensing and quorum sensing, that evolved to help account for the variation in exposure to other strains and species in space and time. These regulatory mechanisms help to reduce the marginal costs of social phenotypes, maximize their fitness impacts, and ensure that the correct recipient cells are targeted.
- Both cooperative and antagonistic behaviors feed back onto population spatial structure by locally altering other cells' growth rates, which alters local biofilm composition. Thus there is a continuous feedback between the spatial structure of biofilms and the social phenotypes of the diverse microorganisms within them.
- Many bacteria and unicellular eukaryotes have evolved strategies for actively altering population structure. They achieve this via selective adhesion that spatially assort biofilms into groups containing one or more specific genotypes, or via secretion of extracellular matrix components that spatially organize biofilm-dwelling cells.

Glossary

Microbiota - A community of microorganisms that live in association with a particular host organism (e.g. the gut microbiota) or abiotic environment (e.g. the soil microbiota).

Social Phenotype - A phenotype that exerts an effect (either positive or negative) on the reproductive output of other individuals, and which evolved in part because of the fitness effect that it exerts (see Box 1).

Siderophore - A low molecular weight compound that binds unavailable iron to be absorbed by cells via a cognate receptor.

Genetic Drift - A change in allele frequency in a population due to random sampling of organisms across generations, e.g. due to stochasticity in reproductive success.

Public Goods - Substances secreted into the extracellular space that provide a benefit to other cells in the vicinity.

Cheating - Occurs when a genotype receives the benefits of an evolved cooperative trait of other genotypes, such as a public good, without contributing to the cooperative interaction itself.

Ecological Productivity - The total biomass produced by a strain or species in a given environmental setting.

Antibiotics - Small molecules produced by various microorganisms that act as toxins against other bacteria or fungi, some of which have been coopted as pharmaceuticals for the treatment of microbial infections.

Bacteriocins - A subclass of antibiotics referring to toxins that are produced by bacteria and specifically target other bacteria. Bacteriocins often occur as toxin-antitoxin pairs that are encoded on the same plasmid or in the same genomic neighborhood.

Contact-Dependent Inhibition (Type V Secretion System) - A mechanism of inhibiting neighbor cell growth by extension of a β-helical structure to contact target cells and delivers toxic effector molecules.

Type VI Secretion System - A mechanism for killing neighboring cells by extension of a phage tail-derives structure to putatively puncture adjacent cells and deliver toxic effectors.

Black Queen Evolution - Regressive evolution in which a genotype loses a catabolic ability because the function is complemented by another species in the vicinity (cheating is a major example).

Syntrophy – Interaction by which one species uses the waste product of another as a nutrient source, such that the producer benefits from the removal of its waste product.

Quorum Sensing - A regulatory mechanism by which bacteria and other microorganisms secrete diffusible molecules and respond to these molecules after they reach a critical concentration, thought to be used for detecting population density and surrounding flow conditions.

Dispersal - The process by which cells depart from a community, either individually or collectively. Dispersal can be active in response to stresses such as nutrient limitation, or passive due to biofilm erosion by fluid flow.

Flocculation - A process by which yeast aggregate to form large multicellular groups that precipitate from liquid cultures and exhibit heightened stress tolerance.

Greenbeard Gene - A gene or a closely linked set of genes that encode both an identifying phenotypic trait and the expression of a cooperative behavior that targets that identifying trait, ensuring that the greenbeard gene bearer only benefits other bearers of the greenbeard gene.