Laboratory Evolution of Microbial Interactions in Bacterial Biofilms

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Microbial adaptation is conspicuous in essentially every environment, but the mechanisms of adaptive evolution are poorly understood. Studying evolution in the laboratory under controlled conditions can be a tractable approach, particularly when new, discernible phenotypes evolve rapidly. This is especially the case in the spatially structured environments of biofilms, which promote the occurrence and stability of new, heritable phenotypes. Further, diversity in biofilms can give rise to nascent social interactions among coexisting mutants and enable the study of the emerging field of sociomicrobiology. Here, we review findings from laboratory evolution experiments with either *Pseudomonas fluorescens* or *Burkholderia cenocepacia* in spatially structured environments that promote biofilm formation. In both systems, ecotypes with overlapping niches evolve and produce competitive or facilitative interactions that lead to novel community attributes, demonstrating the parallelism of adaptive processes captured in the lab.

Since the pioneering discoveries of Robert Koch, most bacteria generally have been studied in planktonic cultures, wherein cells grow dispersed from one another. Recently, the spatially structured environments (definitions of terminology are given in Table 1) of tightly packed bacterial biofilms have been recognized as more appropriate models to study microbial growth and interactions (1), because microbes may live more frequently in biofilms on inanimate or host surfaces (2) and the physiology of biofilm cells differs from that of planktonic cultures (3). The regulation of biofilm formation, persistence, and dispersal has been investigated at the molecular level for many years now, but much more remains to be discovered, particularly related to how biofilm cells interact when attached to one another (4, 5). The increasing interest in biofilm models has revealed the complexity of microbial social interactions (6). Since microbes, to some extent, share the structural components of biofilms (e.g., exopolysaccharides), the process of biofilm construction can be viewed as a form of cooperation, at least within a single lineage of cells (7–9). In addition, biofilm itself provides a structured environment that promotes other types of resource exchange among clonemates or metabolically interdependent strains (10–12). However, biofilm formation may also be triggered as a competitive or defensive response against other strains or species (13). In addition, the structured environment of biofilms creates gradients of nutrients that potentiate the competition for limiting resources, resulting in exploitative competition between cohabiting species (14).

Despite the increasing knowledge of the ecology (13, 15, 16), genetics (17–19), or physical mechanisms (20, 21) of biofilm development and the mechanisms that stabilize cooperation during biofilm formation (7, 9, 10, 22), we still know little about the long-term evolutionary dynamics within biofilm communities. It is unclear how social interactions shape biofilm evolution and how evolution in structured environments shift the balance between competition and mutualism. Long-term serial transfer experiments provide a key approach to fill this gap. Experimental evolution allows studying adaptation under controlled conditions and identification of the evolutionary forces and ecological processes that shape microbial cultures (23, 24). Today, the genomic changes of experimentally evolved bacteria can be easily explored

using next-generation sequencing methods (25) that allow understanding of the molecular basis of evolutionary adaptation.

Here, by reviewing a set of experimental evolution studies, we demonstrate that biofilms are both good models to study the origins of social interactions and to examine how these interactions are shaped in time and space. We focus on the form and strength of interactions between microbial species, ecotypes, or early differentiated lineages cohabiting structured environments of biofilms. Based on the existing literature, we describe how such interactions influence the evolution of microbial communities. Importantly, evolutionary changes in microbial communities may be driven by a set of abiotic factors (e.g., fluctuations in the environment) or biotic components other than microbes (e.g., plant, animal, and human host or symbiont), which are not discussed in the paper. The study of evolutionary dynamics within biofilms and the origin of microbial interactions are significant for several reasons (26). First, biotic interactions among neighbors generate major selective forces that drive the development of many important traits of microbes, such as antibiotic resistance (27). Second, understanding how biofilms evolve can aid in predicting of evolutionary outcomes. Third, tracking molecular evolution in biofilms helps to unravel new regulatory pathways related to biofilm formation, persistence, or dispersal. Here, we present how recent advances in experimental evolution of biofilm systems fulfill this potential.

LABORATORY EVOLUTION OF COOPERATION AND INTERFERENCE IN STRUCTURED ENVIRONMENTS

A pioneering long-term evolution experiment with *Escherichia coli* started in 1988 by Richard Lenski provided direct evidence

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TABLE 1 Description of terms originating from ecology or evolutionary biology that are used in the text^a

Term	Definition	Reference(s)
Competition	Negative interaction among organisms resulting from overlapping resource requirements or chemical warfare, which leads to reduced fitness of the interacting individuals	
Antagonism (parasitism, predation)	Interaction between two organisms in which one profits at the expense of the other (in terms of fitness); the behavior is a derived strategy	
Synergistic interaction/facilitation	Interaction that leads to an increased fitness of the interacting individuals relative to monocultures	35, 66, 72
Productivity	Increase in biomass or cell numbers over time that reflects the efficiency with which a given organism converts energy into biomass	
Niche complementation	When species inhabit functionally complementary niches or environments resulting in reduced competition	71, 73
Generalist	An ecotype that can tolerate a broad range of conditions (e.g., food sources, temperature stress, etc.)	69
Specialist	An ecotype that can tolerate a rather narrow range of conditions (e.g., food sources, temperature stress, etc.)	69
Ecotype	Organism that pursues a certain ecological strategy and differs in some fitness-relevant traits from other organisms	68
Morphotype	Different colony morphology	35, 69
Spatial structure/structured conditions	Environment with low degree of mixing	
Biotic interactions	Any ecological interactions that occurs between two living organisms; in contrast, interactions are termed abiotic when they involve nonliving elements, e.g., sunlight, temp, pH, etc.	
Social interactions	Interaction/behaviors in which the performance of two individuals influences each other's fitness positively or negatively; here, the definition of social interactions refers to microbes	74
Sociality	The ability and extent of a group or an individual to engage in social interactions	
Sociomicrobiology	Research field in which social interactions of microorganisms are investigated	
Cooperation	Costly behavior of one individual that increases the fitness of another individual and which has evolved for this purpose	74, 75
Public goods	Secreted compounds which are freely available for each member of the population, i.e., "public." These substances benefit the population or community yet do not have to be costly to produce	74

^a The definitions explain the context of their usage in the text.

that microbes can be evolved in the lab and their phenotypic and genotypic changes can be tracked over time (28). Lenski's experiment is still in progress, reaching 64,000 generations in January 2016, and many similar studies have been performed by other researchers on different microbial species (not only bacteria but also yeasts, phages, or protists) covering basic scientific questions (29, 30) or even in directly applicative projects (31). While planktonic cultures offer a simple setup to study the evolution of bacteria (23, 28, 31, 32), several studies have shown that the selective pressures in unstructured conditions differ from those in biofilms (33–36). For example, homogeneous, well-mixed environments may select against "social" genotypes that secrete costly metabolites, so-called "public goods," and rather favor fast-reproducing selfish individuals (37). This is not surprising since mixing decreases the likelihood for such metabolites to be retained in the vicinity of the secretor and does not guarantee that the secretions will benefit its closest relatives or offspring cells (38), one of the core preconditions required for the evolution of sociality (39). Recent reports have shown that the situation differs dramatically in biofilms, where the secretors have the primary access to the substances produced, allowing the public good producers to easily outnumber the nonproducers (12, 22, 40, 41). It is therefore expected that structured environments would select for "social" adaptation strategies of microbes.

Experimental evolution studies provide evidence for the prediction that evolution in structured environments can favor ad-

aptations to sociality (34, 35, 42–44). For example, Koch and colleagues demonstrated how the emergence of a strain with an increased level of cell-cell communication can eventually lead to evolution of antibiotic resistance to vancomycin, a last-resort antibiotic (27). Briefly, methicillin-resistant *Staphylococcus aureus* grown in a colony biofilm diversifies sequentially into mutants in which the first clones (named W) are selected due to increased cell-cell signaling, which triggered more surfactin and toxic bacteriocin secretion. Next, bacteriocin-resistant mutants that are also resistant to intermediate levels of vancomycin evolved in response to this new environment (27). Although overall diversification of *S. aureus* was driven by competition, the success of the W variant was due to an increased level of cooperation via the Agr quorum-sensing system that in addition triggered increased levels of bacteriocin secretion (27). This dynamic of evolved resistance depends on the structured environment that promotes both efficient cell-cell communication and intimate interactions between the strains. Bacteriocin producers trigger the appearance of a resistant variant in their vicinity due to local increased exposure to the toxic compound. Another study demonstrated that spontaneous mutants of *Pseudomonas fluorescens* with increased polysaccharide secretions (i.e., mucoid isolates) are able to use these secretions to position themselves on the oxygen-rich surface of the colony and dominate the population (45). The emergence of such mucoid isolates is not observed when the spatial structure of the colony is manually disturbed during development. Therefore,

spatial assortment not only promotes stability of cooperation in biofilms (22, 40) but also supports the evolution of novel public good producers that results in increased biofilm matrix production (34, 45). Spatial structure can also promote diversification into strong and weak secretors as shown in *Pseudomonas aeruginosa* colonies, where repetitive reinoculation of *P. aeruginosa* from the edge or the middle of colony biofilms resulted in clones with reduced or diversified matrix production levels, respectively (34). Structured environments can also support stable coexistence of cooperative and noncooperative strains, thereby promoting diversity within species (46).

In fact, environmental spatial structure is probably the major factor promoting diversity in the microbial world (47). Although spatial structure limits the number of possible interactions with nonrelatives and therefore promotes the evolution of public good production, it also facilitates intimate local interactions between neighboring species. Based on cocultivation studies of various natural isolates, interaction with nonrelatives seems to be dominated by competition (48, 49). Coevolution experiments document how such competition between two closely interacting species can progress into more exploitative interaction (50, 51). For example, an experimental community of *Pseudomonas putida* and *Acinetobacter* sp. was evolved and shown to produce an intimate and specialized association that results in more stable and more productive community than the ancestors. In the ancestral community, *P. putida* was dependent on *Acinetobacter* for benzoate that manifested in an exploitative interaction between the two strains (50). During coevolution, *P. putida* increased its stickiness due to mutations in a gene related to lipopolysaccharide synthesis. This enabled a more intimate relationship between the two species and more efficient exploitation of *Acinetobacter* by *P. putida*. Although coevolution resulted in a more exploitative interaction that was detrimental for one of the partners, the overall productivity of the community was doubled (50).

A similar pattern was recently observed by Kim and colleagues (52) where clonal populations of *P. fluorescens* rapidly diversified into morphotypes M (mucoid) and D (dry and wrinkly). The two morphotypes spread collectively faster than either of them in monoculture, which the authors describe as “division of labor.” Microscopy analyses revealed that D dominates the spreading bulk of the colony sitting at the top of M. The authors proposed that the role of M is to reduce the tension of the solid surface, while D aids M by pushing it outward. Although D constitutes only 90% of the spreading colony and its interaction with M is exploitative, the performance of the whole community is improved as in the case of *P. putida* and *Acinetobacter*. Importantly, in contrast to the preassembled community of *P. putida* and *Acinetobacter*, the exploitative (and at the same time group beneficial) relationship between D and M evolved *de novo* from a monoclonal ancestor (52). In another study, new colony structures observed on agar medium were found to be indicative of altered biofilm formation or adaptation to a particular biofilm niche requirement (53). Studies on *P. fluorescens* microcosms revealed that certain morphotypes (e.g., the wrinkly morphotype) can be selected only under static conditions but not in shaking cultures (36). Similarly, the diversification of the Gram-positive *Bacillus subtilis* is also highly dependent on the condition used in experimental evolution (33). Namely, colony types with intermediate biofilm robustness dominated under static conditions, whereas those of marginal biofilm robustness (very high or very low) were much less

common than shaking cultures (33). This result again highlights the impact of spatial structure on the outcome of laboratory evolution experiments.

EXPERIMENTALLY EVOLVED BIOFILMS OF *P. FLUORESCENS* AND *BURKHOLDERIA CENOCEPACIA*: MODELS OF BIOFILM DIVERSIFICATION

Below, we describe in greater detail studies of the laboratory evolution of *P. fluorescens* and *B. cenocepacia* in spatially structured biofilm environments, in which a mixture of ecological generalists and specialists evolve and coexist. The first project began in 1998 when Rainey and Travisano reported rapid diversification of *P. fluorescens* into 3 different colony types, preferentially inhabiting different niches of static liquid medium (surface, bottom, and the medium) (36). The story of *B. cenocepacia* began 13 years later, when the field of experimental evolution was already more advanced (e.g., the Lenski’s evolution experiment had exceeded 50,000 generations). In 2011, Poltak and Cooper tracked the evolution of a *B. cenocepacia* community in a carefully designed microcosm equipped with floating beads that can be repeatedly colonized and decolonized by the evolving community. These two projects resulted in more than 30 research papers that vastly improved our understanding of ecological and molecular dynamics of adaptation in biofilms, making the two pioneering studies (35, 36) milestones in the field of experimental evolution. In the following sections, we examine each experimental design and findings separately, focusing mainly on the social interactions within the evolving microcosms.

EVOLUTION OF COOPERATION AND EXPLOITATION WITHIN AND BETWEEN ECOTYPES IN *P. FLUORESCENS* STATIC MICROCOSM

When *P. fluorescens* natural isolate SBW25 is incubated in unshaken glass vials (“static microcosms”) in nutrient-rich medium, this strain rapidly diversifies into three main phenotypically and genotypically different variants (36). Each of these variants is specialized for a certain niche in the spatially structured microcosm and forms phenotypically distinct colonies when grown on agar plates (Fig. 1A). The ancestral smooth morphotype (S) grows predominantly in the liquid column of the microcosm, whereas the fuzzy spreader (F) forms aggregates at the bottom of the tube. The third colony variant is called the wrinkly spreader (W) and occupies the air-liquid interface where it forms a robust pellicle or biofilm (36, 44).

The diversification of the ancestral strain is greatly influenced by the oxygen gradient that is created by the ancestor itself shortly after inoculation of the microcosm (54). Close to the air-liquid interface, the oxygen level is high but decreases rapidly with increasing depth. This spatially heterogeneous environment therefore selects for specialist mutants that prevail under different levels of oxygen availability (54). Without such spatial heterogeneity, no morphologically different variants evolved, and this heterogeneous environment was also essential for maintenance of diversity (36). The W variant in particular is very successful in the spatially structured microcosm since it rapidly accounts for 30 to 50% of the whole population (36, 54) and achieves higher fitness than the S morphotype (53). This advantage and the rise of the W variant depends on its ability to access the elevated oxygen levels in the upper zone of the microcosm by forming a floating pellicle, while other variants grow slower in low-oxygen conditions (54).

FIG 1 Experimental setup of biofilm evolution in the *P. fluorescens* microcosm (A) and the *B. cenocepacia* bead selection method (B). Biofilm populations in both setups reveal successive adaptive diversification into three ecotypes, namely, smooth or studded (S), fuzzy (F) or ruffled spreader (R), and wrinkly spreader (W). Note that the evolved smooth variant of *B. cenocepacia* is distinct from the ancestor exhibiting enhanced biofilm formation.

To form biofilms at the air-liquid interface, W mutants adapt by producing more cellulose polymers (53) that constitute the biofilm matrix and provide the robustness and structure necessary for a floating mat. It is important to note that in producing this cellulose pellicle, W mutants inherently become more cooperative by producing a public good, but similarly to other biofilm-based cooperative traits (22, 55), it is costly for the individuals to produce (53). Intriguingly, Rainey and Rainey (43) showed that non-cooperators that were evolved in the presence of cooperators could invade biofilms formed by cooperating cells. These noncooperators showed an S-like morphology with no aggregation properties and their invasion caused biofilm collapse. Brockhurst et al. (42) also showed that the frequency of cooperators was higher when more resources were provided for the microcosm since relatively less energy was invested toward public good production. However, even under such conditions, competition might occur within the biofilm among different W variants.

Most studies addressing microbial cooperation non-cooperators as “cheaters” or “free-loaders” and focus solely on their social exploitation. Yet, a study of Hammerschmidt et al. (56) used *P. fluorescens* to show that noncooperating cells carrying the S phenotype can function as colonists of new habitats. They reported that although S cells cause premature collapse of the biofilm mat formed by the W, they can also found a new microcosm where they diversify again and form W biofilms. Therefore, the relationship between W and S resembles the one between a vegetative cell and a spore, where only the latter can survive harsh conditions and give rise to new population, often at the cost of vegetative cells (57). The noncooperating S cells, therefore, could serve as new founders (germ line), allowing the reestablishment of the biofilm collective in another potential environment. This simple life cycle facilitated the maintenance of cooperative collectives. In addition, rapid switching between phenotypes (W and S variants) can be also interpreted as a bet-hedging mechanism that

benefits simple organisms, like bacteria in fluctuating environments (58).

Genome sequencing of the *P. fluorescens* W variant revealed mutations in the diguanylate cyclases (DGCs) WspR, AwsR, and MwsR that catalyze the synthesis of cyclic diguanylate monophosphate (c-di-GMP) from GTP (59, 60). Initially, the impact of the *wsp* operon was understood only in the evolution of the W variant (53, 59, 61), while the influences of mutations in *aws* and *mws* genes were revealed only after additional evolutionary experiments (60). Specifically, when the static microcosm of *P. fluorescens* was allowed to adapt in a *wsp*-deficient background (using $\Delta wspABCDEF R$ as the ancestor), the *aws*-dependent circuit was discovered, resulting in a similar W variant. The third alternative circuit (*mws*), leading to the evolution of W, was discovered after evolution of *P. fluorescens* that carried both $\Delta wspABCDEF R$ and $\Delta awsXRO$ alleles (60). These mutations in all three loci are mainly responsible for the increased c-di-GMP level that manifests in elevated cellulose production by the W variant (60, 62). Specifically, increased c-di-GMP is responsible for the activation of a membrane-bound enzyme complex responsible for cellulose synthesis that is encoded by the *wss* operon (53).

Initially, a screen of the W variant transposon library for loss of the wrinkly colony morphology supported the fact that the genes of this operon (*wssA-wssJ* and *wspR*) are crucial for the W variant (53). The operon encodes the DGC WspR and several proteins exhibiting similarity to known cellulose biosynthesis components and chemotaxis systems (53). Mutations in two genes of this operon, *wspE* and *wspF*, were also shown to be responsible for WS variant appearance. WspE represents a histidine kinase that activates WspR by phosphorylation, suggesting that certain mutations in WspE might lead to WspR overactivation (44, 60). WspF is thought to be involved in modulation of WspR activity, indicating that a mutation in the *wspF* gene might have effects similar to those in WspE (61). Later analysis of *wsp*-independent circuits

revealed the role of the *aws* locus in W variant formation. The authors discovered deletions in *awsX*, a negative regulator of diguanylate cyclase AwsR. They proposed that this new W variant resulted from overproduction of the acetylated cellulose through the *wss* pathway due to lack of AwsR regulation and increased synthesis of c-di-GMP (60). Another *wsp*- and *aws*-independent circuit resulting in the W phenotype was also related to increased activity of another diguanylate cyclase MwsR, which, in contrast to AwsR, was not caused by mutations in an extragenic negative regulator but by mutations in the *mwsR* gene itself (60). These experiments demonstrate that several pathways can lead to increased production of c-di-GMP and therefore elevated expression of the *wss* cellulose operon in certain members of the population that in turn increases overall fitness.

LONG-TERM EXPERIMENTAL EVOLUTION OF A COMPLETE BIOFILM LIFE CYCLE USING *B. CENOCEPACIA*

Evolution during the full life cycle of a biofilm, from attachment and maturation to dispersal, can be experimentally studied using a simple bead selection method (Fig. 1B) (35). Here, bacteria growing in a test tube must attach to a plastic bead to be transferred to a new tube with fresh medium each day. The new tube also contains a new bead; thus, a new biofilm must form on an uninhabited surface on a daily basis. Because tubes are incubated on a roller drum, established biofilms are continually subjected to shear forces that select for attachment, planktonic growth, and reattachment. Importantly, this cycle enables easy archiving of the evolving populations as well as precise reconstitution to study the ecological and evolutionary forces prevailing at that time (35), which renders the model especially suitable for long-term experimental evolution (LTEE). Additionally, LTEE can be paired with contemporary sequencing methods to identify the mutational mechanisms, enabling adaptation in the laboratory (25, 63, 64).

Adaptation under these conditions with minimal galactose growth medium has been studied for the opportunistic respiratory pathogen *B. cenocepacia*, but such an approach can be also applied for microbes in natural settings or during host infections (65). However, modifications of the bead-based experimental evolution setup, such as changes in the number of beads or in nutrient concentration, may allow the detailed study of social interactions (35), the degree of facilitation or competition among cells in the biofilm (66), and the predicted colonization and dispersal-dependent fitness (67).

With this novel approach, the biofilm population of *B. cenocepacia* revealed sequential adaptive diversification into three classes of heritable colony morphologies distinctive from that of the ancestor (35), similar to the diversification of *P. fluorescens* during biofilm establishment. The *B. cenocepacia* morphotypes were classified as studded or smooth (S), ruffled spreader (R), and wrinkly spreader (W) (35). Morphologically, the *B. cenocepacia* variants are similar to the experimentally evolved *P. fluorescens* colony types (36). As in *P. fluorescens*, these different *B. cenocepacia* morphotypes inhabit different niches in the test tube (35) and were therefore characterized as separate ecotypes (35, 68). The W variant, possessing strong adhesion to the bead and tube walls in monoculture, is an early colonist of the plastic bead. As the R frequency increases, the W variant declines in frequency. S cells, on the other hand, escalate steadily throughout the growth phase, suggesting that the early evolved S variant adheres to the later-evolved R and W variants, which are better surface colonizers. As S variants re-

main the dominant type in the community, their proportion decreases slightly prior to transfer as the frequencies of R and W variants escalate from their low initial occurrence (35).

Intriguingly, the three coevolved ecotypes in such biofilm community reach higher productivity than each one of them in monoculture or their expected productivity in the mix. This increased productivity is attributed to the prevailing facilitative effect of niche complementarity (35, 66), in which the ecotypes inhabit different spatial regions of the biofilm. Further synergy among these morphotypes was revealed in a cross-feeding assay demonstrating that the specialists (R and W variants) profit from metabolic by-products of other communal members but not from their own metabolites. This suggests the evolution of a resilient symbiotic food web wherein the generalist S variant achieves high biomass by superior growth but attaches preferentially to the biofilm produced by R and W cells, which in turn profit from secreted metabolites (35). Nonetheless, only the late S variant increased its productivity in the mix culture relative to that in its monoculture, while total productivities of R and W in the mix were reduced compared to those of their monocultures, although the per capita production of R and W increased (66). This indicates that although the competition in the late community was lessened both by facilitative effects (66) and via cross-feeding between R and W (35), it still affected the system.

The sequencing of evolved *B. cenocepacia* clones and populations revealed that the observed colony morphologies are the result of different mutations (69) that affect the regulation of the “stick-or-swim” decision (66, 67, 69). Surprisingly, biofilm specialists (R and W variants) recurrently evolved from generalist types (S variant) by adaptive mutations in the *wsp* locus homologous to the one first described in *P. fluorescens* (69). Sequencing of both population samples and clones revealed complex dynamics driving the evolution of the three ecotypes. Initially, cells that best attached to the plastic beads achieved high frequencies by way of mutations in genes related to the biofilm lifestyle regulator c-di-GMP. These mutations produced distinct colony morphologies and excluded the ancestor genotype. The independent occurrence of these adaptive changes in parallel populations suggests that changes in c-di-GMP production are sufficient for the adaptive niche partitioning because they generate ecotypes with different tendencies to attach or disperse (69). Subsequent mutations related to central metabolism and polysaccharide biosynthesis influenced the proliferation of the S variant and enhanced its competition against earlier mutants. Surprisingly, new *wsp* mutations evolving from S haplotypes gave rise to new R and W mutants, which invaded these more specialized biofilm niches. Interestingly, competition among ecotypes for limiting iron led to the remodelling of the entire community, as both S and W lineages independently acquired mutations in the promoter sequence of bacterioferritin and excluded all cells lacking either of these mutations (69).

A focused analysis on mutations evolved in replicate population B1 (one out of six independently evolving biofilm populations) determined the molecular basis of ecological differentiation of S, R, and W (69). In addition to the *wsp* mutations discussed above, at least eight independent mutations in the gene *yciR* (also known as *rpfr*) were discovered (69, 70). YciR harbors a PAS sensor domain that binds the quorum-sensing molecule *cis*-2-decenoic acid (BDSF) (70) as well as a diguanylate cyclase (GGDEF) and a phosphodiesterase (EAL) domain. YciR has been found in many bacterial species to function as a phosphodiesterase

that is responsible for degradation of c-di-GMP and the attenuation of biofilm development (66). A single mutation in *yciR* carried by the R ecotype resulted in its increased biofilm formation. Moreover, ecotype S carried a deletion of *yciR* along with 94 other neighboring genes (69). The different phenotypes of the *yciR* mutants imply that altering different domains can produce different ecological strategies that coordinate quorum sensing of BDSF with c-di-GMP metabolism and hence the stick-or-swim cell decision.

Meanwhile, analogous to the *P. fluorescens* system, the genetic causes of the wrinkly phenotype in *B. cenocepacia* are single missense mutations in either *wspA* or *wspE*, the products of which are involved in the biofilm signal transduction cascade related to c-di-GMP synthesis (71). Although the cognate diguanylate cyclase or phosphodiesterase in this bacterium is not yet known, subsequent evolution studies using W mutants as ancestors revealed a new two-component regulator (Bcen2424_1436) that could relate to the c-di-GMP response and explain this phenotype more generally. Further, growth of W mutants in planktonic culture generated strong selection to regain planktonic fitness, often favoring loss-of-function mutations in Wsp (67). Such mutations could limit subsequent evolution of the wrinkly biofilm in fluctuating environments (67).

CONCLUDING REMARKS

These laboratory experimental systems used spatially structured environments to demonstrate how microbial communities can evolve from clonal ancestors and generate complex ecological interactions. In both experimental setups (*P. fluorescens* static microcosms and *B. cenocepacia* biofilms on suspended beads), the spatially heterogeneous environment facilitated the evolution of distinct ecotypes. These systems allow for the study of adaptive diversification in real time and provide direct insights into evolutionary processes that used to be limited to theoretical models, like the positive feedback between niche construction and natural selection. Intriguingly, the diversified communities can be shaped by the range of social and ecological interactions, including antagonism (i.e., social exploitation of the W by S variants observed in *P. fluorescens*), niche complementation, or even synergistic interactions via cross-feeding as observed in *B. cenocepacia*. Finally, the interactions that arose between the ecotypes of the evolved community became a new factor determining biofilm productivity that could not be observed with an isogenic ancestor community. Importantly, parallel evolution experiments performed under unstructured conditions (planktonic cultures) in both species did not show diversification patterns similar to those of the structured environments in the biofilms (Fig. 2) (35, 36). In addition to the basic understanding of evolutionary processes, these studies provide insight into how microbes adapt at the molecular level. In both systems, wrinkly phenotypes emerged from mutations related to *wsp* genes, two-component transcriptional regulators, and polysaccharide production-associated genes. Understanding how opportunistic pathogens (e.g., *Burkholderia*) or beneficial root colonizers (e.g., *P. fluorescens*) undergo adaptive diversification in biofilms could illuminate the ecological forces that drive adaptation during chronic infections or plant biocontrol.

In the future, an experimental evolution approach using constructed microbial interactions might also provide a better understanding of the forces that govern assembly of natural mixed-species biofilms as well as how best to engineer stable, synthetic microbial ecosystems. More broadly, laboratory evolution of bio-

FIG 2 The population genetic dynamics of experimentally evolved bacterial populations differs between planktonic and biofilm cultures. Planktonic adaptation is frequently explained by few beneficial mutations that evolve sequentially on a haplotype that fixes and purges diversity. In contrast, biofilm adaptation involves the rise of multiple contending mutations in a spatially structured environment that preserves genetic diversity. This structure can also facilitate the evolution of ecological interactions that maintain diversity.

film populations can be an important asset in our understanding of ecoevolutionary dynamics within more complex communities of multicellular organisms that are less amenable to manipulation.

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