MICROBIAL BIOFILMS

Spatial structure, cooperation and competition in biofilms

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Abstract | Bacteria often live within matrix-embedded communities, termed biofilms, which are now understood to be a major mode of microbial life. The study of biofilms has revealed their vast complexity both in terms of 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.

Microbiota
The community of microorganisms that live in association with a particular host organism (for example, the gut microbiota) or abiotic environment (for example, the soil microbiota).

Social phenotypes
Phenotypes that exert an effect (either positive or negative) on the reproductive output of other individuals and which evolved, in part, because of this fitness effect that they exert.

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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 indispensable to global biogeochemical cycling[3,4] and the healthy functioning of the microbiota of multicellular organisms[5]. By contrast, biofilms also cause antibiotic-tolerant infections[6] and the destruction of surfaces and flow systems; they are therefore of great concern in medical and industrial settings[7–9].

Biofilm-dwelling cells interact intimately and influence each other's evolutionary fitness through social phenotypes[10,11] (BOX 1). Many of these behaviours are simple forms of cooperation that benefit neighbouring cells, such as the secretion of nutrient chelators[12,13], digestive enzymes[14], surface adhesins[15], wetting agents[16], structural polymers[17] and signalling molecules[18]. For example, outside human hosts, *Vibrio cholerae* forms biofilms on environmental particles of the structural polymer chitin, which it digests through communally beneficial chitinases[19,20]. Diverse biofilm-dwelling bacteria also produce siderophores, which are low-molecular-mass compounds that bind to and solubilize otherwise inaccessible iron, a frequently limiting nutrient in the abiotic environment and within host organisms[12,21]. Owing to their cooperative and collective behaviour, biofilm-dwelling cells have substantial advantages compared with solitary cells, including an increased resilience against external threats and a higher efficiency in digesting complex nutrients[20,22]. Microorganisms are thus fundamentally social organisms, and their cooperative phenotypes are pivotal to how they affect the world around them.

Social interactions can also be competitive however, and cells within a microbial community should not be assumed to work together harmoniously[11]. Competition for limited space and resources is pervasive[23,24], and many social phenotypes harm other strains and species. Antibiotic secretion, the direct injection of toxins into adjacent cells, and mechanisms for displacing or suffocating neighbours all target competitors for elimination and can substantially alter the composition of biofilms[25–28]. For instance, *Pseudomonas aeruginosa* engages in bouts of type VI secretion system (T6SS)-mediated attack specifically in response to antagonism from other bacteria[27,29]. *V. cholerae* and *Pseudomonas fluorescens* produce extracellular matrix materials that give secreting cells a positional advantage over competitors, which are physically displaced[30,31] or cut off from nutrient access[32].

The spatial arrangement of different strains and species within biofilms strongly influences the relative fitness benefits of cooperative and competitive phenotypes. By altering the reproductive rates of neighbouring cells, social phenotypes can cause compositional and structural changes in microbial communities, thus shaping their overall function and, in the case of pathogens, their virulence[33–35]. Therefore, to understand microbial communities, we must consider the balance of cooperation and competition within biofilms, and how this balance influences their macroscopic properties. This goal poses great difficulties, as biofilms are complex, often heterogeneous systems that emerge from an interplay of many physical forces and local interactions among cells[36,37]. A growing body of theoretical and experimental literature has nevertheless begun to dissect the intricacy of biofilms and to identify general rules of cell–cell interaction within these structures. In this Review, we discuss key recent findings, focusing on the central importance of spatial structure for understanding and predicting microbial social behaviours.

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 (the recipients) other than the organism expressing the phenotype (the actor). The field first developed in the context of animal behaviour, seeking to explain now-famous examples of behavioural 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 those recipients. The third factor is often phrased in terms of a relatedness coefficient, which refers broadly to the genetic similarity of an actor and a recipient, relative to the population average[63,66,67,155]. In asexual microorganisms, the effects of relatedness can often be reduced to a simple genotypic view of microbial interactions: if interacting cells have the same genotype at the locus that defines a social trait, then cooperative interaction is favoured, but if they are of a different genotype at that locus, then competition is usually favoured[75]. Cooperation can evolve between different genotypes, and particularly between different species that do not compete for resources, but the conditions for such evolution are more restrictive than those for cells of one genotype[93,156].

An important limitation to the social evolution approach is that, by design, it can 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 specifically to influence other individuals and asocial phenotypes that incidentally affect other individuals. However, there are some common signatures of social phenotypes. Social phenotypes are often energetically costly, such that they incur a net negative fitness effect if they are expressed in the absence of target recipients[11]. For example, a bacteriocin-secreting strain suffers a net fitness loss from bacteriocin secretion if there are no susceptible cells in the vicinity. By contrast, in all communities, individuals coincidentally influence each other's fitness owing to asocial traits that evolved without regard to their effects on conspecifics and heterospecifics. In microbial groups, this phenomenon can manifest as one strain that produces a metabolic waste product that may benefit (for example, by providing a new nutrient source) or harm (for example, by changing environmental pH) other strains. The secreting strain benefits from releasing its waste product regardless of whether other cells are affected. Although such accidental effects on other genotypes can be important for our understanding of a given community and they may precede the evolution of social phenotypes, 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, whereas elephants did not evolve to make dung for beetles.

Spatial structure and 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 attachment sites (reviewed in REF. 38). As surface-adhered cells grow, divide and interact with each other, the structure of their community often changes (FIG. 1). For example, an initially disordered mixture of strains and species can become highly structured such that the final community contains large single-genotype patches that span many cell lengths (FIG. 1b).

In general terms, a social phenotype will be favoured or disfavoured by natural selection depending on its fitness costs, its effects on other cells, and the genotypes of the affected cells (BOX 1). In biofilms, this last factor — the genotypes of those cells that are most strongly affected by a social phenotype — is highly influenced by spatial structure, because microbial social behaviours typically have the greatest effect on immediate neighbours[10,39]. The arrangement of cells in space is therefore crucial to whether competitive or cooperative interactions are advantageous in a given environmental context[40]. 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 technical literature with an intuitive guide, we consider three key scenarios of spatial structure within biofilms and their relationship to patterns of competitive and cooperative behaviour (FIG. 1).

First, cells may be dispersed at low density on a surface, such that they are essentially solitary (FIG. 1a). Although such an organization is crucial as an early phase of biofilm growth[41], it generally disfavours the expression of social phenotypes, many of which are likely to have evolved to influence nearby neighbours. Second, at high population densities, biofilms can contain segregated genetic lineages or, third, mixed lineages. Cooperative and antagonistic phenotypes can have the strongest impact on evolutionary dynamics when population density is high enough for cells to affect each other through either direct contact or the release of diffusible substances. This Review focuses on such high-density conditions, in which cell lineages (that is, different mutants, strains and species) may be segregated, such that cells primarily interact with their own genotype (FIG. 1b), or mixed, such that several genotypes interact with each other (FIG. 1c). We first address how shifts between these spatial structures affect 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 and alter population spatial structure.

Spatial segregation: benefits within genotypes. Owing to the constraint on movement that is common in biofilms, clonal clusters can be generated passively — that is, without active aggregation among clonemates — as cells grow and divide. This phenomenon has been observed *in silico*, *in vitro* and *in vivo*[30,42–44], and causes clonal patchiness as a function of surface colonization, division, death and dispersal rates[45]. Even when different strains or species are initially well mixed, populations that grow towards a limited nutrient pool often experience strong spatial bottlenecks as some cell lineages are cut off by the actively growing front. This process, referred to as gene surfing or spatial genetic drift, induces population subdivision into monoclonal sectors[46] and has been documented in agar colonies of *Escherichia coli*[46,47], *Bacillus subtilis*[48], *P. aeruginosa*[49], *Saccharomyces cerevisiae*[46,50,51] and *Dictyostelium discoideum*[52].

Spatial structure can be crucial for cooperation within species and the evolution of simple public goods, which many microorganisms require to take advantage of the nutrient reservoirs in their natural habitats. For example, pathogens and saprophytes exploit tissues that are composed of large polymers, which must be digested into soluble components by secreted enzymes before they can be imported and catabolized. *Clostridium difficile* uses secreted enzymes to digest host connective tissue[53], and numerous bacteria produce exoenzymes to

Type VI secretion system
(T6SS). A mechanism for killing neighbouring cells by the extension of a phage-tail-derived structure to putatively puncture adjacent cells and deliver toxic effectors.

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, owing to biofilm erosion by fluid flow.

Genetic drift
A change in allele frequency in a population due to random sampling of organisms across generations (for example, due to stochasticity in reproductive success).

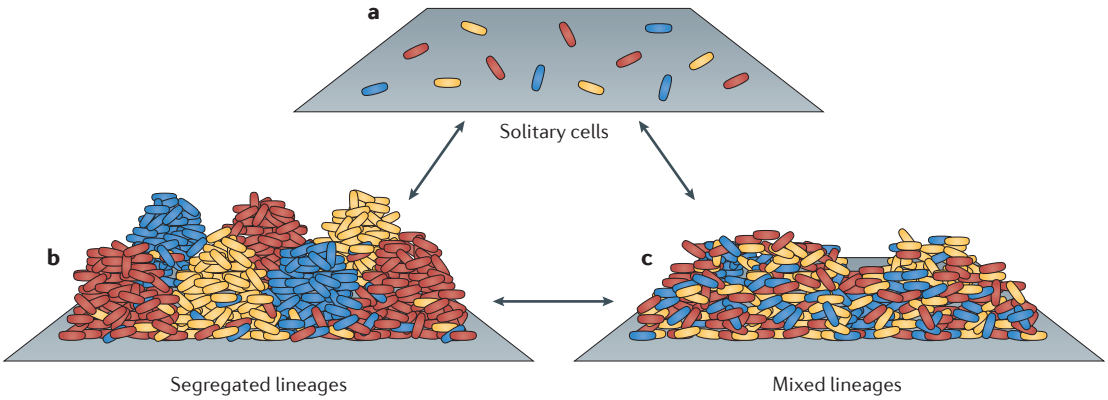


Figure 1 | **Spatial structuring in microbial biofilms and its influence on the evolution of social phenotypes.** Cells of the same colour represent distinct cell lineages (that is, different species or different strains within a species). **a** | When cells are solitary on a surface, their social phenotypes are often downregulated owing to the absence of suitable targets for either cooperative or antagonistic interactions. There are notable exceptions to this pattern, including the secretion of extracellular matrix components[145] and aggregative surface motility[161]. **b** | When biofilms contain segregated genetic lineages at a high population density, cooperative public goods are often favoured, because neighbouring cells (which are often most strongly affected by social phenotypes) are almost exclusively clonemates. **c** | When biofilms contain mixed lineages at a high population density, interactions are expected to be predominantly antagonistic, although interstrain commensalism or mutualism is also possible. Whether biofilms transition from initial surface colonization by solitary cells to a high-density segregated or mixed state depends on numerous factors, but lineage segregation can occur by default as cells divide while spatially constrained. Segregation is further strengthened by spatial bottlenecks that are caused by limited growth along an advancing front, or by mechanisms that support mother–daughter cell attachment. Populations can be shifted towards lineage mixture by physical perturbation, spatially homogeneous growth rates, diffusive cell movement, rapid population turnover due to migration, and mutualistic cross-feeding interactions. Finally, 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.

digest cellulose[54,55], which is a ubiquitous plant structural compound. The nutrients that are released by the activity of extracellular enzymes are potentially available for uptake by nearby cells, a principle that extends to other secreted compounds, including nutrient chelators and communal adhesins[12,21,33,56,57]. These behaviours result in public goods dilemmas: public good-producing cells can be exploited by cheating mutants[18,58]. The production and exploitation of public goods have been most heavily explored in laboratory settings, but recent work suggests that they also have a role in clinical[59,60] and natural environments[21]. A bioinformatic and phylogenetic analysis of natural *Vibrio* spp. populations revealed frequent loss of genes for the production of iron-chelating siderophores, but no loss of the corresponding cognate receptors, which is consistent with a producer–cheater dynamic for siderophore secretion[21].

When secreted cooperative compounds are costly to produce, their evolutionary fate is determined by whether or not they benefit clonemates rather than competing strains and species[10,14,39,61]. This will depend on how far the secreted public good travels, which is affected by its production, its transport by advection and diffusion, its uptake and its decay[10,39,61,62]. When the spatial scale of public goods sharing is similar to the spatial scale on which clonal clustering occurs, public goods dilemmas can be resolved[14,39,61,63,64]. Therefore, clonal clustering tends to promote the evolution of public good production[10], as long as the public good does not rapidly diffuse throughout the system[20,65]. The logic of this prediction dates back to the birth of social evolution as a field and was originally conceived with animal behaviour in mind[66,67], but it is also upheld for microbial systems with cooperative phenotypes[68]. For example, in *V. cholerae* biofilms that are growing on chitin particles[20], which the bacterium digests using secreted chitinases, the production of the extracellular digestive enzymes is more strongly favoured as clonal cluster size increases. Similarly, competition experiments that were carried out on agar plates have shown that siderophore secretion by *P. aeruginosa* is more strongly favoured as agar concentration is increased, because this decreases public good diffusivity and limits the receipt of cooperative help to neighbouring clonemates[64,69]. When *P. aeruginosa* is grown on glass, siderophore exchange becomes limited to direct neighbours; in combination with clonal clustering, this strategy largely prevents exploitation by cheating mutants[70]. Finally, recent work using colonies of *B. subtilis* showed that stronger cell lineage segregation promotes the secretion of an extracellular polymer that cooperatively helps cells to spread along agar surfaces[48,71].

Computational simulations of biofilm growth (BOX 2) predict that the lineage segregation that occurs in expanding populations can favour behaviours that benefit neighbouring cells, because this segregation generates clonal clusters that are large relative to the scale of diffusion of cooperative secreted compounds[39,44,52] (FIG. 2a). This prediction is supported by experiments that use different cooperative phenotypes and model organisms, including yeast[50] and bacteria[48,49,72–74] (FIG. 2b). By contrast, the clonal clustering that emerges

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

Cheating mutants
Genotypes that gain a relative fitness advantage by receiving the benefits of an evolved cooperative trait of other genotypes, such as a public good, without contributing to the cooperative interaction themselves.

Ecological productivity
The total biomass produced by a strain or species in a given environmental setting

spontaneously owing to spatial genetic drift can destabilize cooperation between different strains or species by separating the mutually beneficial partners from each other[68] (see below).

Box 2 | **Individual-based modelling of biofilms**

The formation of biofilms involves many processes, including cell–surface and cell–cell adhesion, physical shoving among cells as they grow and divide, solute diffusion, bulk fluid transport, shear forces that are exerted by local flow, cellular secretion of various compounds into the extracellular space, and biofilm matrix rheology[37]. Developing predictive theory for biofilm behaviour and community dynamics is difficult, but engineers have progressed this field using specialized individual-based simulation techniques[157,158]. These techniques implement idealized microorganisms as independent agents that respond to their local microenvironment, which is continually modified by cells as they consume and secrete 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, and the results are often supported experimentally.

Over the past 10 years, spatial biofilm simulations have been co-opted for the study of evolution in microbial communities. The first study of this kind[159] 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 that concern the boundary between biofilm microbiology and social evolution; the table below depicts the chronology of this field, along with a growing body of empirical support for the key predictions.

Summarized predictions	Theory (references)	Experiments (references)
Spatial structuring in biofilms favours yield-maximizing metabolic strategies with group-level benefits	159	No direct tests
Secreted matrix provides cell lineages with greater access to locations with higher nutrient availability	79	30,32
Quorum sensing-mediated regulation of matrix secretion fine-tunes a trade-off between biofilm competition and dispersal	113	No direct tests
Genetic drift in expanding cell groups is proportional to the width of their active layer; spontaneous lineage segregation favours the evolution of diffusible public goods as a function of population structure and public goods transport	10,44,62	48–50,52,72
Bacteriocin secretion is favoured when lineages are mixed and nutrient competition is local; toxin-sensitive strains can coexist with or outcompete secretors under conditions that lead to segregation of different cell lineages	47,85,87	47,86,87
Competition with other species can socially insulate cooperators against cheating; lineage mixing favours the evolution of mutualistic secretion behaviours, whereas segregation does not	68	51,95
Cross-feeding and detoxification mutualisms both induce spatial mixing of mutualists	68,94,102	102
Self-organized mixing of cross-feeding mutualists can protect them against invasion by cheating strains	68,95,128	95,128
Host-supplied nutrients can select strongly for microorganisms at the epithelium	160	No direct tests
Cells with higher cell–surface and cell–cell adhesion properties can physically displace less-adhesive strains from biofilms and outcompete them	31	31

Many complementary studies to date show that the spatial segregation of cell lineages in biofilms increases the frequency of interactions between cells of the same genotype; generally speaking, these conditions favour investment into cooperative behaviours that heighten the ecological productivity of clonal patches and, as a result, the biofilm as a whole.

Spatial mixing: conflicts between genotypes. Although clonal clustering occurs readily in biofilms owing to limited movement, it is not universal. Cell lineages may become spatially mixed for many reasons, including frequent dispersal and recolonization, diffusive cell motility and homogeneous nutrient abundance[44]. When several strains and species encounter each other often, the default expectation is that competitive phenotypes will predominate, as the primary action of natural selection is to favour genetic lineages that benefit themselves more than others[66,67,75–77]. Such competition has led to the evolution of diverse competitive strategies, which range from rapid growth and resource acquisition[78] to the use of adhesion and matrix production to seize nutrient-rich locations within biofilms[31,79] (see below). One of the most common modes of intermicrobial competition is the secretion of broad-spectrum and narrow-spectrum toxins, coupled with privatized antitoxins that prevent self-poisoning.

The production of antibiotics in general, and of bacteriocins in particular, is widely documented in microorganisms[25] and has been studied for some time in the theoretical ecology literature[80]. Although it has been suggested that antibiotics can function as cooperative signals between species at subinhibitory concentrations, the evolutionary basis for this idea is unclear, and parsimony suggests that the primary role of antibiotics is to kill competitors[77,81,82]. Most simply, antibiotics — and other secreted toxins — benefit the lineages that have toxin resistance by eliminating cells that do not. Lysed neighbours may also be directly exploited for raw materials, including their genetic content[83]. Theory predicts that microbial toxins will be most strongly favoured when competition for resources is localized and competing cell lineages are moderately well mixed in space[34,84,85]. When community mixture is too high, the density of each toxin-secreting strain may be too low to launch an effective attack. By contrast, when communities are clonally segregated, there may be no susceptible cells of other genotypes in the vicinity for toxin secretors to target. Indeed, simulations and experiments show that when cell lineages are segregated, toxin-sensitive species readily coexist with or even outcompete toxin-secreting species within the same biofilm[47,80,85–87] (FIG. 2c,d).

Whereas classic bacteriocins and other antibiotics are exported into the extracellular space[25], other toxins are directly secreted into or onto neighbouring cells by type V secretion systems (T5SSs; responsible for contact-dependent inhibition) or by T6SSs. *Bacteroides fragilis*, a common symbiont of the gut microbiota, uses T6SSs to compete and persist in the mammalian intestine in a manner that is predicted to be dependent on spatial genotype mixing[88]. The opportunistic pathogen

Proteus mirabilis expresses a T6SS along with the motility machinery that is required for collective movement on agar surfaces, and thus deploys a pre-emptive attack against susceptible competitors as it prepares to migrate[89]. This behaviour maintains the clonal structure of a growing cell cluster; in fact, many social phenotypes have a strong reciprocal influence on spatial structure (see below).

Spatial mixing: benefits between genotypes. Although microbial antagonism is common, spatial population mixing can enable cells to receive benefits from other strains or species[68] (FIGS 1c,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. For example, *Bacteroides* spp. digest host-ingested polysaccharides and excrete acetate as a metabolic waste product. The excreted acetate is used as a carbon source by other members of the microbiota that do not, as far as we know at present, produce anything useful in return[90]. When the released factor is costly to produce (that is, if it is not a waste product; see BOX 1), the recipient of such unidirectional benefits is essentially a cheating strain, as discussed above.

A recently introduced idea that is qualitatively similar to cross-species cheating is that of black queen evolution: one species survives the loss of a catabolic capacity because another species in the vicinity leaks complementary metabolites into their shared environment[91]. This process is thought to have occurred for the marine bacterial group '*Candidatus* Pelagibacter ubique', which depends on reduced sulfur that is released by cohabiting microorganisms[92]. Cheating and black queen effects both depend on a cell density that is high enough to generate usable concentrations of the exchanged compound, and on a sufficiently mixed community structure in which recipient cells can access the compounds released by producers[93,94].

Spatial mixing of cell lineages also enables reciprocal benefits and the evolution of cooperation between species[68,94,95] (FIG. 2e–h). A potential evolutionary trajectory to such mutualisms is through syntrophic relationships[96] in which a secreted waste product of the first species renders one of its core metabolic reactions thermodynamically unfavourable. If this waste product is absorbed and used as a nutrient source by a second species, the second species can help the first species to grow[94]. Such an interaction occurs within oil-degrading microbial communities: the recently sequenced species *Desulfatibacillum alkenivorans* metabolizes alkanes when paired with *Methanospirillum hungatei* JF-1, which absorbs the hydrogen and formate that are produced by *D. alkenivorans* as a result of this metabolic process[97]. This form of exchanged benefit can occur 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.

In principle, between-species cooperation that requires costly investment from each party may also arise, including cross-feeding partnerships whereby metabolites that are produced by one species mitigate the auxotrophy of another, and vice versa[98]. Several groups have constructed synthetic obligate mutualisms, including a pair of *E. coli* amino acid auxotrophs that complement each other when cultured together[99]. Recent work has also found evidence for evolved cooperation between *Bacteroides* spp. in the human gut[100], 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 by-product consumption rates and the extent of interspecific competition among interacting partners[93,101]. Theory and experiments with synthetic systems agree that some mixing of cell lineages is essential for mutualisms to evolve[68,94,102] (FIG. 2f–h). By contrast, theory suggests that overly homogeneous mixing can undermine mutualistic interactions by exposing them to cheating genotypes, or to passive genotypes that 'socially insulate' mutually beneficial partners from interacting with each other[51,68,93].

In summary, spatial mixing of genotypes can favour strong antagonism, as is widely seen in antibiotic warfare, but it also enables dependencies to evolve between strains, whereby one strain uses the beneficial products of another. Under specific conditions, these dependencies may further evolve into mutualistic cooperation, although the spatial mixing of many different genetic lineages can lead to cheating and social insulation, and can thus compromise between-genotype cooperation.

Regulation of social phenotypes

Above, we discuss cooperative and competitive traits within biofilm communities under the assumption that these traits are constitutively expressed. However, social phenotypes are often strictly regulated in response to biotic and abiotic inputs. The evolution of these regulatory strategies depends on how the reproductive costs and benefits of a particular trait change as a function of the chemical and biological environment of a cell.

Reducing the reproductive cost of social phenotypes (that is, minimizing their negative effect on survival and division rates) is one of the broadest principles that underlies their regulation. For example, *P. aeruginosa* regulates the synthesis of the iron-scavenging molecule pyoverdin depending on iron availability in a manner that minimizes its marginal production cost[103,104]. Pyoverdin is durable over several bacterial generations; as the compound accumulates locally, *P. aeruginosa* reduces its investment into pyoverdin, thus decreasing the trans-generational cost of production[104,105]. *P. aeruginosa* also secretes copious rhamnolipid surfactants, which are thought to improve motility and resource acquisition at the edge of expanding colonies. Although the production of rhamnolipids involves substantial resource allocation, its mode of regulation has little negative impact on the cell division rate, as rhamnolipids are synthesized only by cells with access to more carbon than they need for growth[16]. This strategy of metabolic prudence seems to apply to many secretion phenotypes[16,106].

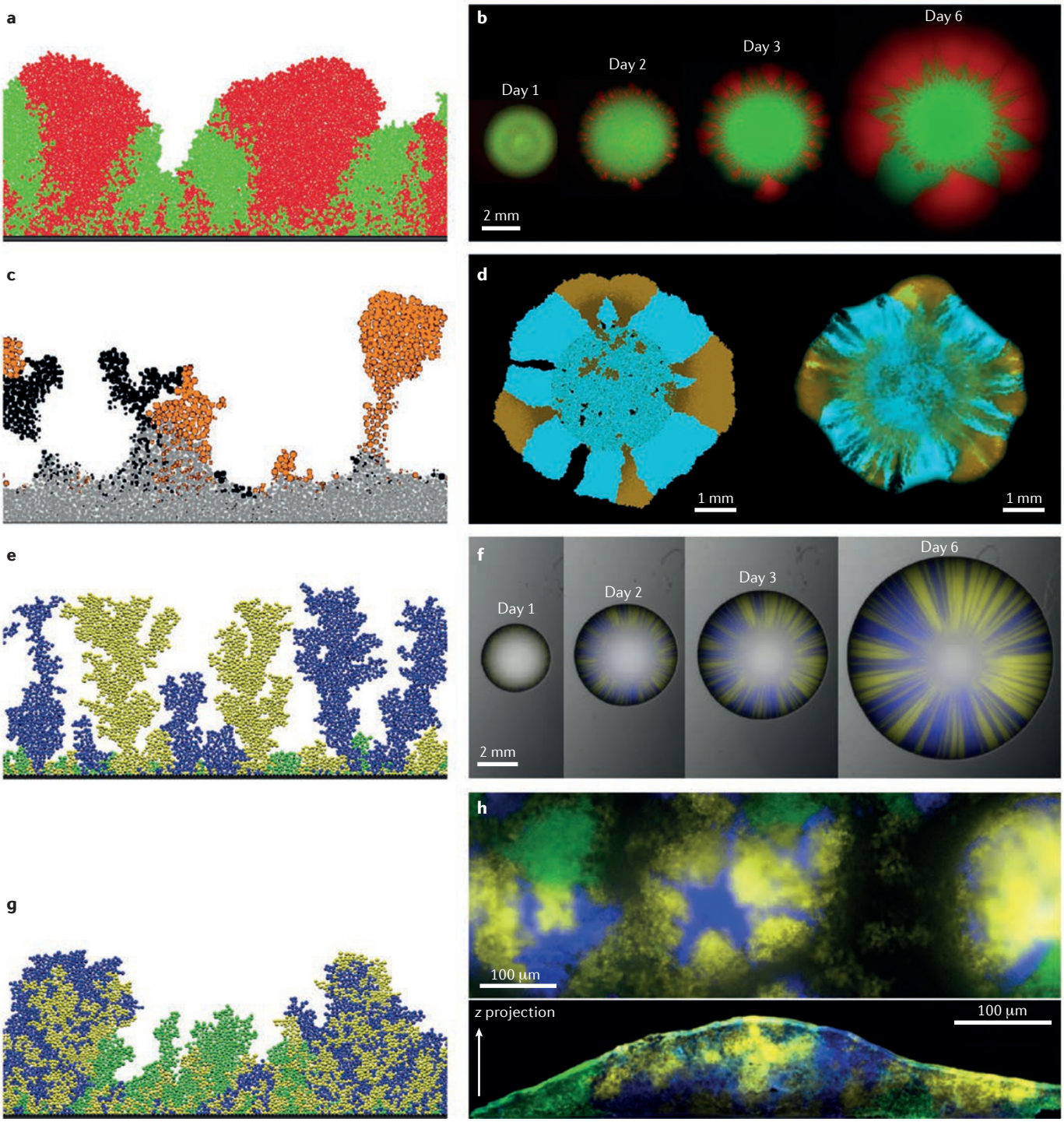
In addition to reducing the cost burden of social traits, their regulation can increase the likelihood that their associated fitness effects are delivered to the appropriate

Antibiotics
Molecules that are produced by various microorganisms and act as toxins against other microorganisms; some antibiotics have been co-opted as pharmaceuticals for the treatment of microbial infections.

Bacteriocins
Antibiotics 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 neighbourhood.

Contact-dependent inhibition
A mechanism of inhibiting the growth of neighbouring cells by the extension of a helical structure to contact target cells and deliver toxic effector molecules.

Syntrophic relationships
Interactions in which one species benefits by using the product of another as a nutrient source; the producing species may in turn benefit from the removal of this product.



target cells. As discussed above, the evolutionary fitness consequences of a particular secretion phenotype depend heavily on the presence and genetic identity of other cells nearby. Consequently, natural selection might favour regulatory networks that predict both the density and identity of cells in the vicinity[107] — that is, networks that differentiate between the population structure scenarios described in FIG. 1. Cells sense various signals to distinguish biofilm spatial structures, including environmental stressors and molecules that correlate with cell density.

Many cooperative secretion phenotypes are controlled by quorum sensing, a microbial regulatory mechanism that involves the secretion of and response to diffusible molecules termed autoinducers[108,109]. Quorum sensing is typically considered to be a means of assessing local cell population density and monitoring fluid transport processes in the immediate environment[110–112]. Biofilm modelling and experiments with *V. cholerae* in microfluidic devices indicate that quorum sensing could also be used to fine-tune the timing of extracellular matrix

Figure 2 | **Simulations and experiments exploring social phenotypes in biofilms.** **a**,**b** | Cell lineage segregation on the front of expanding cell groups enables cells that secrete public goods (red) to gain a competitive advantage and outcompete non-secreting cells (green). This was first predicted using biofilm simulations[44] (part **a**) and was verified by several studies, including a public goods system (part **b**) using wild-type cells (red) and invertase-null mutants (non-producing cells; green) of *Saccharomyces cerevisiae*[50]. **c**,**d** | Biofilm simulations (part **c** and left image, part **d**) illustrate the potential for coexistence between toxin-secreting cells (black) and susceptible cells (orange) when cell lineages segregate in space. The simulations in part **c** also show quiescent, nutrient-deprived cells in grey. The study depicted in part **d**[47] also includes resistant but non-toxin-secreting cells (teal) and experimentally verified the model of coexistence in the left image by using bacteriocin-secreting (unlabelled and thus appearing black), bacteriocin-sensitive (orange), and non-secreting but bacteriocin-resistant (teal) strains of *Escherichia coli* (right image, part **d**). **e**,**f** | Simulations[68,94,95,102] predict that mutually beneficial strains spatially segregate on expanding fronts when mutualism is weak relative to competition (part **e**; mutually beneficial strains are shown in yellow and blue; non-producers are shown in green). This prediction is borne out in experiments with synthetic yeast strains (yellow and blue), which do segregate over time when mutualism is negligible relative to competition[51]. **g**,**h** | When mutualism is strong relative to competition, simulations (part **g**) predict that mutually beneficial strains (yellow and blue) spatially assort together and exclude non-producer strains (green)[68]. Spatial mixing of beneficial genotypes and exclusion of non-beneficial genotypes has been demonstrated experimentally[51,95,102] (part **h**; colours are as in part **g**). Part **a** is adapted from REF. 44. Part **b** is reproduced with permission from REF. 50, Elsevier. Part **c** is adapted with permission from REF. 85, © 2011 by The University of Chicago. Part **d** is adapted from REF. 47, Royal Society. Parts **e** and **g** are adapted with permission from REF. 68, National Academy of Sciences. Part **f** is reproduced with permission from REF. 51, National Academy of Sciences. Part **h** is adapted from REF. 95. The colour schemes in panels **a**, **c**, **d**, **e**, **g** and **h** were altered from the original to facilitate comparison.

secretion, as the matrix confers an advantage in competition for limited space but reduces dispersal ability[30,113]. Quorum sensing and the phenotypes that it regulates are themselves susceptible to exploitation by mutants that either do not produce or do not respond to autoinducers, as has been observed *in vivo* for *P. aeruginosa*[33]. However, biofilm simulations indicate that quorum sensing might act to predict when clonal patches occur along cell group fronts, enabling cells to time the secretion of public goods to avoid exploitation[114].

Quorum sensing has also been found to regulate competitive traits, including the production of bacteriocin by *Streptococcus* spp.[115,116] and *Lactobacillus* spp.[117]. This is consistent with the hypothesis that toxin-secreting strains can mount effective attacks only at a sufficiently high cell density or under restricted fluid transport conditions[107]. Regardless of their population density, toxin secretors cannot gain a net benefit from their antagonistic behaviour without the presence of susceptible target cells. Toxin secretors can detect nearby target cells by sensing many other diffusible cues that are not canonical quorum sensing autoinducers but still correlate with population density[107,118]. For example, *P. aeruginosa* releases the toxin pyocyanin in response to *N*-acetylglucosamine that is shed from the cell walls of Gram-positive bacteria[119].

Another mechanism for detecting the presence of competitors is through the stresses they induce when they are in close proximity. Such 'competition sensing' can manifest as a response to nutrient limitation or, perhaps more reliably, to cell damage[107]. One potentially general way to detect cell damage is through reactive oxygen species, which occur in *E. coli* cells in response to T6SS-mediated aggression and antibiotic treatment[120]. In support of the competition sensing idea, the secretion of antibacterial toxins is indeed commonly upregulated after the recognition of stresses that are associated with competitors (for example, starvation or cell wall degradation), but not stresses that are strictly abiotic in origin (for example, heat or osmotic shock)[107]. For instance, the *P. aeruginosa* T6SS is activated following heterologous T6SS attack from *V. cholerae* and *Acinetobacter baylyi*[29]. Biofilm formation itself also seems to be a response to competition sensing[77]. Increasing evidence suggests that biofilm production confers a competitive advantage to matrix-secreting strains[30–32,79], and natural isolates of *P. aeruginosa* upregulate biofilm production after encountering bacteriocins that have been secreted by competing cells[77]. A recent study suggested another, similar mechanism of competitor detection: *P. aeruginosa* upregulates the T6SS after detecting the solutes released by lysed clonemates[121]. This gives rise to the intriguing idea that bacteria can sense when clonemates have been killed by competition in the vicinity and can respond accordingly (an example of 'danger sensing')[118].

Collectively, these studies show that bacteria experience highly variable chemical and social environments, and that their regulatory networks have evolved to respond to this complexity. The upregulation of social traits is a function of their fitness costs, their benefits, and whether there are cells in the environment, be they friend or foe, that can be targeted appropriately.

Social interactions alter population 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 social phenotypes also feed back and influence the arrangement of genotypes within biofilms. These feedback mechanisms fall into two general categories. First, any of the cooperative or competitive phenotypes discussed above can modify the fitness of neighbouring cells, thereby changing population structure by increasing or decreasing the local abundance of different strains (FIG. 3). Second, many microorganisms can modify their interaction neighbourhoods through adhesion-driven spatial assortment or the secretion of matrix components that organize biofilm architecture.

Fitness modification. Ecologists and evolutionary biologists have long recognized that social interactions influence population structure by locally altering reproductive rates[122–124], and theoretical and experimental work has demonstrated that the same principle applies for microorganisms (BOX 2). For example, public good secretion together with restricted movement and nutrient limitation within a biofilm can lead to patches of a single genotype[44,68,125]. This is partially an amplification of the structure that is caused by limited dispersal. In addition, biofilm growth is often limited to individuals on the advancing edge of a cell group, such that the reproductive fitness of a cell depends strongly on its proximity to the biofilm front[126]. Theory has shown how public good secretion can enable a cooperative genotype to grow

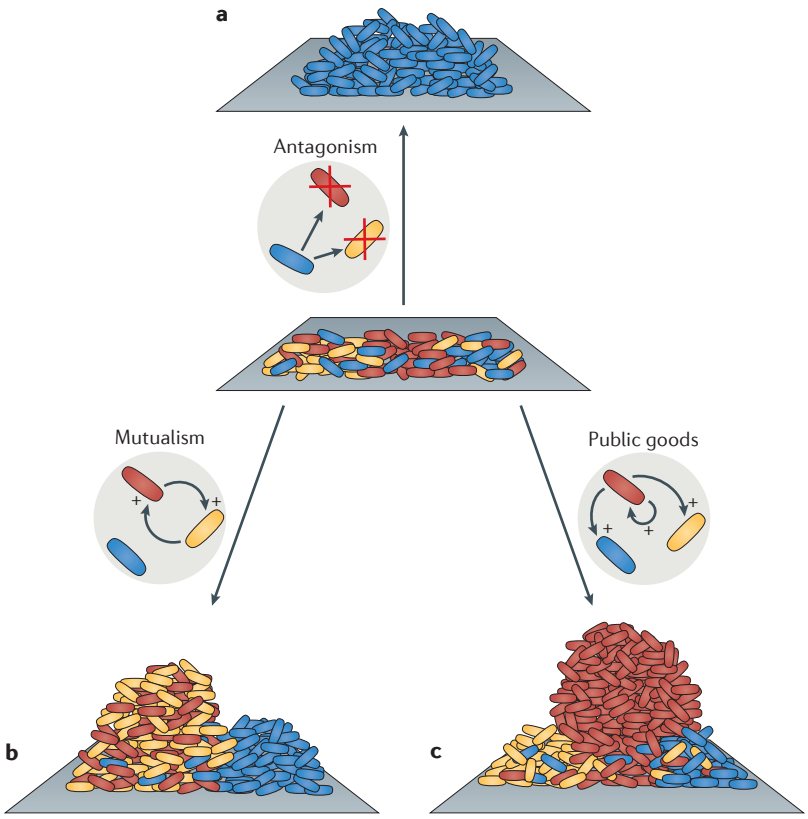


Figure 3 | **The influence of social phenotypes on the spatial structure of biofilm communities.** Cells of the same colour represent distinct cell lineages (that is, different species or different strains within a species). **a** | From an initially well-mixed population, antagonistic phenotypes, such as the secretion of toxins or the expression of the type VI secretion system, can eliminate susceptible cells in the vicinity, culling the population to one genotype. **b** | Mutualistic cell lineages tend to become entangled, as their growth rates are proportional to their spatial proximity. This can result in spatial mixing of the mutualists and exclusion of cheating or non-interacting third parties. **c** | If populations contain limited early clonal clustering (for example, clustering caused by spatial constraint or spatial genetic drift), then cells that secrete public goods can preferentially benefit nearby clonemates (as these cells are closer), which proliferate more rapidly than neighbouring lineages and thus cut these lineages off from the actively growing front of the biofilm.

locally, expand, and propel itself to the colony front at the expense of other strains in the vicinity[44]. This effect cuts off non-cooperative cell lineages from further access to nutrients, which prevents them from replicating for the duration of biofilm growth[10,44] (FIGS 2a,3c). In a recent study, co-inoculation of wild-type *S. cerevisiae* and an invertase-null mutant on agar surfaces provided direct support for this prediction[50] (FIG. 2b). Invertase digests sucrose 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 owing to spatial genetic drift, enabling wild-type invertase secretors to preferentially benefit their clonemates. Clusters of invertase secretors expand more rapidly than clusters of cheating mutants and eventually dominate the entire colony front[50].

Antagonistic and mutualistic interactions also strongly affect the distribution of genetic lineages within biofilm communities. For example, bacteriocin production and T6SS expression can destroy susceptible competitor cells in the vicinity. Some of the earliest experiments that explored antagonistic interactions among bacteria growing on agar surfaces showed local clearance of susceptible cells by bacteriocin-secreting *E. coli*[127]. Consequently, interstrain antagonism can increase genetic segregation by locally eliminating all but one cell type[85]. This finding has the interesting implication that toxin secretion, by reducing the local abundance of other genotypes, breaks down the well-mixed population structure that favoured this secretion in the first place (FIG. 3a). It is perhaps not surprising then that bacteriocidal toxin secretion is often tightly regulated based on the 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[68,94,95,102,128] (FIG. 3b). Mutualistic cell types grow faster in proportion to their proximity and can become entangled as they divide, which can spatially exclude potential cheating strains that do not contribute to the mutualism[68,95,102,129]. This theoretical prediction was first experimentally verified using strains of *S. cerevisiae* that were engineered to behave as obligate mutualists, including an adenine-secreting lysine auxotroph, a lysine-secreting adenine auxotroph and a cheating lysine auxotroph[95,102]. In liquid culture, the cheating strain can exploit the two mutualists, but on solid surfaces, colonies of the two mutualistic strains spontaneously interdigitate, spatially excluding the cheating strain and obtaining a collective competitive advantage (FIG. 2g,h).

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 enable cells to bias their interactions towards preferred partners of the same or other genotypes. Second, it can enable cell lineages to collectively alter their location within biofilms and gain optimal access to limited resources.

Many examples of genotypic assortment are now known in microorganisms and can evolve rapidly in laboratory settings[130]. In this Review, we focus on examples that are most relevant to biofilm-like growth (see REF. 131 for a broader discussion). Different cell lineages of *Neisseria gonorrhoeae* can self-assort from initially mixed populations owing to variation in the density and chemical properties of cell surface pili[132]. The yeast *S. cerevisiae* associates with cells of the same genotype by flocculation under physical and chemical stresses[133]. The resulting flocs, similar to bacterial biofilms, are far more resistant to various environmental assaults than individual cells. Yeast cell aggregation is dependent on the expression of flocculation protein 1 (Flo1), a surface protein that binds to the cell wall of other cells. Cells that lack Flo1 are predominantly omitted from flocs and killed under stressful conditions. In the vernacular of

Flocculation
Aggregation of yeast cells to form large multicellular groups that precipitate from liquid cultures and exhibit heightened stress tolerance.

Greenbeard gene
A gene (or a set of closely linked genes) that is responsible for both an identifying phenotypic trait and a cooperative behaviour that targets that identifying trait, ensuring that the greenbeard gene bearer preferentially benefits other bearers of the greenbeard gene.

social evolution, Flo1 is a greenbeard gene that identifies copies of itself in other cells and selectively confers a cooperative benefit to them[134].

Cells can also increase their chances of residing next to clonemates simply by remaining attached to their progenitors following cell division. Such mother–daughter cell adhesion is pronounced in several facultatively unicellular prokaryotes and eukaryotes, and it is widely thought to be a primary driver of evolutionary transitions to multicellularity[135–137]. Natural strains of *S. cerevisiae* form small multicellular clonal clumps, and laboratory strains that lost this phenotype during domestication can re-evolve it rapidly[138,139]. Moreover, clusters of yeast cells can use cooperative digestive enzymes more efficiently than single cells, which lose most digestion products to advection and diffusion[139,140]. Similarly, bacteria control their population structure using surface adhesion strategies and even their cell shape. Numerous species, such as *Anabaena* spp. and *Streptomyces* spp.[137], undergo incomplete cell division to produce multicellular filaments or clusters that confer protection against environmental stresses, especially predation by protists and the phagocytic cells of host immune systems[141]. The lake-dwelling bacterium *Caulobacter crescentus* exploits its curved shape and a surface-adhesive polar holdfast to increase the likelihood that daughter cells are deposited onto substrata directly adjacent to mother cells under fluid flow. This behaviour creates a foundation on which clonal microcolonies are subsequently built[142].

The secreted matrix, a ubiquitous and defining feature of biofilms, has a central role in organizing local and global biofilm architecture[143] as well as the spatial arrangement of cell lineages[2,17,144]. Shortly after initiating biofilm growth, *V. cholerae* secretes the matrix protein RbmA to enforce tight binding of mother and daughter cells to each other and to the surrounding polysaccharide matrix[145]. Moreover, cell clusters bound by RbmA are protected from invasion by cells in the surrounding planktonic phase, promoting local genetic similarity within the biofilm[146,147].

In addition to genotypic assortment, a second and parallel function of matrix-driven population structuring is to achieve favourable spatial positions within a biofilm community relative to competitors. Individual-based modelling (BOX 2) has identified at least two ways by which cell lineages can improve their spatial position in such contexts (FIG. 4). Secreting extracellular matrix can expand cell cluster volume more rapidly than cell

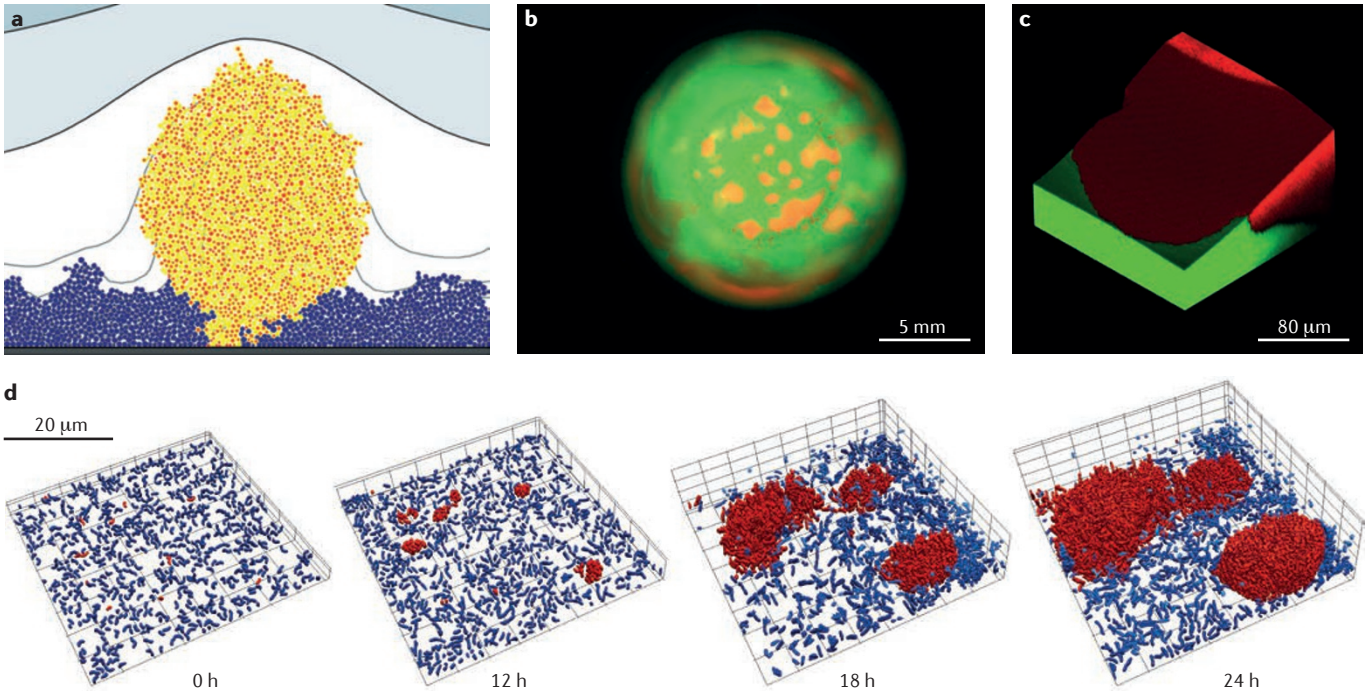


Figure 4 | **Effects of structural matrix secretion on competition in biofilms. a** | Simulations[79] first predicted that if extracellular matrix (yellow) were spatially retained by secreting cells (red), this would enable producer cell clusters to expand in volume more rapidly than non-producing cell clusters (blue), thus propelling the producing cells into regions of higher nutrient availability. **b**,**c** | This prediction was experimentally confirmed[32] in laboratory evolution experiments with *Pseudomonas fluorescens*. When wild-type cells (green) are inoculated on agar, mutants (red) consistently emerge that hyper-secrete matrix relative to wild type. Part **b** shows a top-down fluorescence micrograph of the colony, and part **c** shows a confocal 3D reconstruction of a segment of an agar colony. **d** | Experiments using biofilms grown in microfluidic chambers[30] demonstrated that matrix-secreting pathogenic *Vibrio cholerae* cell clusters (red) also expand in volume over time and gain a competitive advantage against isogenic non-secretors (blue); here, we have re-analysed the published experimental data and rendered these results using single-cell biofilm imaging techniques introduced in REF. 143. This system supports additional simulation predictions[31] that indicate that cell–cell and cell–surface adhesion can enable matrix-secreting cells to physically displace competitors from surfaces. Part **a** is adapted with permission from Xavier, J. B. & Foster, K. R. Cooperation and conflict in microbial biofilms. *Proc. Natl Acad. Sci. USA* **104**, 876–881 (2007). Copyright (2007) National Academy of Sciences, U.S.A. Parts **b** and **c** are adapted with permission from REF. 32, National Academy of Sciences.

division alone, placing a secreting strain at the edge of advancing fronts in a manner analogous to plants competing for access to light[79] (FIG. 4a). This result has been observed experimentally in agar-grown *P. fluorescens* biofilms[32], in which mutants arise that hyper-secrete matrix components and position themselves on the outer surface of colonies (FIG. 4b,c). Another mechanism for improving spatial position within biofilms is simply through strong adhesion to substrata[31]. This result was confirmed in *V. cholerae* biofilms, in which matrix-secreting strains displace non-secreting strains from biofilms through increased cell–cell and cell–substratum adhesion[30,31] (FIG. 4d).

The links between microbial social behaviour and biofilm spatial structure are strongly reciprocal. Phenotypes that help or impair neighbours can alter biofilm structure through their effects on local population dynamics. Microorganisms have also evolved to use specialized adhesins and the extracellular matrix to alter biofilm structure and thereby tip the balance of social interactions in their favour.

Outlook

Our appreciation of the ubiquity of biofilms has dramatically shifted our understanding of microbial natural history[148]. Despite the complexity of biofilm communities, the application of ecological and evolutionary ideas has identified core principles that underlie many of their key properties and phenotypes. Central among these principles is the importance of spatial structuring for the evolution of cell–cell interactions.

Many challenges remain, however. Studies of spatial organization in microbial communities have relied mostly on laboratory assays that do not always replicate natural environments[149]. Advances in microfluidics and microscopy, including single-cell imaging of biofilm-dwelling bacteria[143], have greatly improved our ability to study complex biofilm microhabitats[37,150]. Nonetheless, we know relatively little about the spatial details of cell–cell interactions in ecologically realistic settings (but see REFS 38,42,151–153 for new advances on this front). Thus, several important questions remain to be answered. 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. New theoretical and experimental tools are required to determine how the ecological and evolutionary dynamics that occur within biofilms relate to the compositional changes in community structure that have been 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 to promote the function of such microbial communities is a priority for modern microbiology. What, then, is the key to making or breaking a productive biofilm? We predict that, all else being equal, clonal patchiness will often increase ecological productivity by stabilizing local cooperative traits and reducing 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 will promote productivity. However, highly connected networks of mutualism may be unstable because the loss of a small number of species can compromise the whole community[154]. How to shape microbial interactions to control biofilm productivity and stability is a fundamental question for the future.

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Competing interests statement
The authors declare no competing interests.