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Sociomicrobiology and pathogenic bacteria

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Abstract

The study of microbial pathogenesis has been primarily a reductionist science since Koch's principles. Reductionist approaches are essential to identify the causal agents of infectious disease, their molecular mechanisms of action and potential drug targets, and much of medicine's success in the treatment of infectious disease comes from this approach. But many bacterial caused diseases cannot be explained by focusing on a single bacterium. Many aspects of bacterial pathogenesis will benefit from a more holistic approach that takes into account social interaction within bacteria of the same species and between different species in consortia such as the human microbiome. I discuss recent advances in the emerging discipline of sociomicrobiology and how it provides a framework to dissect microbial interactions in single and multispecies communities without compromising mechanistic detail. The study of bacterial pathogenesis can benefit greatly from incorporating concepts from other disciplines such as social evolution theory and microbial ecology where communities, their interactions with hosts and with the environment play key roles.

INTRODUCTION

Microbiology has gathered much attention in recent years thanks to major scientific advancements in the microbiome field. Large-scale projects such as the NIH funded Human Microbiome Project [1-3] provide extensive catalogues of the microbes that live in and on the human body. Statements like “the human body is home bacteria that outnumber human cells by more than 10:1” or that “the genetic content of these bacteria can be 100x the that of the human genome” are popular in mainstream media and even relatively well known to the general public now. Vast explorations of the human and non-human microbiomes are to large extent boosted by recent breakthroughs in DNA sequencing and community metagenomics [4-6], and the many studies that emerged reveal an expanding role of multispecies host-associated microbial communities in many host functions [7, 8]. Arguably one of the most notable functions of commensal microbiota, i.e. non-pathogenic microbes, is in protecting the host against colonization by microbes [9]. This is an exciting area of research that helps explain many puzzles in pathogenesis such as why individuals exposed to the same pathogen can differ in the level of infection. It can also explain why patients can have increased risk of infections after antibiotic therapy when antibiotics have the undesired effect of destroying the commensal microbiota that would naturally protect against pathogen invasion.

Understanding how microbiomes protect against colonization by pathogens and other related aspects of microbial pathogenesis requires a new set of experimental and theoretical tools.

The focus must broaden beyond the single pathogen as the cause of disease, and start to consider also the host resident microbiota and its important role in modulating infection. Understanding how microbial communities function, how they are assembled and how they change in time after perturbations like antibiotics or diet changes, is a complex problem that is best suited to an integrative approach. Fortunately, there is an extensive body of knowledge on the functioning of complex biological consortia in the fields of ecology and evolution that we can learn from.

Here we start by reviewing the findings of sociomicrobiology, a discipline that aims to address how bacteria function in communities [10]. Then, we analyze how seemingly cooperative microbes may actually be driven by selfish motives even within communities where every microbe is of the same species. We move on to multispecies communities, a more complex scenario where both conflict and cooperation can occur, and in fact may both be essential components of the robust behaviors that micro-ecosystems often have. We end with an ecologist's view of the human microbiome, and a discussion of how resistance against pathogen colonization is best interpreted as a problem in ecology.

BIOFILMS, QUORUM SENSING AND THE DAWN OF SOCIOMICROBIOLOGY

Bacteria are rather social organisms. Biofilms, dense communities of bacteria, are a common cause of persistent infections, and the list of biofilm forming pathogens includes common threats such as *Pseudomonas aeruginosa* [11], *E. coli* [12], *Salmonella enterica* [13], *Klebsiella pneumoniae* [14], *Vibrio cholerae* [15, 16] and *Clostridium difficile* [17]. Microbiologists came to realize the importance of biofilm formation in pathogenesis in part because bacteria once in biofilms have much higher tolerance to antibiotics, and the mechanism of this tolerance appears to be distinct from conventional antibiotic resistance [18, 19].

Biofilms saw a surge in interest among the microbiology community in the late 1990's. Even though it was well known that microbes formed dense surface attached films and that these films have medical implications, the topic seemed to get more interest from engineers who were interested in the mechanics of biofilm formation and their role in engineering problems such as industrial biofouling and beneficial applications such as wastewater treatment [20, 21]. When experiments showed that quorum sensing played a role in regulating biofilm formation [22, 23] the search for genetic mechanisms of biofilm formation became a very hot topic. The excitement in the field quickly grew as new molecular mechanisms of biofilm formation came to light [24, 25]. The growing field generated a new model (Fig. 1), primarily inspired by experiments in *Pseudomonas aeruginosa* but later supported by other species, where biofilm formation follows a developmental program with different stages and phases, each one potentially driving expression of a distinct set of genes, much a like the developmental programs of multicellular eukaryotic organisms [26].

The excitement felt at the time was understandable. If a genetic program similar to developmental pathways in multicellular organisms controls biofilm formation then this would open the way to new therapies. Anti-biofilm drugs such as quorum sensing inhibitors

[27] would be a huge new opportunity for medicine when resistance to traditional antibiotics is a growing problem at the global scale, and pharmaceutical companies invest less in new antibiotic discovery [28]. Could we find ways to fight bacteria by jamming their cell-to-cell communication channels and preventing them from organizing themselves in communities that make them harder to treat?

The years that followed the onset of sociomicrobiology were a boom for biofilm research, leading to important findings. A notable example is the role of intracellular signaling molecule cyclic-di-GMP in regulating the transition from motile to biofilm modes [29]. This molecule, which regulates transition to biofilm in addition to other functions in many species of bacteria, informs the cell that it should down regulate genes for motility and up regulate biofilm genes. In *P. aeruginosa,* there is an emerging picture where the bacterium mechanically senses a surface using the transmenbrane Wsp system [30]. This system is a multi-protein complex composed of WspA, WspB, WspC, WspD, WspE and WspF. The transmembrane WspA protein possibly changes conformation when cells contact an attachment surface. This triggers a phosphorylation of a response regulator called WspR that then leads to cyclic-diGMP production. Downstream of the Wsp complex, the transcriptional activator FleQ regulates expression of flagella genes when cyclic-diGMP levels are low, but switches to expression of biofilm matrix genes (the *pel* operon) when cyclic-diGMP levels are high. The ability of FleQ to bind to c-diGMP and regulate motility-to-biofilm transition properly depends on a protein-protein interaction between FleQ and the antiactivator FleN [31, 32]. Knocking out FleN produces *P. aeruginosa* cells that are multiflagellated but immotile [33], whereas point mutations in that protein can produce multiflagellated mutants that are hypermotile, the so-called hyperswarmers [34]. Hyperswarmers are locked in a perpetual motility mode and cannot make proper biofilms. Similar, a range of mutants in c-diGMP related genes have been found to be locked in either motility or biofilm modes [35]. In this context, c-diGMP is emerging as a central player in the molecular decision making process to transition from the planktonic mode to the surface attached mode of bacterial living (Fig. 1). Work on this area promises to reveal molecular mechanisms that can become targets to prevent pathogens from forming biofilms [36].

Molecular biology studies often seem to take for granted the view of biofilms as highly organized communities. It is not uncommon to find descriptions as ‘city of microbes’ [37] where bacteria would live together in synergy, communicate via cell-cell signaling and share secreted resources. But how realistic is that view? Natural selection is a selfish process where the fittest survive and leave more offspring. Could we expect biofilms with millions of individual bacteria to be immune to the evolution of exploitative mutants that benefit from the cooperation of others? In the next section I discuss the arrival of social evolutionary theory to the field of microbiology, and how the view of natural selection acting primarily at the level of the gene can help clarify some of these issues and shed light on microbial interaction.

BACTERIAL SOCIAL INTERACTION: COOPERATION OF CONFLICT?

Social evolution theory is a field that aims to dissect the evolutionary mechanisms of social behaviors such as altruistic cooperation. The evolution of cooperative behaviors is an old

problem, yet only ten years ago this problem was recognized as one of the top ‘125 unknowns’ by the journal Science [38]. Around that time, the field of social evolutionary theory started its foray into bacterial pathogenesis [39, 40].

A landmark paper at the time looked at the production of iron scavenging siderophores under the lens of social evolution [39]. Siderophores are compounds secreted by bacteria that have high affinity to iron. Once in the extracellular space, siderophores scavenge iron that would otherwise be inaccessible to the bacteria and frees it up to be taken up by bacterial cells (Fig. 2). This allows bacteria such as *P. aeruginosa* to grow in iron limited environments like host tissues, were extracellular iron is normally maintained at very low concentrations preventing the growth of pathogens. The problem from an evolutionary perspective is that siderophores are what is called a ‘public good’ in a bacterial society. A public good is a concept taken from economics that means a resource that is available to all individuals within a population irrespective of who's producing. When the production of a public good is costly there is a strong incentive for cheating, meaning for individuals to not produce the public good and just exploit the public goods produced by others. In these situations, what prevents the collapse of the population?

The study by Griffin and colleagues [39] first showed experimentally that siderophores of *P. aeruginosa* are costly public goods. They compared the growth of a siderophore producing strain and a non-producing strain in iron limited conditions where siderophores are key to bacterial growth. As expected, they saw that the siderophore producing strain grows much better than the non-producing strain when the two are compared in monocultures. However, when mixed together in a co-culture, the non-siderophore producing strain grew better than the producing strain because it could use the siderophores without paying a metabolic cost of their production. Importantly, the final numbers in the population where lower for the co-culture than for the monoculture of siderophore producers. The non-producing strain benefited from being mixed with the producer but the whole population suffered as a consequence (Fig. 3A).

This dramatic outcome is a hallmark of cheating and captures the essence of the problem of the evolution of cooperation [41]. Cheaters gain a selfish benefit from being in the mix with cooperators, but the whole population suffers from it. So how can we observe so many cooperative traits in nature, where organisms seem to altruistically sacrifice their own fitness for the benefit of others? This question has been on the minds of evolutionary biologists for a long time. One answer is: kin selection. J.B.S. Haldane, one of the architects of the modern synthesis, reportedly joked that he would altruistically give his life for two brothers or eight cousins, reflecting the Mendelian inheritance probability of 1/3 of sharing a gene with a brother and 1/8 of sharing a gene with your first-degree cousin. Kin selection explains that a cooperative strategy can evolve if the fitness costs that the cooperative behavior has to the actor is less than the fitness benefit to the recipient multiplied by a relatedness coefficient between actor and recipient. This relationship, $r \times b > c$, is known as Hamilton's rule in honor of William Hamilton who proposed a theory for the genetical evolution of social behavior [42, 43]. The insight behind this rule is that selection acts at the level of the genes, and a gene encoding for a cooperative behavior will still increase in frequency within a population if its function is to make the organism that carries it help other carriers of that gene. Since

fitness concerns the increase of gene frequency in a population, a gene may be fit if it increases other copies of itself, and social evolutionary biologists often use the term 'inclusive' fitness to account for these type of social effects. The concepts were popularized in the book "The Selfish Gene" by Richard Dawkins [44].

How is this relevant to microbial pathogens? Spontaneous mutants that lose function in a gene are common due to the large population sizes and the relatively high mutation rates of bacteria. If a mutant has a loss of function in a cooperative gene, for example in the gene *pvdA* that catalyzes a key step in the synthesis of the siderophores pyoverdin [45], then this mutant could become a cheater. A way for cooperation to be maintained in the face of cheaters is if the remaining cooperators cooperate only with individuals that still carry a functional copy. This would be the case where r would have a high value.

In the experiments of Griffin and colleagues [39] they tested out this prediction by mixing bacteria in different ways to manipulate relatedness. As expected, they saw that conditions of high relatedness favored siderophores producers (cooperators), whereas conditions of low relatedness, where strains mixed more frequently with other clones, favor non-producing strains (cheaters).

Mechanisms stabilizing cooperation in bacterial pathogens

Mixing with other clones reduces the relatedness in a social interaction. However, in nature and in the clinic we expect that bacterial strains and species will often be in mixed communities. Are there other mechanisms stabilizing cooperation in communities where relatedness is low?

The extracellular polymeric substances of biofilms could naïvely be viewed as a public good. These substances, which make up the gooey matrix that sticks bacteria to each other and to the solid substratum in a biofilm (Fig. 1), require significant metabolic resources to be produced. Mutants that do not produce matrix could still benefit from the matrix produced by others. This was shown not to be the case by a series of studies, first with computer simulations [46] and later with experiments with *Vibrio cholerae* [47] and *P. fluorescens* [48]. The reason for this is that bacterial biofilms have very steep gradients of nutrients and other solute substances. For example in biofilms of aerobic bacteria and in colonies growing on agar plates diffusional gradients are often so steep that bacteria in the interior layers cannot grow due to oxygen depletion. In a mixed biofilm of polymer producers and non-polymer producers the producers gain an advantage because the polymers allow them to be pushed on top and reach higher concentrations of nutrients. By secreting polymers, a polymer-producing bacterium benefits itself and its lineage, and literally suffocates non-polymer producers in the inner layers of the biofilm. This mechanism of 'competitive smothering' presents polymer production as a competitive strategy. What could at first glance be perceived as a cooperative trait reveals a selfish motive.

Bacteria have alternative ways push the balance of Hamilton's equation in their favor, even when a trait is clearly cooperative. *P. aeruginosa* colonies are capable of a remarkable collective motility behavior called swarming behavior [49, 50]. Swarming allows the colony to spread across large surfaces in a way that single cells cannot and this way benefits the

population. However, swarming requires that cells produce and secrete larges amounts of biosurfactants, called rhamnolipids, that lubricate the surface and allow the bacteria to slide on top of it [50]. Rhamnolipids are a public good like siderophores. Strains that do not produce surfactants, such as an *rhlA*$^{-}$ mutant, cannot swarm on their own but will do so when mixed with surfactant producers [51]. However, unlike with siderophores there is no public good dilemma. A 1:1 mixture of wild-type bacteria a *rhlA*$^{-}$ remains at the 1:1 even though the wild-type is producing copious amounts of surfactants and the *rhlA*$^{-}$ strain is benefiting from them. How do we explain this conundrum?

The *rhlA* gene is an operon (*rhlAB*) that is tightly regulated by a combination of quorum sensing and metabolic sensing. The regulatory circuit ensures that *P. aeruginosa* wild-type cells express rhamnolipids only when they have reached a quorum but also when they have carbon source in excess of that needed to grow. This regulation, called metabolic prudence, ensures that *P. aeruginosa* delays expression of cooperation to times when it becomes affordable because it has an abundance of carbon. By doing this, *P. aeruginosa* is using transcriptional regulation to decrease the cost of cooperation, the c in Hamilton's equation [51]. Metabolic prudence allows cooperation even in situations of low relatedness where constitutive cooperation is not possible [52].

Cheating can explain clonal diversity in infections

In the absence of a mechanism to reduce cost or increase relatedness, a social trait that provides a benefit to others may be doomed. This is the case in opportunistic infections by *P. aeruginosa* where virulence mediated by type III secretion system is an altruistic trait. Type III systems are important factors in pathogenesis that consist of huge needle–like transmembrane protein complexes that inject toxins into host cells. The system is essential for pathogenesis but the protein complex is thought to be costly to produce. Using a mouse model of lung infection by *P. aeruginosa* Czechowska and colleagues provide evidence for cheating by mutants lacking type III secretion [53]. Although these mutants fail to infect mice on their own, they do well when co-infected with type III positive isogenic strains at a 1:1 ratio. When co-infected at different ratios the type III advantage was high when they were the minority in the mix, but this advantage decreased when they were in the majority. This is what evolutionary biologists call frequency-dependent selection [54], which is in this case where fitness decreases with increasing frequency is another hallmark of cheating (Fig. 3B). The public goods in this case are likely the metabolic products released by the killing of eukaryotic host cells which should benefit all individuals in a bacterial population irrespective of which ones have a type III system. Type III secretion mutants are often found in patients with *P. aeruginosa* and the study proposed that their rise is due to cheating [53]. In the absence of a mechanism to protect against cheating, cheaters are fated to dominate and cooperation would be doomed to extinction. Perhaps this type of phenomenon could be exploited in the development of ‘Trojan horse’ approaches [55] where engineered cheaters exploit wild-type pathogens?

MULTISPECIES COMMUNITIES AND THE MICROBIOME

In the previous section we saw examples cooperation and conflict between pathogens of same species. However, many bacterial communities are multispecies and the number and richness of interactive behaviors can grow exponentially making it harder to dissect experimentally. Computer models of multispecies biofilms suggest that the presence of competing strains can have a strong influence on within-species cooperation and that both within-species and between-species interactions are highly influenced by environmental conditions [56]. In spite of this complexity, understanding how bacteria interact within multispecies communities can be an essential step towards a mechanistic basis of host associated microbiomes relevant for many aspects of host health [57-60] and even host behavior [61].

Low biodiversity in the gut microbiota seems to increase the risk of enteric infection [9]. We can learn a lot from extreme examples, and here the infectious diseases of bone marrow transplantation patients is providing an insightful model. Patients receiving bone marrow transplants typically have a blood or bone marrow cancer such as leukemia or lymphoma and they are hospitalized during the procedure. During this time the patients become immunocompromised and can receive significant doses of antibiotics to prevent opportunistic infectious. In many cases the patients are diagnosed with infectious by pathogens such as *Clostridium difficile* and Vancomycin Resistant *Enteroccus* (VRE) following administration of antibiotics.

Resistance against pathogen colonization

A recent large-scale study analyzed the gut microbiota of a cohort of 94 allogeneic hematopoietic stem cell transplantation patients at Memorial Sloan Kettering [62]. Metagenomics from fecal samples taken at several time points relative to the day of the transplant showed many cases where the biodiversity of the microbiota falls sharply during antibiotic treatment. This drop in biodiversity is typically due to the expansion of a single member of the gut microbiota. Events where a single member dominates the microbiome boost the risk of infection.

The observation that intestinal domination in bone marrow transplants increases risk of infection suggests an ecological view of the microbiome where the commensal gut microbiota is a biodiverse ecosystem that naturally resist invasion by a foreign species, in this case by a pathogen. When the host takes antibiotics this affects the natural composition of the gut microbiota causing a cascading loss of species that interact with each other and a drop in biodiversity that opens the way to invasions (Fig. 4). This is supported by a mathematical model that takes into account the dynamic social interactions between species. Simulations with this model show that sudden shifts in microbiota composition can lead the system to a state of dysbiosis that is difficult to recover from [63]. The same effect was replicated in mouse models, where antibiotics are given to mice to perturb their gut microbiota before they received a dose of pathogens. The procedure has been tested in a range of antibiotics and at least two pathogens, *C. difficile* and VRE [64, 65]. In these mouse models pre-treatment with antibiotics increases infection rates dramatically.

Understanding resistance to invasion in the gut microbiota is a problem in ecology, and it makes sense to apply the tools of mathematical ecology to dissect its mechanisms. A recent approach used the classical model of predator-prey dynamics, called the Lotka-Volterra model, to describe the interactions between microbes in the gut [66-68]. In its most detailed form, the model includes three terms that describe (i) the intrinsic growth rate of each microbe, (ii) the pairwise interactions between two microbes and (iii) the effects of external factors such as antibiotics on each microbe. These models have a large lumber of parameters and determining the values of the parameters is technically challenging. It is nonetheless possible by using large enough datasets and machine learning approaches that avoid parameter overfitting. Once a model is correctly parameterized it can reveal the networks of interactions occurring between microbiota members, which is valuable information that allows investigating mechanisms of resilience to antibiotic perturbations but also identify microbes that protect against pathogen invasion [67].

The Lotka-Volterra approach was applied recently to model the microbiota of human patients and mouse models following antibiotic treatment and *C. difficile* infection. The model revealed that a single commensal microbe, *Clostridium scidens*, explained a significant part of the protection in both mice and humans. However, a community was always better than a single microbe. Experimental follow up studies unveiled the mechanism by showing that the capability of *C. scidens* to metabolize secondary bile acids is key to hinder *C. difficile* colonization [69].

CONCLUSION

Microbes have rich and diverse social lives [70], and pathogenic bacteria are not an exception. In many cases, pathogens invading a human host encounter a commensal community that may be viewed as the first line of defense against pathogen invasion. Understanding how these communities function requires an investigation of its social interactions, both cooperative and competitive, and how they produce a biodiverse and robust microbiota.

These are exciting times for the field of the human microbiome. Although the millions of bacteria that live in and on our bodies have been long recognized to play important roles in health and disease, their study has been traditionally hampered because most microbes are difficult to cultivate in laboratory conditions. Recent advancements in DNA sequencing, metagenomic analysis and culturing of microbial communities [71, 72] enable the direct and mechanistic analysis of gut microbiome dynamics. The booming filed of metagenomics-based analysis of the microbiota is opening a new perspective that presents new challenges and many opportunities. We can anticipate that microbiome analysis of patients may become routine in the future, enabling the use of mathematical ecology models to assist medicine for example in the rational design of antibiotic therapies [73].

Before this is possible we must gain a better understanding of the ecology and evolution of social interaction in microbial communities. As we discussed here, even monospecies communities have the potential for conflict and cooperation. Analyzing these features of microbial communities requires new frameworks that expand the field of sociomicrobiology

to include concepts form evolution. There is a tremendous potential for the field of microbial pathogenesis here, as social interactions may reveal new therapeutic targets against microbial infections.

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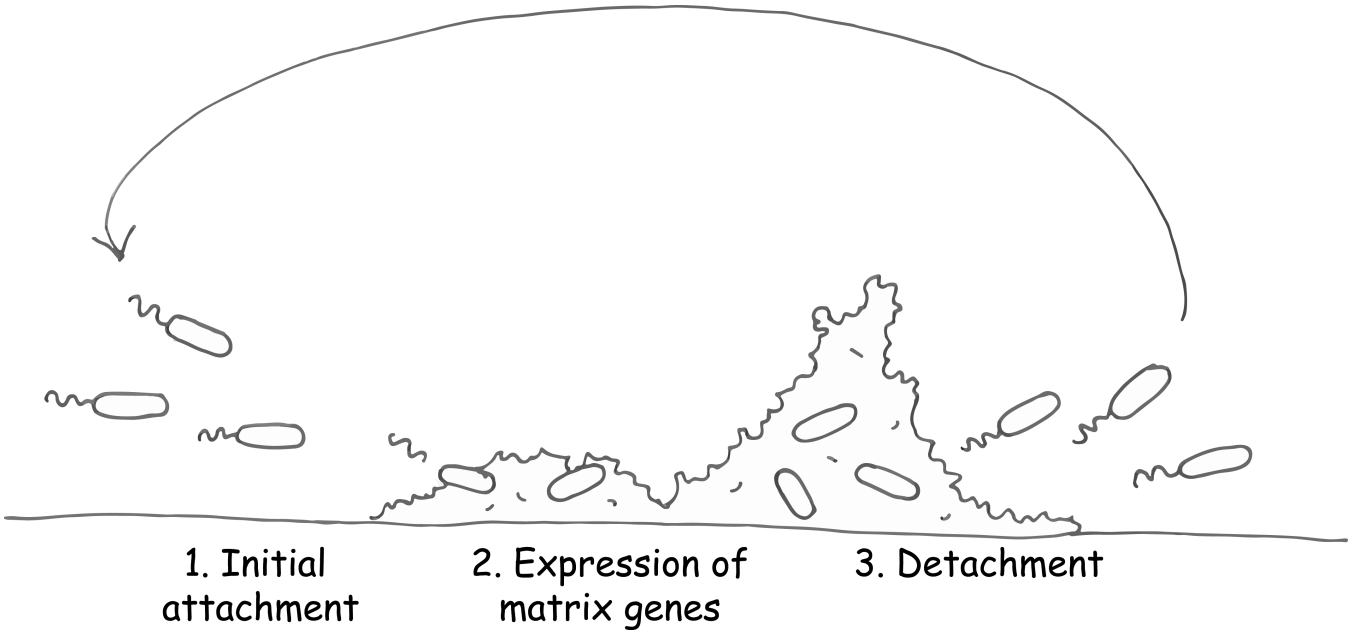

Figure 1.

A model of biofilm development and life cycle proposed in [18]. Planktonic bacteria attach to surfaces, initiate expression of biofilm genes such as synthesis of extracellular polymeric matrices and grow a biofilm. Cell can detach from a mature biofilm back to the planktonic state, closing the biofilm life cycle.

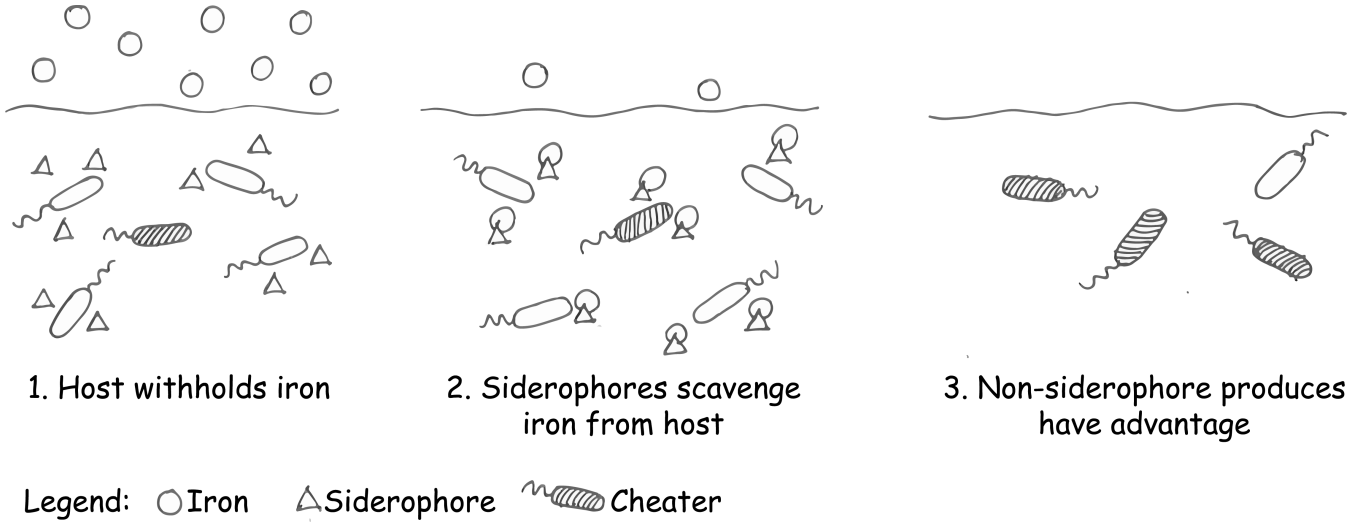

Figure 2.

Siderophore production as a cooperative trait [74]. Bacterial pathogens such as *P. aeruginosa* can secrete siderophores to scavenge iron in iron-limited environments such as host tissues (panel 1). The siderophores have high affinity to iron and can be taken up by bacteria including non-siderophore producers that still have the siderophores receptors (panel 2). Non-siderophore producers exploit wild-type producers by not paying the cost of siderophores production, but this can lead to the extinction of siderophores production in the population (panel 3).

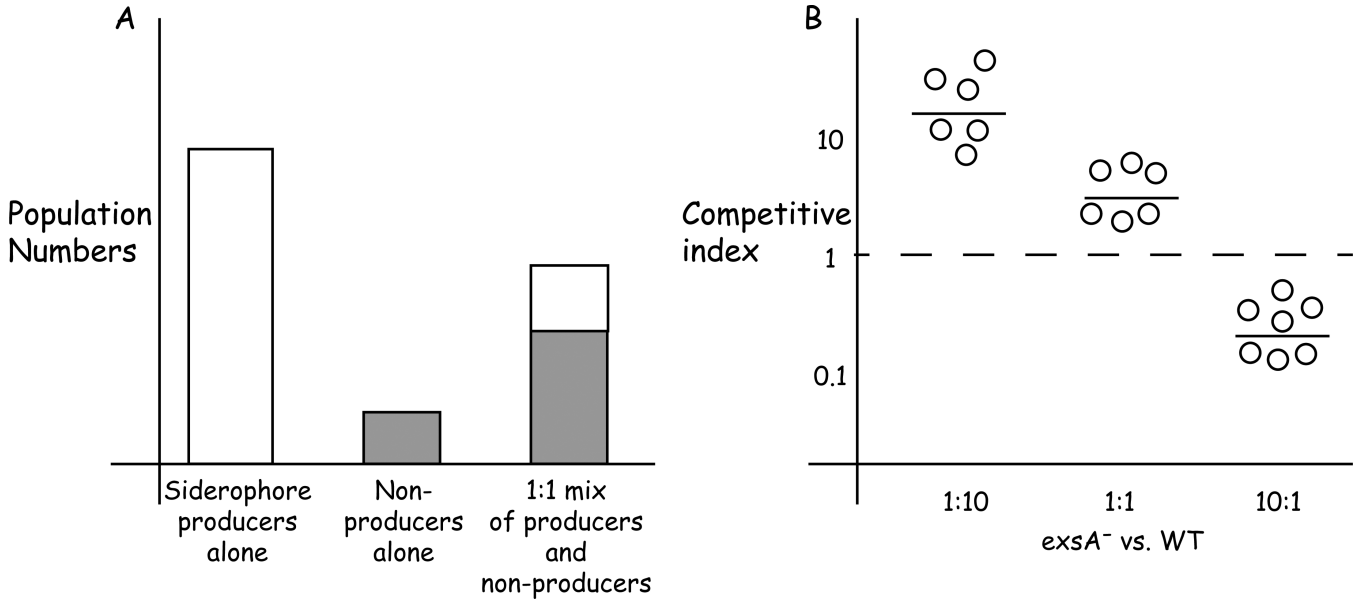

Figure 3.

Laboratory experiments that reveal the hallmarks of cheating. A) Siderophore producing *P. aeruginosa* grow reasonably well in iron-depleted media by increasing iron uptake thanks to sideophore scavenging (Fig. 2). Non-siderophore producers (cheaters) grow poorly in the same environment when alone, but do better when mixed with producers by not paying the cost of siderophore production. The advantage of non-producers comes at the expense of the whole population [74]. B) The competitive advantage of cheaters decreases as their frequency increases because there are less cooperators to exploit in the population. This example is taken from a study of type III secretion systems in where *P. aeruginosa* where *exsA*$^{-}$ mutants lacking the type III system could cheat over wild-type bacteria (WT), but their measured competitive index decreased as cheater numbers increased in the population [53].

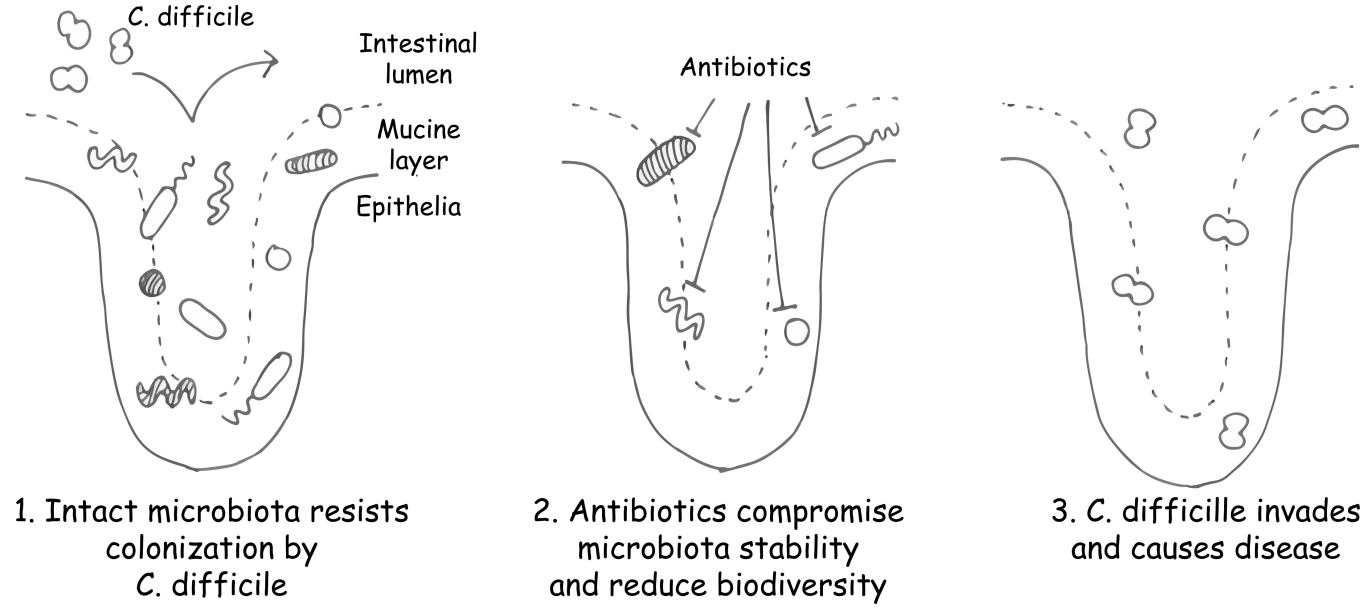

Figure 4.

Colonization resistance in the gut microbiota and the harmful effect of antibiotics. 1) The gut microbiota can resist colonization by pathogens such as *Clostridium difficile*. 2) Antibiotics disrupt the ecology of the commensal microbiota. 3) Antibiotic challenged microbiota open the way to colonization.