Mechanisms of Competition in Biofilm Communities

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ABSTRACT Bacterial biofilms are dense and often mixed-species surface-attached communities in which bacteria coexist and compete for limited space and nutrients. Here we present the different antagonistic interactions described in biofilm environments and their underlying molecular mechanisms, along with ecological and evolutionary insights as to how competitive interactions arise and are maintained within biofilms.

One general law, leading to the advancement of all organic beings, namely, multiply, vary, let the strongest live and the weakest die.

CHARLES DARWIN, The Origin of Species

INTRODUCTION

The rapid development of new sequencing technologies and the use of metagenomics revealed the great diversity of microbial life and enabled the emergence of a new perspective on population dynamics. Moreover, it has highlighted the central role of social interactions in ecological and evolutionary processes. Microbes living in multispecies communities are prevalent in nature and have been shown to extensively cooperate and compete. Both intra- and interspecies interactions are instrumental in major geochemical cycles and are important in human health and homeostasis (e.g., the human microbiome has been associated with several diseases) and in industrial and clinical settings (2). Few studies have addressed the role of individual species within mixed communities (3), and they generally focus on cooperative interactions and increased benefits of community life (4, 5). However, recent work pointed out that most interactions are competitive rather than cooperative, suggesting that adaptation is more likely achieved by competitive success (6).

A further degree of complexity in understanding multispecies interactions and dynamics is brought by increasing evidence suggesting that phenomena occurring in complex communities cannot be predicted by the observation of single-species communities (7).

How do biofilm characteristics contribute to shape the evolution of competitive social interactions? What are the different competitive strategies deployed by bacteria when forming biofilms? Here, we describe exploitative and interference competition strategies, with a special focus on the underlying molecular mechanisms involved; we offer some insights on the evolutionary origins and ecological consequences of competition and reflect on new venues for this exciting multidisciplinary field.

ECOLOGICAL AND EVOLUTIONARY PARAMETERS OPERATING WITHIN BIOFILMS

Our current understanding of microbial physiology is mostly based on studies performed in homogeneous batch cultures. This reductionist approach enabled an experimental simplicity that has led to major discoveries but has largely neglected the complexity of the microbial...
world. Indeed, extrapolating characteristics observed in liquid to traits potentially relevant to a community context could be misleading. In nature, bacteria display complex multicellular behaviors and influence each other, enabling them to perform a great diversity of tasks that they would not otherwise accomplish in liquid monocultures. The following is a review of some important parameters for the study of competition within biofilms (Fig. 1).

**Consequences of Clustering: Group Effects**

Life in groups or aggregates is a common trait found in all forms of life, from animals to bacteria. The Allee effect suggests that there is an inherent benefit to grouping, evidenced by a positive correlation between population size or density and mean individual fitness (8). This phenomenon has been extensively studied; for instance, it was demonstrated that grouping provides animals with enhanced resistance to predation (9), affects mating and reproduction efficiency in mobile organisms (10), and increases prokaryote resistance to desiccation (11). In bacteria, it has been shown that survival of *Streptococcus mutans* growing on the surface of teeth and experiencing acidic stress is strongly density-dependent (12). Other examples of the Allee effect include trade-offs between dispersal and survival in *Escherichia coli* populations (13) and an increased tolerance to several antibiotics when bacteria are at higher cellular densities. This tolerance to antibiotics is independent of biofilm formation, quorum sensing (QS), and antibiotic resistance pathways (14).

Although grouping is often beneficial, the physiological consequences of increased cell density in bacterial clusters can enhance competition. In the case of biofilms,
bacteria are encased in a self-produced biofilm matrix that acts as a molecular sink or reservoir due to limited outward diffusion and/or retention of compounds by the matrix. Therefore, antagonist molecules released by bacteria are more concentrated in local areas and lead to increased efficiency against neighboring competitors (15). Finally, grouping enhances competitive interactions, the effects of which are strengthened when cellular densities are high and resources low, for example, via QS-dependent regulation and other strategies controlled by positive feedback loops (16).

**Cooperation in Multispecies Biofilms**

In contrast to competitive forces, spatial proximity may also allow for cooperative interactions between microbes (17). Coaggregation, or recognition and adhesion between genetically distinct bacteria (18), can be advantageous and develop into mutualistic behaviors that impose few, if any, associated costs to the interacting partners. Examples of such interactions are (i) cross-feeding of two species (19, 20), (ii) more efficient degradation of herbicides by a three-species biofilm than comparable single-species or dual-species biofilms (21), and (iii) altruistic and/or synergistic degradation of toxic compounds, allowing growth of other sensitive species (22, 23).

However, more often than not, there are costs associated with cooperation, and understanding the evolution of such a social investment in Darwinian terms can be challenging, in particular, when the burden of cooperative behavior is costly enough to impact the individual reproductive fitness of the cooperators. During cooperative interactions, “cheaters,” (individuals that benefit from the cooperation but do not pay the costs associated with it), can emerge and eventually invade the population due to higher individual fitness than cooperators. The dilemma of cooperation is also relevant during biofilm formation and matrix production, which strongly rely on the cooperative secretion of shared compounds, also referred to as “public goods,” such as exopolysaccharides (EPSs) and iron siderophores, and to whose production all members should contribute (24).

Hamilton proposed that the evolution of cooperation can be explained by an indirect fitness effect, which occurs when cooperation is directed toward kin, individuals with high genetic relatedness (25). Consequently, perfect cooperation in biofilms should only occur in niches populated by one genotype or highly related genotypes. In this situation, there is no evolutionary competition, and genotypes behave optimally for the group (26). Additionally, several mechanisms have been described to control cheaters (27, 28) and to ensure the maintenance of cooperation (29). This includes cheater policing (30) or preferentially directing cooperation benefits toward kin (i.e., kin discrimination and green beard effect (mechanism by which natural selection favors altruistic behavior toward kin) [31]).

Interestingly, as predicted by theoretical models (32), biofilm characteristics such as proximity, limited dispersal, and spatial structure could enhance cooperation *per se* by increasing genetic relatedness between the interacting partners and avoiding the spread of cheating genotypes. This is supported by experimental work showing that cooperative bacteria have greater fitness than cheaters in biofilms (33). More particularly, thick biofilms limit the diffusion of public goods through the biofilm so that only producers and their immediate neighbors have access to it (15, 33).

**Kin Competition in Biofilms**

When resources are scarce, biofilm bacteria have to compete not only against other species but also against their own kin, therefore limiting the benefits of cooperative behaviors (34, 35). Reports of the persistence of cooperation found in nature, either by establishment of lifelong symbiosis or isolated collaborations, indicate that the biofilm lifestyle presents evolutionary advantages despite competition (kin or not) and/or cheater apparition (36). In addition to Hamilton’s rule (see above) and aforementioned mechanisms, the evolution of cooperation can be favored by ecological factors influencing both benefits and strengths of the competition, even when kin competition is harsh (37). For instance, the spread of cheaters and their invasion is strongly determined by the scale of the competition (34). Moreover, cooperation is facilitated over competition if the public good increases group productivity (37). Taylor revisited Hamilton’s rule and proposed a more integrative model for kin selection including competition, patch structure, and their effects on the evolution of cooperation (17). Such a model might more accurately represent biofilm communities.

A widespread weakness when studying kin competition is that only one social trait is usually taken into account (for instance, siderophore secretion). Reports that consider several cooperative traits (e.g., siderophore secretion, bacteriocin production, virulence factors, matrix production) at once and analyze how they are impacted by social interactions are scarce (38). Additionally, most studies investigating the emergence of cheaters are performed in single-species communities, without the additional level of complexity added when nonrelated species are introduced into the community (39, 40) (see below).
Genetic and Phenotypic Diversity

Biofilms are highly heterogeneous environments in which different microniches can coexist. They are characterized by a vertical heterogeneity where the deeper layers of the biofilm are thought to be under strong starvation conditions, reduced oxygen diffusion, and accumulation of waste products. There is also a heterogeneity corresponding to the presence of small patches of tightly packed cells separated throughout the biofilm by the extracellular polymeric matrix and water channels (41).

Hence, biofilm bacteria might have different growth rates, suffer from different stresses, and accordingly, adopt different physiologies (2). The existence of small and diverse microniches within biofilms provides an excellent landscape for adaptive radiation and, consequently, genetic and phenotypic divergence. Several experimental studies indicate that structured habitats lead to increased diversity due to frequency-dependent selection and to adaptation of to unexploited microniches generated by spatial heterogeneity (42). Indeed, key innovations to exploit new niches in a biofilm rapidly and readily arise given the opportunity (43, 44) (see “Experimental Evolution in Biofilms,” below).

Ecological competition plays a key role in maintaining diversity in biofilms, because it is unlikely that one genotype is well adapted to all environmental conditions of heterogeneous structures. Consequently, potential trade-offs across the different microniches of biofilms allow for the coexistence of different adapted genotypes (43). The resultant self-diversification ensures that in changing conditions a subset of the population will still be adapted and have an advantage (43). For example, Pseudomonas aeruginosa biofilm cells diversify into different genotypes that show enhanced dispersal or faster formation of biofilms (43).

Biofilms have two inherent characteristics that maintain diversity: limited dispersal of genotypes (clones remain in the neighborhood) and the existence of defined spatial structure (i.e., heterogeneity). This results in the coexistence of different competing genotypes, whereas mixing and enhanced diffusion results in the establishment of the fittest genotype (45). Other mechanisms such as negative frequency-dependent selection also allow variants adapted to a microniche to be maintained (44). Finally, the existence of high genetic diversity also implies that the ability of one genotype to overtake an entire population is slowed down because it has to displace competitors with different beneficial mutations. Taken together, there is increased genetic diversity caused and maintained by the ecological competition and spatial structure found within biofilms.

Understanding the diversity within a community is a useful first step to elucidating its distribution patterns in a given environment and identifying mechanisms of competition within multispecies communities. Since the launch of the Human Microbiome Project, next-generation sequencing has made tremendous progress and considerably lowered the detection limits for underrepresented taxa in microbial populations. The use of new diversity indexes taking into account both high and low abundant taxa (46) will likely improve our understanding of ecological diversity in host (i.e., mouth, intestine, skin), and environmental biofilm niches across individuals or between populations.

Genetic Expression Profiles

Biofilm bacteria have very different gene expression profiles compared to planktonic lifestyles, with up to a 10% difference between transcriptomes (47, 48). Aside from genes involved in general metabolic function, these studies show that stress response genes, energy production, and envelope-associated genes are upregulated in biofilm bacteria (47, 49, 50). Differences between biofilm and planktonic cells may also be relevant during competition because many upregulated loci during biofilm development still have no known function (47, 50).

Moreover, recent studies reported that biofilm gene expression profiles are highly dynamic and vary throughout the developmental stages of biofilm. For instance, dispersal of Streptococcus pneumoniae biofilm completely changes its gene expression profiles compared to planktonic biofilm and bacteria in early stages of biofilm formation (51). These changes in expression include genes involved in the synthesis of rhamnolipids and other molecules already known to have antibiofilm effects on other bacteria (52, 53). These novel biofilm gene expression patterns could lead to biofilm-specific functions associated with a potential role in ecological competition (54).

MECHANISMS OF BIOFILM COMPETITION

Ecological competition can be classified into two groups. The first type, known as “exploitative competition,” refers to indirect interactions between organisms, by which one organism prevents access to and/or limits the use of resources by another organism (55, 56). Exploitative competition is common across the biological spectrum; however, it is particularly acute within biofilms, which are already nutrient-depleted environments (57). The second type of ecological competition is direct or “interference competition,” which corresponds to
specific mechanisms which damage competitors’ survival or their access to an ecological niche or specific resources (Table 1). This can be illustrated by the production of antibiotics and other growth-inhibiting mechanisms that lower bacterial fitness. In the context of biofilms, numerous interference strategies that do not alter growth but do alter the ability to colonize a niche have been recently described (see reference 58 for review) (Table 1). Both exploitative and interference competition mechanisms strongly affect evolutionary outcomes and population dynamics. Interactions between both mechanisms remain largely unaddressed; nevertheless, theoretical studies predict that the combination of these two strategies should result in increased biodiversity or the coexistence of genotypes rather than exclusion or extinction (59).

**Exploitative Competition**

The current general view considers that most biofilm bacteria live under severe environmental conditions, characterized by low nutrient concentrations and low rates of gas renewal or exchanges. This situation is particularly common in the deep layers of a biofilm, where diffusion is difficult (41). Because growth is dependent on the concentration of limiting resources (iron, carbon, oxygen, etc.) in this biofilm environment, the availability of such resources drives competition fate.

In air-liquid interface biofilms (also called pellicles), competition for oxygen can determine the success or failure of one species to cocolonize the surface. For instance, *Brevibacillus* is an obligate aerobe that can grow in pellicles in a multispecies consortium composed of four species including the facultative aerobe *Pseudoxanthomonas*. However, in pair-wise competition with *Pseudoxanthomonas* spp., *Brevibacillus* initially dominates, but its viability rapidly decreases due to severe competition for oxygen (60). These results show that competition between aerobes in the air-liquid interface is strongly driven by access to oxygen. Moreover, population dynamics can also be further altered by other ecological and biotic factors, such as the presence of other species in the consortium (60).

In addition to oxygen, exploitation of a variety of essential elements, including carbon, phosphorous, and especially iron have been reported (61–63). Iron is a key element in microbial physiology, controlling many bacterial functions including virulence and biofilm formation. Due to its low bioavailability, bacteria have evolved many iron-scavenging strategies, such as high-affinity iron-chelators or siderophores. Theoretical models have pointed out the advantages of producing such public goods (i.e., iron-sequestering molecules) during competition. Siderophore-producing genotypes increase their own growth rate while they deplete iron in their surroundings and potentially limit access to it for other genotypes (64). Besides iron limitation and competition for iron acquisition, iron sequestration can be used by bacteria to manipulate coexisting microorganisms. For instance, a *Burkholderia* sp. is able to alter the physiology of neighboring *P. aeruginosa* bacteria through the production of the ornibactin siderophore. *P. aeruginosa* specifically responds to ornitobactin secreted by *Burkholderia* and induces genes usually expressed under genuinely low levels of iron (63). Whether ornitobactin affects the fitness of *P. aeruginosa* is still unclear. Taken together, these results suggest that iron might drive not only intra-species competition, but also interspecies interactions between *Pseudomonas* and *Burkholderia*.

**TABLE 1** interference competition. Summary of the interference strategies described in this chapter

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ASMscience.org/MicrobiolSpectrum
Interference Competition

Different interference competition strategies, which are also relevant in biofilm conditions, have evolved; for instance, some target bacterial ability to form a biofilm, whereas others are less specific and kill or limit the growth of competing bacteria within mixed biofilms (Table 1). Many studies addressing interference competition usually start with the identification of antagonist molecules produced by the interacting strains in liquid monocultures. Although this might not be the optimal approach to understand the reality of ecological competition in natural settings, it has certainly been successful, for instance, leading to the identification of antibiotics and many other antibacterial toxins. However, this approach also potentially misses other competitive possibilities. As previously stated, changes in gene expression associated with the biofilm lifestyle (47, 48) can lead to production of biofilm-specific metabolites and polymers (54), some of which can display antagonist activities against other microorganisms in mixed species contexts (65–67). In addition to competitive interactions induced by the biofilm lifestyle, interference mechanisms might also be triggered in response to the presence of competition in the surroundings, also known as the competition-sensing hypothesis (57). Hence, bacterial defense mechanisms would only occur when bacteria are under stress due to the harmful action of other competing bacteria rather than being constitutively expressed. This hypothesis also differentiates harm imposed by other bacterial species (nonkin) from harm originating from cells with the same genotype (kin) for which there is no evolutionary competition because all bacteria seek the same interests (57). In the context of competition, kin could be potentially differentiated from nonkin by self-produced molecules such as QS signals. Furthermore, Cornforth and Foster propose that stress response regulatory networks could integrate the source of the competition (kin or nonkin) with the level of stress/damage experienced by the sensing bacteria and subsequently respond in an appropriate manner (57).

Here, we describe ecological interference mechanisms within biofilms (Table 1 and Fig. 2). Although most mechanisms are not biofilm-specific, selected examples gain special relevance in biofilms or were shown to play a role in mixed communities.

Interference mediated by growth inhibition

Environment alteration

Bacterial fitness, or survival, is environment-dependent. One mechanism by which bacteria can negatively impact competitors is by changing their local environment either directly or as a consequence of their secondary metabolism and physiological by-products. Lactobacilli spp. produce lactic acid that lowers environmental pH and hence limits the growth of other bacterial species. Indeed, growth of Neisseria gonorrhoeae in mixed cultures with vaginal Lactobacilli spp. was severely reduced due to the acidification of the environment. When a suitable pH is sensed, N. gonorrhoeae can resume growth (68). Other examples of molecules able to alter surrounding conditions are volatile compounds such as trimethylamine secreted by E. coli and other

![FIGURE 2](ASMscience.org/MicrobiolSpectrum)
Gram-negative bacteria in intestinal or precursor-rich environments. These compounds increase environmental pH and affect biofilm formation and stress resistance (69). Although volatile compounds can potentially be secreted in liquid conditions, their production is strongly density-dependent, and therefore their effect in biofilms is magnified.

**Toxic metabolic byproducts**

Bacteria also use low-molecular-weight compounds derived from their own metabolism to kill and gain competitive advantage over surrounding bacteria. One of the best-described effects is hydrogen-peroxide-mediated killing. Hydrogen peroxide (H$_2$O$_2$) is a byproduct of aerobic metabolism from a reaction usually mediated by a pyruvate oxidase (70). The role of hydrogen peroxide in competition has been demonstrated in several environments. In the human nasopharynx, *S. pneumoniae* produces hydrogen peroxide against *Neisseria meningitidis* and *Moraxella catarrhalis* (70), whereas *Streptococcus sanguinis* and *Streptococcus gordonii* are able to inhibit *S. mutans* in dental plaque (71). However, this competitive advantage is strongly dependent on abiotic conditions. Levels of oxygen and glucose modulate the production of hydrogen peroxide by SpxB pyruvate oxidase in *S. sanguinis* and *S. gordonii* as well as the production of other growth-inhibiting factors, such as bacteriocins by *S. mutans* (71).

**Small antimicrobial compounds**

**Colicins.** A widespread mechanism of defense used by 50% of the *E. coli* biodiversity against closely related genotypes is the production of colicins and microcin (72). Colicins are typically encoded by small plasmids that enable horizontal transfer of colicin production. Colicinogenic bacteria are resistant to colicin biocidal action via a constitutively expressed immunity gene coded downstream of the colicin gene. For lethal activity, most colicins are released into the extracellular medium by cell lysis, presumably encoded by the *kil* gene carried in the plasmid. Colicins interact with target cells by direct binding to cell surface receptors and are subsequently internalized. Colicins display two distinct mechanisms of action: they can either form pores in the inner membrane (activity encoded in the C-terminal of the colicin) or display an enzymatic activity that will degrade DNA or RNA, both of which will result in cell death (for a review on colicin biology, see reference 73). Both theoretical and experimental work showed that toxin producers always outcompete the nonproducer.

In addition, colicin production also enables the invasion of pre-established microbial communities and resistance to other species invasions (74, 75).

Recently, a study described a new pore-forming colicin, colicin R, specifically produced within biofilms (65). Colicin expression is tightly regulated by the SOS response (76), which has been shown to be highly induced in mature biofilms (77). This stress response leads to the production of colicin R and subsequent local concentration of colicin within biofilms due to limited diffusion. The release of colicin R conferred a strong competitive advantage in mixed biofilms. Finally, colicin release by one strain within mixed communities induces the colicin production by a second strain and vice versa (78).

Other Gram-negative bacteria also produce small antimicrobial peptides similar to colicins, such as pesticins from *Yersinia pestis*, klebicins from *Klebsiella* spp., pyocins produced by *Pseudomonas* spp., or limicins of *Photorhabdus luminescens* (73).

**Bacteriocins.** A wide range of antimicrobial peptides are generically and misleadingly termed bacteriocins, but strictly speaking, bacteriocins are proteinaceous toxins produced by 3 to 23% of Gram-positive bacteria, notably, lactic acid bacteria (79). They are synthesized in the form of premature peptides with an N-terminal signal sequence, typically accompanied by an immunity protein to protect the producers. As observed for interference molecules, some bacteriocins can be produced in a biofilm-specific fashion, for instance, mutacin I of *S. mutans* (80) or bacitracin produced by *Bacillus licheniformis* (66). Bacitracin was only detected within biofilms, but addition of spent biofilm supernatant to planktonic cells of *B. licheniformis* triggered bacitracin production, suggesting the presence of some inducer in the biofilm supernatant (66).

**Contact-dependent growth inhibition.** Contact-dependent growth inhibition (CDI) is a mechanism of interbacterial competition originally described in uropathogenic *E. coli* strains (81) and now shown to exist in many species such as *Pseudomonas fluorescens* and *Bibersteinia trehalosi* (82). CDI functionality is encoded by three genes, *cdiBAI* (in *E. coli*), and mediated by a two-partner secretion system. The effector, CdiA, needs cell-to-cell contact to inhibit growth and be released into the cytoplasm of the targeted bacteria. Toxic activity, often due to a nuclease, is encoded in the terminal residues of the CdiA. CdiB aids the secretion across the outer membrane of CdiA, whereas CdiI encodes the immunity protein (81). One of
the targets of CDI is the outer membrane protein, BamA. Further, acrB mutants, which fail to produce a protein that acts downstream of BamA, are also resistant to CDI (83). An analogous CDI system (BcpAIOB proteins) was recently reported in Burkholderia thailandensis. These proteins are involved in the biofilm formation process, suggesting a cooperative role. However, biofilm cocultures showed that BcpA also mediated intraspecific competition and was involved in community exclusion of nonkin (84).

Additionally, other CDI systems have been revealed by an experimental evolution setup using E. coli as a model. After serial passaging of independent E. coli populations, parallel mutations in the glgC gene emerged. This resulted in strong inhibition and subsequent domination of ancestral/competitive strains (85). This competition mechanism was induced during stationary phase (and termed SCDI, stationary contact-dependent inhibition), a physiological state that resembles biofilms, in which cells are not required to actively grow (86).

Another widespread contact-dependent system that recently received a lot of attention is the type 6 secretion system (T6SS), which is able to inject directly a toxic compound not only into eukaryotic cells, but also into other competitor bacteria (87, 88). Although it has been more studied for its role in virulence, T6SS fulfills several roles including bactericidal effect, kin recognition, and competitive growth (89). T6SS of B. thailandensis is required for colonization and persistence within biofilms. Moreover, in mixed flow cell biofilms, T6SS conferred an ecological advantage because it protected B. thailandensis from invasion by other competitor species, for example, Pseudomonas putida (90).

**Predation.** Several predation mechanisms have been described, including (i) phagocytosis by the unicellular amoeba Dictyostelium discoideum, (ii) cell invasion via penetration through the outer cell wall such as Bdellovibrio bacteriovorus mediated by type IV pili, (iii) predation by diffusible factors (secondary metabolites such as antibiotics) such as Streptomyces spp., and (iv) community-dependent predation displayed by the soil-dwelling bacterium Myxococcus xanthus (91). M. xanthus, often described as a “bacterial wolfpack” (91), hunts cooperatively by tightly coordinating community movement (rippling) and then releasing a myriad of secondary metabolites (92), antibiotics (93), polyketides, and degrading enzymes into the local environment. Despite these observations, the precise molecular mechanisms of predation remain to be elucidated.

Myxobacterial ubiquity in soils world-wide and the ability to prey on a broad spectrum of microorganisms (bacteria and fungi) suggest that M. xanthus may have a great impact on population and evolutionary dynamics in the soil (94).

**Interference mediated by alteration of biofilm development**

The increase of fitness in biofilms relies on the ability of a given strain not only to adhere, settle, and develop as a biofilm, but also to inhibit others from doing so. Bacteria have evolved different strategies that prevent other bacteria to form or colonize existing biofilms. All steps of biofilm formation can be targeted: from inhibition of initial adhesion to matrix degradation to jamming of cell–cell communications, and induction of biofilm dispersion (58). Biofilm-inhibiting strategies have gained interest recently for their potential applications in the industrial and medical settings as an alternative to the use of antibiotics (for review see references 95–97) (Fig. 2).

**Inhibition of cell-to-cell communication**

Biofilm formation triggers quorum sensing (QS), a density- and dose-dependent communication system that coordinates gene expression at the community level (98, 99). Disruption of QS-regulated genes was shown to interfere with the ability to form a biofilm (100). Some bacteria have evolved mechanisms by which they degrade QS molecules; for instance, Gram-negative bacteria use acyl homoserine lactones that can be digested by several enzymes synthesized and released by competing bacteria (101–103). Spent bacterial supernatants containing phenolic groups and aliphatic amines inhibit biofilm formation of P. aeruginosa PAO1 (104), or production of AiiA, an AHL-lactonase, by Bacillus cereus inhibits Vibrio cholerae biofilm formation (105).

In mixed P. aeruginosa and E. coli biofilms, production of indole determines E. coli fitness, since mutant bacteria unable to synthesize indole are rapidly out-competed by P. aeruginosa. It was shown that indole is able to block production of the pyocianin toxin and other QS-related phenotypes of P. aeruginosa, suggesting that indole affects QS signaling and alters population dynamics both in liquid cultures and in biofilms (106).

The oral environment provides several ecologically relevant examples of secreted degrading enzymes that inhibit communication signals of coexisting species. Colonization by S. mutans, the primary etiologic agent of human dental caries, relies on a competence-stimulating peptide (CSP), an essential QS molecule. However,
competing streptococci, such as *S. gordonii*, *Streptococcus salivarius*, *S. sanguinis*, *Streptococcus mitis*, and *Streptococcus oralis*, are all capable of secreting degrading enzymes that inactivate CSP and inhibit *S. mutans* biofilm formation (107, 108). These CSP-degrading enzymes were shown to be relevant in mixed biofilms, because *S. salivarius* had a strong competitive advantage over *S. mutans*. Moreover, *S. mutans* resistance to bacteriocin is also regulated by CSP.

Although the prevalence of QS-inhibitory molecules has been documented in different marine and soil environments (109, 110), assessing the importance of QS-inhibitory strategies in nature remains difficult, because resistance can emerge readily (111, 112). Some authors suggest that bacteria could even escape from QS inhibition without undergoing genetic changes (113).

**Inhibition of adhesion**
The first stage of interaction between bacteria and surfaces (initial adhesion) are crucial for colonization and biofilm development. Mechanisms interfering with these first steps generally involve (i) secreting specific molecules that alter surface physico-chemical properties of the surface itself, (ii) downregulation of key bacterial adhesion factors, or (iii) modulation of microbe-microbe interactions (Fig. 2).

**Modification of adhesion by surface-active compounds.**

Surface-active compounds (SACs) produced by microorganisms or biosurfactants are amphipatic lipid-based molecules that lower interfacial tension (114, 115). SACs are diverse in their biochemical nature and include glycolipids (rhamnolipids, trehalolipids, sophorolipids), lipopeptides and lipoproteins, fatty acids and phospholipids, and other polymeric biosurfactants (115). Some of these surfactants display antimicrobial properties. However, in recent years, many SACs have also been described to inhibit biofilm formation without affecting growth (for review, see reference 116). These antibiofilm effects are likely due to alterations in the wettability and surface charges induced by SACs on treated surfaces (114). These modifications impact bacterial interactions and bacteria-surface interactions and weaken the bacteria’s ability to adhere and form a biofilm. For instance, polysaccharides specifically produced within biofilms formed by *E. coli* natural isolates (Ec300 and Ec111) were shown to inhibit biofilm formation by Gram-positive bacteria (*Staphylococcus aureus*, *Streptococcus epidermidis*, *Enterococcus faecalis*) but not by Gram-negative bacteria (*E. coli*, *Enterobacter cloacae*, *P. aeruginosa*, *Klebsiella pneumoniae*) (117). These polysaccharides were also shown to alter the properties of abiotic surfaces by lowering interfacial energy and increasing the hydrophilicity of treated glass surfaces, leading to inhibition of bacterial adhesion (117). The production of such bacterial surfactants protected *E. coli* biofilms from colonization by other biofilm-forming species (*S. aureus*), showing a competitive advantage for the production of otherwise costly polysaccharide (117). Other SAC polysaccharides, such as group 2 capsules produced in both planktonic and biofilm conditions by uropathogenic bacteria, affect abiotic surfaces by increasing their hydrophilicity and biotic surfaces by altering Lewis base properties (118, 119).

**Downregulation of adhesion factors.** Bacteria secrete different molecules into the extracellular medium including digestive enzymes and polysaccharides, both of which were shown to regulate gene expression of ecological competitors. For instance, released polysaccharides of *Lactobacillus acidophilus* are able to inhibit biofilm formation of a broad range of bacterial strains including enterohemorrhagic *E. coli* (EHEC), *Salmonella enteritidis*, *Salmonella enterica* serovar Typhimurium, *Yersinia enterocolitica*, *P. aeruginosa*, and *Listeria monocytogenes* and modify gene expression of *E. coli*. Exopolysaccharides downregulated genes involved in chemotaxis (cheY) and adhesion (curli-associated genes *crl, csgA*, and *csgB*) and resulted in a dramatic decrease of *E. coli* biofilms in mixed cultures with *L. acidophilus* (120).

Similarly, *Streptococcus intermedius* releases an arginine deaminase, which affects expression of different fimbriae of *Porphyromonas gingivalis*, a coexisting strain in the oral microbiota (121).

**Matrix degradation**
The extracellular matrix of a biofilm is an important element involved in cohesion and structure, resistance to both physical and chemical aggressions, and other functions (122). The biofilm matrix is composed of proteins, nucleic acids, polysaccharides, amyloid fibers, vesicles, and ions. Several compounds have been described as targeting the expression, assembly, or integrity of matrix components and can negatively impact biofilm fitness. Dissolution of the biofilm matrix results in reduced biomass, increased cell exposure to other growth-inhibiting molecules, bacterial dispersal, and ultimately, liberation of an ecological niche. Many broad-spectrum nucleases targeting DNA and RNA (123–125) as well as polysaccharide-degrading enzymes (126–128) have been described. More species-specific
enzymes have also been studied; for instance, Esp secreted by S. epidermidis inhibits colonization and degrades matrix-associated proteins of S. aureus (129, 130). Similarly, Streptococcus salivarius, a commensal bacterium covering the oral epithelium, tongue, and throat produces an exo-beta-d-fructosidase or fructanase (FruA) shown to inhibit biofilm development of other oral coexisting bacteria, such as S. mutans, the primary etiologic agents of human dental caries (127).

Biofilm dispersal
Biofilm dispersal is a natural step in the biofilm lifecycle by which cells leave the biofilm and re-enter the planktonic lifestyle. Dispersal is triggered by different environmental cues such as nutrient and oxygen levels, physiological cues, and other cellular signals such as second messengers and QS (see reference 131 for a review). Dispersal often results in cell death, induction of bacterial motility systems, downregulation of adhesins, and secretion of matrix-degrading enzymes. Despite many published studies, dispersal remains one of the most complex and less understood steps of the biofilm cycle, which was mainly studied in single-species contexts. Hence, it is difficult to determine whether any of the identified dispersal molecules are primarily involved in biofilm self-control mechanisms or contribute to competitive strategy against other biofilm-forming bacteria.

Several diffusible signal factors have been described to trigger biofilm disassembly in different bacterial species. First described in the plant pathogen Xanthomonas campestris, small cis unsaturated fatty acids can induce biofilm dispersal of a broad range of bacteria and are also involved in cross-kingdom interactions (132). Nevertheless, these molecules also have a physiological impact on the strain producing them, and failure to produce diffusible signal factors can result in altered biofilm phenotype (133). Similarly, P. aeruginosa is also capable of producing other antibiofilm molecules such as cis-2-decenoic acid known to disperse K. pneumoniae, E. coli, Bacillus subtilis, and S. aureus (134) or rhamnolipids able to disperse Bordetella bronchispetica biofilms (135, 136).

Another well-studied dispersing signal is nitric oxide, which is released in the deeper layer of the biofilm, where oxygen is scarce. Nitric oxide upregulates a phosphodiesterase that degrades the second messenger c-di-GMP, resulting in low intracellular levels, which in turn trigger dispersal mechanisms of both monospecies and multispecies biofilms (137). Additionally, nitric oxide was reported to, among other things, downregulate the expression of adhesins in P. aeruginosa (138).

Recently, several other signals were reported to trigger biofilm disassembly in B. subtilis: D-amino acids and norspermidine (139, 140). These signals were shown to have biofilm-inhibitory activity against other competing strains (140, 141). However, whether D-amino acids and norspermidine are self-produced by B. subtilis and play a role in biologically relevant contexts is controversial (142, 143).

Motility-based interference
Bacterial motility per se or in combination with other mechanisms is a competitive strategy that is often neglected, and its impact on bacterial evolution has only recently received some attention (144, 145). In non-motile organisms, adaptation ultimately occurs through increases in the intrinsic average reproductive rate (growth rate), exploitation of novel resource niches (exploitative competition), or hindering direct competitors (interference competition) as shown in many experimental evolution studies. However, mobile organisms can additionally adapt by modifying the rate, pattern, or energetics of their movements, which could lead to a fitness increase. Moreover, motility allows microorganisms to escape from competition, or from an exhausted niche, and most remarkably, motility is instrumental in bacterial predation. Additionally, several elements implicated in motility are also instrumental in the early stages of biofilm formation, such as type IV pili (146).

Several mechanisms have been described to illustrate the importance of motility in competition within biofilms. First, An and colleagues described a strategy called “surface blanketing” (147). In this case, bacteria compete not for a limited resource but for access to an appropriate surface and subsequent colonization. In cocultures with Agrobacterium tumefaciens, P. aeruginosa displays an increased growth rate, which enables it to rapidly consume available resources and occupy the ecological niche. P. aeruginosa spreads through the surface via swarming and twitching motility, preventing A. tumefaciens adhesion. Consistently, a P. aeruginosa flgK motility-deficient mutant unable to spread quickly over a surface was no longer able to exclude A. tumefaciens (147). In addition to surface blanketing, in iron-depleted situations, P. aeruginosa is also able to secrete a compound(s) that inhibits and disperses A. tumefaciens independently of QS (148). Bacteria can also force out competitors by stimulating their motility. E. coli produces BdcA, a protein that binds cyclic di-guanosine monophosphate, c-di-GMP, a ubiquitous bacterial second messenger, regulating both dispersal and motility. Upon BdcA binding, intracellular levels of c-di-GMP are
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reduced and motility is stimulated, forcing bacteria out of the biofilm. In a multispecies biofilm composed of *E. coli*, *P. aeruginosa*, or *Rhizobium meliloti*, *E. coli* is able to transfer BdcA by conjugation, resulting in increased dispersal of the biofilm via enhanced motility (149).

Finally, motile bacteria can create holes or tunnels in the matrix of mature biofilms, increasing the susceptibility of those drilled biofilms to toxic molecules from the environment (150). Houry and colleagues screened motile bacteria for their ability to destabilize biofilms from other species, and in most cases, motile bacteria were able to penetrate and move through foreign biofilms (150). Such strategies, in combination with other competitive strategies, could lead to important ecological advantages. For instance, motile *Bacillus* spp. expressing antimicrobial compounds were able to tunnel through *S. aureus* biofilms and release the bioactive molecule directly into the inner core layers of the biofilm. This resulted in a complete clearing of the *S. aureus* biofilm and establishment of the *Bacillus* biofilm (150).

Resistance to colonization

One major fitness parameter in biofilm development is the ability to resist colonization of other genotypes that could invade the biofilm and eventually take over. Moreover, invasive genotypes can lower the relatedness of the interacting cooperators and threaten the maintenance of cooperation. Several of the previously discussed mechanisms can also be implicated in colonization resistance, for example, the aforementioned surfactant produced by *E. coli*, Ec300p, that not only inhibits initial adhesion of competing strains, but also provides a shield against invading bacteria (117). Similarly, T6SS in *B. thailandensis* protected the biofilm from competitors (90).

Due to its medical relevance, there has been increased interest in the ecology of host-associated microbiota and how colonization resistance plays a role in maintaining health and homeostasis. However, due to the complexity of multispecies communities, very few studies have shed light on the specific mechanisms. Recently, a transcriptomic study investigated how commensal populations of *E. coli* reacted when challenged with two pathogens, either invasive *E. coli* or *K. pneumoniae* (151). This approach revealed specific responses upon invasion and highlighted the role of several genes in limiting pathogen settling and spreading, including two genes, *viaF* and *bssS*, implicated in *in vivo* resistance to colonization. However, the precise mechanisms by which these genes reduce colonization remain to be elucidated (151).

Similarly, in experimental oral biofilms, a microbial consortium of 10 species was able to resist colonization of *E. coli*. The consortium specifically sensed the presence of *E. coli* and responded by increasing H$_2$O$_2$ secretion that has a bactericidal activity and results in the killing of *E. coli* (152). In a later study, it was shown that three members of this consortium played very distinct and fundamental roles in this colonization resistance pathway. First, one species, named “the sensor,” *Staphylococcus saprophyticus*, detected the presence of *E. coli* via cell-to-cell interactions, possibly through lipopolysaccharide detection. Then, “the mediator,” *Streptococcus infantis*, responded to diffusible signals produced by the sensor *S. saprophyticus* and induced the production of H$_2$O$_2$ in a third bacterial species, “the killer,” *Streptococcus sanguinis*. In this bacterial cascade, and in the absence of *E. coli*, *S. infantis* repressed H$_2$O$_2$ secretion of *S. sanguinis* (153). This example supports the competition-sensing hypothesis (57) and provides more evidence that some competitive outcomes observed in multispecies biofilms cannot be predicted by observing pair-wise competitions.

**EVOLUTION OF COMPETITIVE INTERACTIONS**

Most of them [species] are doomed to rapid extinction, but a few may make evolutionary inventions, such as physiological, ecological, or behavioral innovations, that give these species improved competitive potential.

Ernst Mayr, “Speciational Evolution or Punctuated Equilibria” (154)

Microorganisms are instrumental in the study of general evolutionary theory. Understanding the evolution of competitive interactions, the selective forces acting on multispecies communities, and the nature of adaptation mechanisms provides the opportunity to analyze (and predict) evolutionary fate and the distribution of possible outcomes. This is especially relevant for biofilms, which not only are a prevalent lifestyle in nature, but also play a central role in major natural processes as well as in human health and homeostasis.

**Theoretical Modeling of Species’ Competitive Interactions**

Modeling of single-species communities was commonly used as a tool to gain insight into the evolution of bacterial interactions, namely, cooperation and competition (155–157). Few studies have directly taken into account the complex dynamics and heterogeneity...
of multispecies communities (158–160). Moreover, most models specifically addressing species interactions within biofilms remain to be experimentally tested (161, 162).

Xavier and Foster created a computational model to analyze competitive outcomes between two genotypes from a single species with various levels of exopolysaccharide (EPS) production within a biofilm. Counterintuitively, the model predicted that, although EPS producers have lower individual fitness due to associated costs of polymer production, they would prevail in the biofilm over the nonproducers by suffocating the latter cells in the bottom of the biofilm, where environmental conditions are harsher. EPS-producing strains were predicted to ascendency the biofilm and gain access to more oxygen-rich niches (163). Recent experimental work confirmed the model’s predictions. Work carried out with V. cholerae showed that EPS-producing bacteria benefit from their clone-mates and gain competitive advantage against other nonproducers (164). However, the advantage in the biofilm environment came at a cost since, upon biofilm dispersal, the EPS producers were hindered in their dispersal capacity and subsequent colonization of new niches compared to nonproducers (164).

Another model predicts that when nutrient concentrations are low, cooperative bacteria secreting public goods, in this case a growth-promoting compound, would cluster together. This would result in the segregation of nonproducers, and because the benefits are preferentially directed toward other producers, the latter will have higher fitness than the nonproducers (32). By contrast, in nutrient-rich conditions, the different genotypes (producers and nonproducers) would more readily mix (32). However, this competitive outcome between genotypes of the same species can change when the complexity of the biofilm is increased (i.e., chimerism). In another study, Mitri and colleagues analyzed how the addition of a totally different species affects competition between producer and nonproducer genotypes from the same bacterial species (40). They showed that in low-nutrient environments, the new bacterial species could undermine the benefits of cooperation between the producer and nonproducer and eventually take over the biofilm (40). Moreover, competition imposed by the incoming bacterial species is more detrimental to the producers than it is to the nonproducers. This was shown in a study in which S. aureus was added to a P. aeruginosa culture in which some cooperator cells secreted iron siderophores and others did not. S. aureus increased nonproducers’ fitness over that of the producers (39).

Experimental Evolution in Biofilms

Experimental evolution is a powerful tool to decipher mechanisms of adaptation, including gain in competitiveness. This strategy is based on observing the effect of time on a given community. It is generally carried out by serial passaging of replicate populations for long periods of time under controlled conditions (163). Nevertheless, emergence of competitive traits might happen very fast (44). Experimental evolution approaches have been mostly used with single-species batch cultures. For example, the long-term experimental evolution set-up with E. coli has shown that key innovations result in the novel use of another carbon source (citrate) (166) and that fitness can increase for very long periods of time in an unchanged environment (167), among other important evolutionary findings. Recently, Poltak and Cooper described an experimental model that enables experimental evolution in biofilms (19). This method is based on the use of beads to which bacteria can adhere. Each day, a colonized bead is transferred into fresh media and a new bead is colonized, allowing cells to adhere, form mature biofilms, and disperse. This approach showed that self-diversified phenotypes became synergistic and significantly increased group productivity (19). Such new techniques should increase studies that experimentally address evolution in biofilm population dynamics and long-term consequences of biofilm selection.

Early work by Rainey and Travisano showed that when there is ecological opportunity in microcosms, exploitative competition arises readily and generates diversity in a population (44). After three days, the initial population of P. fluorescens, a generalist genotype, diverged into three stable specialist morphotypes that occupy different niches. This radiation was driven by competition for oxygen and achieved by several mutations. One morphotype evolved by mutations in the cellulose-like operon, resulting in the overexpression of the polymer and the formation of a self-sustainable biofilm in the air-liquid interface (168). The repeatability of the evolutionary outcome suggests very strong pressure for diversification. Such diversity did not evolve in homogeneous batch cultures with reduced ecological opportunities (44).

The study of more complex communities also revealed the rapid emergence of competitive interactions between different species (169), not only within species, as a result of self-diversification (44). In a dual-species evolution experiment, it was shown that spatial structure led to the emergence of exploitative interactions by a single mutation altering lipopolysaccharide biosynthesis of one of the interactants (169). More precisely,
P. putida was initially grown with an Acinetobacter sp. in an environment in which P. putida was dependent on the Acinetobacter sp. After several days of coevolution, the dynamics and biofilm morphology changed; P. putida grew closer to and eventually overgrew Acinetobacter (169). The P. putida population developed a rough morphotype in response to the presence of Acinetobacter and as a result of developing within a biofilm, probably due to enhanced oxygen competition.

More recently, a study using Burkholderia cenocepacia as a model organism showed that there are several possible routes to adapt via interference competition within a population. This study showed that increased fitness by bacteria compared to their ancestor was achieved either by enhanced iron storage, enhanced metabolic efficiency, and/or alteration of polysaccharide and lipopolysaccharide structures (170). Moreover, this study highlighted the role of competition in maintaining genetic diversity within complex communities (170), in contrast to results obtained in batch cultures or more homogeneous environments in which competitive mutants quickly take over the population (170). This study also showed that for a given genotype to sweep the population, more time and more mutations are required, probably due to high cell density and absolute population numbers (171).

Finally, a recent study showed that the complexity of a community and the number of interacting partners strongly influences evolution in ways that were unpredicted by single-species experiments (7). More specifically, interspecies competition led to the use of alternative resources and shifted the population from generalists to specialists. This resulted in increased productivity of the whole community due to the complementarity in the use of resources. Interestingly, several species were able to feed on waste products of other members of the community. This and other examples suggest that some evolutionary pathways are only available when within multispecies communities and not in simpler settings (172, 173).

CONCLUDING REMARKS
The studies and experimental data presented in this article were carried out under lab-controlled conditions, so the question remains as to whether these processes are meaningful in the natural world. Indeed, given the inherent complexity of natural ecosystems, competition could be harsher and selective pressures stronger and applied through longer periods of time than the ones created or used in laboratory conditions.

Besides the ecological and evolutionary importance of understanding microbial competition, knowledge of corresponding underlying mechanisms could be particularly useful in dealing with specific applied problems, such as the ubiquity of antibiotic resistance in clinical settings (58, 174). Interference interactions have already inspired the design of alternatives to antibiotics in the war against pathogenic microorganisms (97). Additionally, well-defined antagonisms could be exploited to adequately use probiotics to prevent infection (175) or to restore and preserve the equilibrium of the flora to efficiently resist pathogen colonization (176).

Several studies highlighted that complex communities interact differently depending on their degree of chimerism and the diversity of their genotypes. Moreover, they have shown that several adaptive pathways and outcomes are only available with high levels of complexity and that they cannot be predicted by observing a simplification of the ecosystem in either single-species or dual-species studies (7, 153, 172). Recently, a new integrative approach, community systems (CoSy) biology, was proposed to further explore how social interactions in multispecies communities evolve (3, 177), and different alternative strategies based on multispecies cultures are emerging (178, 179). New multidisciplinary approaches combining population genetics, evolutionary and molecular biology, and bioinformatics are needed to decipher the genetic networks underlying community interactions and place them in their ecological and evolutionary context. Additionally, the spatial scale at which interactions occur is also an important parameter to take into account. Hence, the integration of physical and mathematical models of biological processes will advance our ability to predict evolutionary outcomes of bacterial competition in heterogeneous environments. Finally, genotypic diversity within naturally occurring biofilms should inspire future experimental work to progressively move away from simplified microbial systems toward the study of complex populations including more genotypes. This will lead to a broader understanding of bacterial biology and could expose new mechanisms of competition within multispecies communities, with potential impact on applied systems biology and control of colonization resistance in medically or industry-relevant biofilms.

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