Exploiting social evolution in biofilms
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Bacteria are highly social organisms that communicate via signaling molecules, move collectively over surfaces and make biofilm communities. Nonetheless, our main line of defense against pathogenic bacteria consists of antibiotics–drugs that target individual-level traits of bacterial cells and thus, regrettably, select for resistance against their own action. A possible solution lies in targeting the mechanisms by which bacteria interact with each other within biofilms. The emerging field of microbial social evolution combines molecular microbiology with evolutionary theory to dissect the molecular mechanisms and the evolutionary pressures underpinning bacterial sociality. This exciting new research can ultimately lead to new therapies against biofilm infections that exploit evolutionary cheating or the trade-off between biofilm formation and dispersal.

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Public good cooperation
*P. aeruginosa* is a gram-negative bacterium notorious for causing diverse infections in multiple anatomic niches, including wounds, chronic lung infections in cystic fibrosis, sepsis, bacterial keratitis and urinary tract infections. This opportunistic pathogen is also a highly social organism. *P. aeruginosa* lives in close interaction with microbial strains of the same and other species and is becoming a model system for microbial social evolution.

Much of the social activity of *P. aeruginosa* involves cooperation through the secretion of public goods. ‘Public good’ is an umbrella term used in the microbial social evolution literature to refer to a resource, such as a secondary metabolite, that is secreted by bacteria and becomes publically available to other cells within a population [8]. Many public goods are costly to produce, making them susceptible to exploitation by cheater strains that benefit from the public good without producing it themselves. For example, the siderophores of *P. aeruginosa* are iron-scavenging molecules that are costly to produce but are necessary for colonization of iron-limited environments such as eukaryotic tissues [10,11]. The potential for cheater exploitation makes public good production an attractive target for therapies that select against resistance. The prediction on the basis of social evolutionary theory is that in contrast to antibiotic-resistant mutants, mutants that resist a drug to prevent public good production would not be favored by natural selection. Because drug-sensitive bacteria would get a small initial growth advantage due to not producing costly public goods when the drug is present, drug-sensitive cheaters would eventually outcompete the drug-resistant producers. In the case of siderophores, a cheater population would be weakened without iron and more easily cleared by the host (Figure 2b).

This conceptual example illustrates how a drug targeting a microbial social trait may succeed where antibiotics fail by enabling cheaters to outcompete more virulent strains.
However, the reality can be more complex. For example, social evolutionary experiments have shown that less virulent quorum sensing signal-blind mutants, which do not produce a range of public goods, outcompete virulent *P. aeruginosa* strains *in vitro* [12–14]. The selective advantage may explain why signal-blind mutants are often found in long-term chronic cystic fibrosis infections [15] and suggests that quorum sensing inhibitors would be a suitable therapy targeting population-level traits [13]. Nevertheless, another recent study tested a quorum sensing inhibitor in patients and the results were quite the opposite: instead of attenuating the *P. aeruginosa* infection the quorum sensing inhibitor aggravated infection by decreasing the relative advantage of cheats [16*]. The exact selective pressures involved in social disruptions should be thoroughly investigated.

**Social evolution in biofilms**

Biofilms are a continuing problem in the clinic because biofilm bacteria are often more robust against antibiotic and metabolic stresses than planktonic bacteria [17]. Biofilm formation is itself a social trait that requires the production and secretion of shared substances. For example, extracellular polymeric substances, (EPS) that encase bacteria in biofilms [18] are shared products, which, like siderophores, are potentially available to neighboring cells after they have been secreted. Unlike siderophores EPS production seems not to be exploited by cheats. A mechanism for this protection was recently proposed on the basis of computer simulations that modeled the dynamics of EPS production and nutrient diffusion in biofilms [19]. The model showed that even when EPS-producers pay a large cost of EPS production, EPS-producers are capable of outcompeting EPS non-producers in the same biofilm. EPS-producers are able to overcome their growth disadvantage because daughter cells of EPS-producers (unlike daughter cells of non-producers) are pushed up above the focal cell in the EPS matrix, allowing the EPS-producer cells to access superior nutrient conditions such as higher oxygen levels. As the cells grow, divide and secrete EPS, EPS-producers form high, tower-like structures within the biofilm, smothering the neighboring non-producer cells, which remain close to the substrate (Figure 3).
Nutrient limitation is key to the EPS-producer advantage. In simulations where there is no nutrient gradient, the non-producers win due to their higher growth rate. However, conditions without nutrient gradients are unrealistic as nutrients are transported into the biofilm by diffusion, which can be a slow process compared with the fast rates of nutrient uptake by bacteria [20]. As a consequence, biofilm bacteria rarely grow in conditions where nutrients or oxygen levels are non-limiting. In realistic conditions, EPS-producers are able to overcome the cost of production and outcompete cheaters by their ability to take a more advantageous location within the biofilm [19].

While the computer simulations were originally conducted with *P. aeruginosa* in mind [19], the proposed role of EPS in the competition between cell lineages within a biofilm was confirmed by experiments in *Vibrio cholerae* [21**]. In these experiments, the EPS-producers (ΔflaΔ*hapR*) were more competitive and increased in population fraction during the course of co-culture competitions with non-producers (ΔflaΔ*hapRΔcpsL*), even though EPS-producers pay a substantial production cost and have a slower maximum growth rate compared to non-producers. As seen in the *in silico* study, *V. cholerae* EPS-producers formed high towers in the biofilm while the non-producers remained in a flat layer [21**]. Taken together, these studies demonstrate the importance of social interaction for the success of bacterial strains in a community and illustrate that rather than being the result of a purely competitive process, the formation of complex biofilms can involve a balance between cooperative and competitive interactions [22].

Another recent study investigated the role of quorum sensing cheaters on *P. aeruginosa* biofilm stability. The experimentalists demonstrated that cheaters reduce the overall productivity of a biofilm and render biofilms more susceptible to antibiotics [23]. Importantly, the effects of cheaters were more severe in biofilms than in planktonic populations, suggesting that biofilm infections are particularly susceptible to social disruption strategies. With both competitive and cooperative traits playing important roles for biofilm stability, the next question is which social traits are appropriate targets for biofilm disruption therapies.

**Inducing biofilm dispersal**

Inducing dispersal of unwanted biofilms is an appealing strategy [17,24]. However, the use of extrinsic detachment promoting agents can be limited by a slow diffusion of the agent into the biofilm [25]. An alternative is to manipulate the bacterial regulatory mechanisms for different modes of growth to make bacteria less successful.
at forming biofilms or even disperse established biofilms. In *P. aeruginosa*, biofilm formation and motility behaviors are inversely regulated [26,27]. The *sad* genes, chemotaxis genes and intracellular c-di-GMP levels have been shown to play roles in this inverse relationship between biofilms and motility [27]. As more molecular mechanisms are uncovered (Table 1), such systems could be manipulated to direct cells away from forming a stable biofilm.

*P. aeruginosa* produces rhamnolipid biosurfactants that promote detachment of its own biofilms [28]. The ability to secrete the right amount of rhamnolipids is crucial for proper biofilm architecture and stability. Therefore, strains that overproduce rhamnolipids are deficient in biofilm formation, forming thin, flat biofilms compared to the wild type’s voluminous towers. Artificially induced production of rhamnolipids, on the other hand, leads to detachment of cells within the biofilm [28]. Rhamnolipid-induced dispersal is not limited to *P. aeruginosa* making these biosurfactants appealing candidates for dispersal of multi-species biofilms in both medical and industrial settings [29]. Rhamnolipid secretion has the potential to induce biofilm dispersal *in vivo* and make bacteria more vulnerable to clearing by the host or by traditional antibiotics.

Here, again, proper caution must be taken. The rhamnolipids of *P. aeruginosa* have detrimental effects on eukaryotic cells, lysing red blood cells and causing necrotic cell death in polymorphonuclear leukocytes (PMNs) and macrophages [30]. This function may have an important role in protecting biofilms from the host immune cells such as PMNs, which are critical to clearing *P. aeruginosa* *in vivo* [31]. Biofilms of rhamnolipid-deficient strains cause significantly less necrosis in PMNs [30]. Additionally, experiments using implants colonized by *P. aeruginosa* in mice showed that robust implant colonization requires biosurfactant production [31,32]. Rhamnolipid-deficient strains can be phagocytosed and cleared by predating PMNs, thereby failing to form biofilms comparable to those of wild type rhamnolipid-producing strains. The role of *P. aeruginosa* rhamnolipids is therefore multifactorial and their use as a therapeutic target requires further investigation.

**Table 1**

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<th>Examples of intrinsic mechanisms for dispersal of bacterial biofilms</th>
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<td>Bacillus subtilis — D-amino acids</td>
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<td>Pseudomonas aeruginosa — rhamnolipids</td>
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<td>Staphylococcus aureus — extracellular proteases</td>
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<td>Bacteriophage engineered delivery of dispersal enzymes</td>
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Rhamnolipid-targeting drugs may take advantage of a complex transcriptional regulation of rhamnolipid synthesis that integrates metabolic and quorum sensing signals. Rhamnolipids are produced by the action of three sequentially functioning enzymes: RhlA, RhlB and RhlC [33]. RhlA is required for any rhamnolipid synthesis in the cell and catalyzes the initial conversion of β-hydroxyacyl-ACP to 3-(3-hydroxyalkanoyloxy) alconic acids (HAAs) [34]. After this conversion, RhlB and RhlC are sequentially required to add rhamnose units, producing mono-rhamnolipid and di-rhamnolipids, respectively. Quorum sensing signals are necessary but not sufficient for the expression of the rhlAB operon, the rate-controlling step for rhamnolipid production. New studies suggest that bacteria require excess carbon in addition to quorum sensing signals to trigger the synthesis of rhamnolipids [35*]. Thus, the bacterial cells carry out an integration of metabolic and quorum sensing signals in order to initiate rhamnolipid production (Figure 4). The mechanism of metabolic and quorum sensing integration is called metabolic prudence, since it prevents wild type strains from being outcompeted by cheaters even though rhamnolipid production requires significant metabolic resources [35*,36]. Through metabolic prudence, rhamnolipid synthesis is delayed until other nutrients such as nitrogen have been depleted and excess carbon used in rhamnolipid synthesis is free to be diverted away from the production of biomass.

In addition to biofilm dispersal, targeting rhamnolipid production with social disruption strategies could have substantial impacts on the ability of bacteria to compete successfully in a biofilm or infection. Engineered rhamnolipid-producer strains, which have rhamnolipid synthesis under an inducible promoter and therefore lack metabolic prudence, are highly susceptible to cheaters and are quickly outcompeted in co-culture swarming colonies [35*]. Manipulating rhamnolipid secretion will require a more complete understanding of the system, as key molecular details of the integration between quorum sensing and metabolic sensing are still unknown. Further study into the molecular basis of metabolic prudence may reveal new avenues to exploit the intrinsic mechanism of biofilm dispersal of P. aeruginosa.

Conclusion

There is a pressing need for alternatives to antibiotics, our main defense against bacterial pathogens that is increasingly threatened by the emergence of resistance. The solution may come from targeting population-level traits such as biofilm formation and quorum sensing. Microbial social evolution can help identify novel therapeutic targets and assist in the rational design of therapies that avoid selection for resistance. The coming years are sure to bring more insights from the fascinating interface between molecular microbiology and social evolution theory.

Acknowledgements

We thank Karina Xavier and Carlos Carmona-Fontaine for comments on the manuscript. We acknowledge support from the National Institutes of Health (grant DP2OD008440 to JBX) and a James S. McDonnell postdoctoral fellowship to SH.

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• of special interest

** of outstanding interest

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35. Xavier JB, Kim W, Foster KR: A molecular mechanism that stabilizes cooperative secrections in Pseudomonas aeruginosa: Mol Microbiol 2011, 79:169-179. This paper shows that P. aeruginosa uses a mechanism of metabolic prudence to delay production of rhamnolipid biosurfactants, a public good required for swarming migration, until nitrogen is depleted and growth has slowed. Metabolic prudence reduces the cost of producing the public good, making the wild type more competitive against cheaters.