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Does Social Exploitation within Pathogen Populations Pose an Opportunity for Novel Therapeutic Approaches?

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Abstract

Bacterial virulence (damage to host) is often cooperative, with individual cells paying costs to promote collective exploitation. This chapter reviews how cooperative virulence traits offer novel therapeutic avenues involving either the genetic introduction or chemical induction of "cheats" that can socially exploit cooperative wild-type infection. Issues of efficacy and evolutionary robustness are discussed, and evidence of an evolutionarily robust therapeutic that targets bacterial social behaviors is highlighted.

Social Evolution in Microbes and Pathogens

Cooperative Microbes

Many species of microbes are now recognized to be highly social, with individual microbes working within large collectives to engineer their environments to allow further growth and dispersal. Collective behaviors include investments in extracellular foraging, collective shelter, dispersal, signal-mediated coordination, and the production of chemical and biological weapons (Crespi 2001; West et al. 2007a; Brown et al. 2009a; McNally and Brown 2015).

Investment in these collectively beneficial behaviors often bears an individual cost (typically the cost of producing and secreting extracellular molecules) and therefore poses a basic social dilemma: Why should a cost be paid to benefit others? More directly, how can cooperative producer lineages survive in competition with nonproducer "cheat" lineages, which take the benefits but do not pay the costs? In the terminology that has been adapted in this book,

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cooperative producer cells can also be viewed as *investors* in microbial common goods (producing and consuming common goods), while nonproducer cheats can be viewed as *exploiters* of these goods (less production and/or more consumption) (see, e.g., Brown and Taylor 2010).

Solutions to the classic social dilemma posed by cooperative behaviors are various in detail but can all be placed within two classes: nepotism and self-interest (West et al. 2007b). Self-interest may favor cooperative investments if the returns on investment to the actor outweigh the costs of investment. Nepotism (or kin selection) may favor cooperative investments if the return on investment falls preferentially to other individuals carrying copies of the allele(s) that code for the cooperative trait. Both classes of solution can be captured by Hamilton's rule (Hamilton 1964), reviewed in detail by Gardner et al. (2011).

Pathogenic (and opportunistically pathogenic) microbes can also be highly social. For instance, pathogenic and nonpathogenic strains of *Escherichia coli* show broadly equivalent levels of investment in secreted molecules (Nogueira et al. 2009). However, these collectively beneficial secreted factors are now likely to come at the expense of the host and are typically labeled "virulence factors" in the biomedical and microbiology literatures (Allen et al. 2014). For a recent review of microbial sociality in infections, see Leggett et al. (2014).

Pseudomonas aeruginosa as a Model System

The opportunistic pathogen *P. aeruginosa* is a leading experimental model system for bacterial sociality. *P. aeruginosa* is an impressive environmental generalist, able to grow in diverse soil, aquatic, and host environments. As an opportunistic pathogen, it displays an extraordinary host range, from protists to plants to animals. In humans, *P. aeruginosa* infects burns, cuts, catheters, implants and, most notoriously, the lungs of cystic fibrosis patients. This incredible environmental range is also associated with an extensive battery of secreted factors (McNally et al. 2014).

Iron Scavenging by Secreted Siderophore Molecules

One of the first bacterial social traits (and noted virulence factor) to receive extensive experimental attention was the collective capture of limiting iron via secretion of the *P. aeruginosa* siderophore, pyoverdine. Secreted pyoverdine molecules bind to insoluble ferric iron(III), with the resulting pyoverdine-iron complex now accessible to uptake by any cell that expresses an appropriate receptor. Exploiting the ability to construct "cheat" strains by knocking out the ability to produce siderophores (but leaving uptake intact), Griffin et al. (2004) were able to grow cooperative (producer) strains with nonproducer cheats under different metapopulation structures, to test basic social evolution theory on the conditions maintaining costly cooperative traits. They demonstrated that

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the cooperative lineage was able to survive and approach fixation across a metapopulation only when relatedness was high (each subpopulation founded by a single clone) and the scale of competition was global (more productive patches were able to export their productivity via greater propagule release into a general migrant pool) (Figure 7.1). Since this work, many studies have extended our understanding of siderophore-mediated social interactions (discussed further below, under the section on Chemical Cheat Therapy and Antivirulence Drugs). For a critical exchange of views on the merits of social interpretations of siderophore production, see Zhang and Rainey (2013), Kummerli and Ross-Gillespie (2014), and Rainey et al. (2014).

Extracellular Proteins and Quorum-Sensing Control

In addition to siderophores and other small molecules, bacteria invest up to 3% of their genome coding for proteins that are directly secreted from the cell, with secretome-rich genera including *Bacillus* and *Staphylococcus* (McNally et al. 2014). While intracellular proteins can be efficiently recycled, secreted proteins are likely to be lost to the environment and therefore have imposed selection for the use of cheap amino acids in their construction (Nogueira et al. 2009). Cost management can also be seen in many bacterial species through the use of complex regulatory circuits to control investment in secreted proteins,

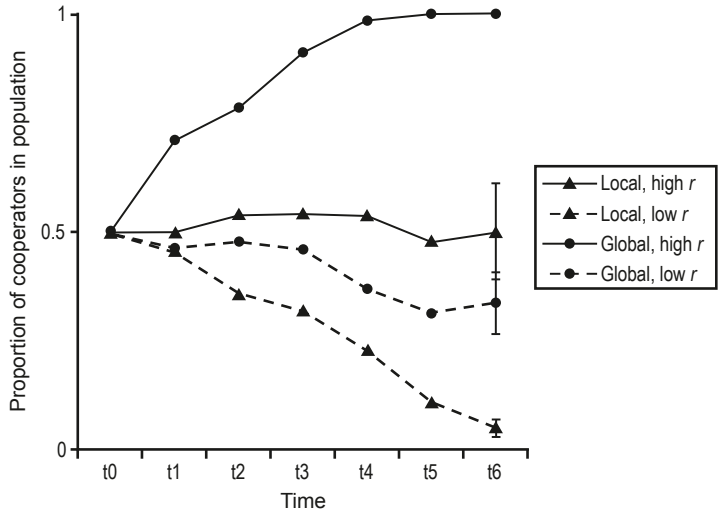

Figure 7.1 Bacterial cooperator versus cheat dynamics *in vitro*. Metapopulation dynamics of cooperators (siderophore producers) versus cheats (nonproducers) as a function of relatedness (high r = single clone founder per subpopulation; low r = two clone founders), and scale of competition (local = all subpopulations contribute equally to migrant pool; global = subpopulations contribute in proportion to their productivity). From Griffin et al. (2004), reprinted with permission.

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in particular via the widespread use of quorum sensing to control secretome expression (Popat et al. 2015).

Quorum sensing is a form of cell-to-cell communication in bacteria, mediated by diffusible signal molecules (Rutherford and Bassler 2012; Schuster et al. 2013). The accumulation of high signal levels (due to high densities and/or low environmental removal rates) then triggers the expression of secreted proteins in *P. aeruginosa* and other quorum-sensing species (Popat et al. 2015). Diggle et al. (2007) presented the first experimental analysis of the social dilemmas posed by signal-mediated control of a costly cooperative trait, again using *P. aeruginosa* as a model system. Using isogenic wild-type (cooperator) and signal-deficient (cheat) strains, Diggle et al. demonstrated that while signal cheats showed attenuated growth in monoculture (as their signaling defects resulted in a loss of cooperative digestive enzyme production), they increase in frequency in mixed culture due to their ability to benefit from extracellular protein degradation by the wild type.

Rumbaugh et al. (2009) subsequently demonstrated similar results *in vivo*, in a mouse model. The signal cheat strains (both "deaf" and "mute" mutants) grew poorly in monoculture and showed attenuated virulence to their host, compared to wild-type infections. However, wild-type coinfections and cheat mutants showed a rapid enrichment of the "cheat" lineages from their intially rare state , and a concurrent attenuation of host mortality (Figure 7.2).

Figure 7.2 provides a clear illustration of the cooperative nature of bacterial virulence, as cooperative investments among pathogen individuals increases exploitation of the host. In the following discussion, I review the potential to control cooperative bacterial infections, either through the introduction of genetic "cheats" or through the chemical induction of a phenotypic cheat state.

Controlling Infections with Genetically Engineered Cheats

The results from Rumbaugh et al. (2009) immediately point toward a novel therapeutic intervention: the presence of initially rare "cheats" (*lasI* or *lasR* mutants) attenuated the infection (Figure 7.2), suggesting that we can reduce virulence simply by adding "cheat" genotypes to a pathogen population to undermine the collective production of host-damaging virulence factors (Brown et al. 2009b).

The notion of "cheat therapy" (Brown et al. 2009b) depends on several complementary processes:

1. Cheats can be genetically engineered to display minimal virulence, in particular through the deletion of secreted virulence factors.
2. The loss of individually costly yet collectively beneficial virulence factors (e.g., siderophores, secreted enzymes, toxins) implies that a relatively small inoculum of cheats will increase in frequency within the

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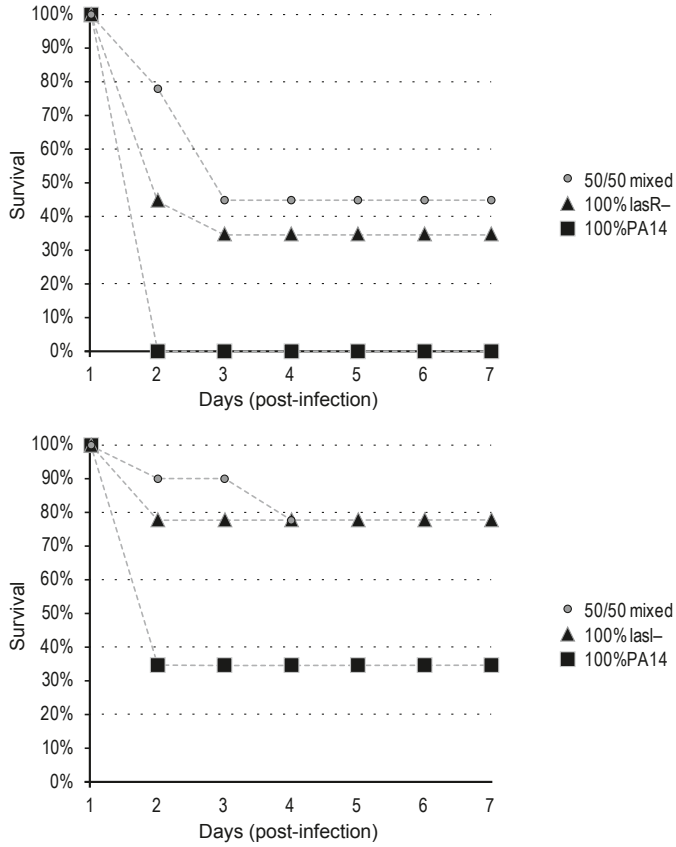

Figure 7.2 Signaling cheats attenuate virulence in mice. PA14 is a cooperative *P. aeruginosa* wild-type strain, with intact quorum-sensing control of secreted virulence factors. *lasR*– is a "deaf" mutant, unable to respond to the primary signal molecule in *P. aeruginosa*. *lasI*– is a "mute" mutant that is unable to produce the primary signal molecule in *P. aeruginosa*. Data from Rumbaugh et al. (2009).

site of infection and therefore attenuate the infection (Rumbaugh et al. 2009; Figure 7.2). For similar amplification kinetics, see phage therapy (Levin and Bull 2004).

3. As the introduced cheat lineage enriches, the total population density is predicted to decline due to a "tragedy of the commons," because fewer individuals contribute to the collective good.
4. The infection can then be fully controlled so long as the introduced cheat is sensitive to an appropriate antibiotic (the "Trojan cheat" strategy, adding a "recall" function to the introduced strain).

While attractive in theory, the potential efficacy of cheat therapy is likely to be limited by a number of processes that can block the ability of cheats to exploit

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cooperators. Below I briefly review four key obstacles: within-host spatial structure, regulatory control of secreted factors, infectious cooperation (horizontal gene transfer), and concurrent strong selective forces.

Within-Host Spatial Structure

In the idealized *in vitro* setting of cooperator–cheat competition (Griffin et al 2004), cheats and cooperators encounter each other in a well-mixed, shaken flask. This well-mixed regime maximizes the ability of cheats to exploit the cooperative activities of the wild type, favoring the local (within flask) enrichment of cheats. As hosts are clearly not well-mixed flasks, it is no longer evident that cheats will be enriched within a host, due to spatial segregation of the two lineages (increasing the average distance between cooperator and cheat cells). The results of Rumbaugh et al. (2009) illustrate *in vivo* that cheater lineages can increase in frequency. However, this occurred under the specific condition of joint inoculation: the cheat and cooperative lineages were mixed and injected together, and thus likely ended up in a shared within-host environment permitting social exploitation by the cheats. Subsequent sequential inoculation experiments did not show the same enrichment effect, most likely due to sequestration of the initial cooperator lineage away from the subsequent cheater inoculation (Griffin, pers. comm.).

In terms of the potential limitation on cheater invasion posed by within-host spatial structure, it has been suggested that in infection contexts with high spatial structuring, invasion of a therapeutic lineage could be enhanced by the addition of "spiteful" anticompetitor traits, such as the production of bacteriocins and antibiotics by the therapeutic strain (Brown et al. 2009b). The use of anticompetitor adaptations thins the distinction between cheat therapy and the broader concept of competition therapies, such as fecal microbiota transplants to treat *Clostridium difficile* infections (Gough et al. 2011).

Regulatory Control of Cooperative Virulence Traits

The motor to cheater invasion in the models of Brown et al. (2009b) and related theory are the costs of cooperation paid by the wild type: the higher the cost, the faster the invasion by cheats. However, as mentioned briefly above, microbes display multiple, complex adaptations to limit the effective costs of cooperation, for instance, using the cheapest amino acid building blocks when constructing secreted proteins (Nogueria et al. 2009) and complex regulatory rules to limit the potential for exploitation (Kummerli and Brown 2010; Xavier et al. 2011; Allen et al. 2016). The use of regulatory control can ensure that cooperative investments are limited to initial and punctual "start-up costs," which once paid can remove any phenotypic difference and therefore selective differential between cooperator and cheat genotypes (Kummerli and Brown 2010). Regulatory control can also limit investments to environments where

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costs are negligible (Xavier et al. 2011) or where cooperators are enriched; that is, bacteria can implement simple reciprocity rules of "cooperate only with cooperators"(Allen et al. 2016).

Mobile Genetic Elements and Infectious Cooperation

Bacteria, like any other cellular life form, are vulnerable to molecular parasites or "mobile genetic elements" (MGEs), such as plasmids and phages. Strikingly, as well as damaging their hosts, bacterial MGEs can also confer novel and cooperative phenotypes (Smith 2001), with secretome genes enriched on MGEs versus the chromosome (Nogueria et al. 2009). The presence of cooperative genes on MGEs poses a problem for cheat therapy, and for cheat invasion more generally, as cheat lineages can now become infected with cooperative behavior via horizontal gene transfer, protecting the cooperative phenotype from local extinction (Smith 2001; Nogueira et al. 2009; Dimitriu et al. 2014).

Strong Nonsocial Selection and Local Adaption

The ability of cheats to invade a resident population of cooperators hinges on "all else being equal." In theoretical and experimental treatments, care is taken to ensure that the two strains are isogenic, and thus equally adapted to the experimental conditions or host environment. In practice, a resident wild-type population is likely to be better adapted to the local host environment than any introduced strain. If not, then both strains are likely to be undergoing strong nonsocial selection that is likely to overwhelm any social selection mediated by differences in secreted factor production.

Morgan et al. (2012) demonstrated the potential for strong nonsocial selection to swamp social selection, in an experiment which pitted cooperators (siderphore producers) against cheats in an environment to which neither strain was well adapted. They found that the invasion of rare cheats could be halted and even reversed due to the greater evolvability of the larger cooperative lineage. For example, under strong phage selection, the more numerically dominant cooperator lineage is more likely to experience a beneficial phage resistance mutation. As this rare resistance mutation sweeps, it will then carry the (hitch-hiking) cooperative allele to fixation and exclude the rare cheat lineage.

Chemical Cheat Therapy and Antivirulence Drugs

In addition to introducing genetically engineered nonproducer cheats, the phenotypic state of nonproducers can also be induced chemically via an array of drugs that turn off secreted factors or limit their extracellular function.

The quest for drugs to turn off secreted virulence factors has become a major theme in medical microbiology due to the growing urgency of the antibiotic

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resistance crisis. "Antisecretion" drugs form a part of a broader theme of "antivirulence" drugs, which aim to disarm rather than kill or cripple our bacterial pathogens. The goal is that by blocking the production of toxins, exoenzymes, and other virulence factors, the pathogen will either return to a commensal state or be more readily cleared by host immune responses (Allen et al. 2014).

Because antivirulence drugs do not directly kill or cripple their bacterial targets, it has been argued that antivirulence drugs confer little or no selection for resistance (Clatworthy et al. 2007; Rasko and Sperandio 2010). The claim of no resistance was first dealt an apparent blow by Maeda et al. (2012), who demonstrated, using a transposon mutant library screen, that several mechanisms of resistance could be found in a novel quorum-sensing interference drug, and that these mutants could be enriched in a specific selective environment. In a recent review, I argue that in light of our experience with antibiotic resistance, mechanisms of resistance will inevitably exist and that the critical question for antivirulence drugs is whether they will increase in frequency under the action of antivirulence drug selection (Allen et al. 2014). Here I will briefly outline the major predictions on the direction of selection as a function of class of virulence factor targeted.

Redundant Virulence Factors and Evolutionarily Robust Drugs

In one scenario, we predict that antivirulence drugs will directly select *against* any resistant mutants, simply because the virulence factors being turned off by the drug are of no benefit to the pathogen's growth or survival within the host (Allen et al. 2014; Figure 7.3a). Under this scenario, any mutant that can restore expression of the virulence factor in the presence of the drug will simply pay the costs of expression without any benefits and be outcompeted. In other words, there is a coincidence of interests between the patient and the pathogen. Both gain by chemically turning off an inappropriate virulence factor.

The broader question becomes: Why would any organism carry a trait that is purely redundant, providing no benefit (direct or indirect) to the individual expressing the trait? Here the answer lies in an understanding of the ecology of bacterial pathogens. The great majority of bacterial pathogens are opportunistically pathogenic in humans, in the sense that their major mode of replication is not in the sites of human disease but instead in some commensal compartment of humans (e.g., *Streptococcus pneumonia*), or some environmental reservoir (e.g., *P. aeruginosa*). What biomedical researchers refer to as "virulence factors" are potentially shaped by distinct functions in these diverse environments (Brown et al. 2012). Due to a lack of broader ecological study of bacterial opportunistic pathogens, we currently lack clear examples of redundant virulence factors, but Allen et al. (2014) propose some candidates.

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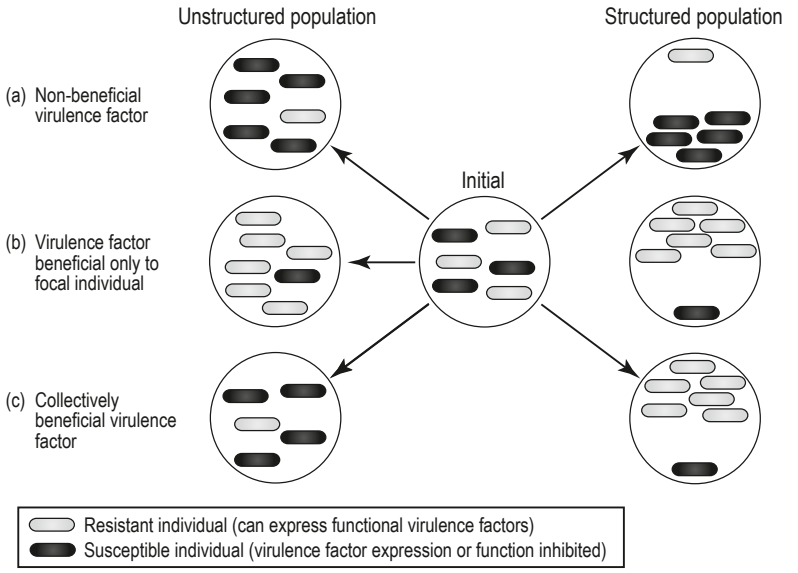

Figure 7.3 Predicted direction of selection on resistance to antivirulence therapeutics. Central circle: an initial population of resistant (gray) and susceptible (black) cells. Left circles: the impact of selection in a well-mixed environment. Right circles: the impact of selection in a structured environment. (a) Virulence factors are redundant at the site of infection; (b) individually beneficial virulence factors; (c) collectively beneficial (cooperative) virulence factors. From Allen et al. (2014), reprinted with permission.

Directly Beneficial Virulence Factors and Evolution-Prone Therapeutics

In the second and opposing scenario, the expression of virulence factors is directly coupled to the growth and survival of a focal cell in the site of infection. In this scenario, much like for antibiotics, any cell that has innovated a mechanism of resistance will experience a direct reproductive benefit (Figure 7.3b). Allen et al. (2014) outline several example traits that fall into this class.

Collectively Beneficial Virulence Factors and Mixing-Contingent Therapeutics

The final scenario addresses the set of virulence factors that generate collective benefits. What is the fate of a rare resistant mutant that is able to restore expression of an individually costly yet collectively beneficial trait? As sketched in Figure 7.3c, the answer depends on the degree of spatial mixing of the wild-type (now a phenotypic cheat) and resistant (cooperator) cells. When the drug-targeted population is well mixed, then any drug-resistant cells will produce, at a cost, the cooperative phenotype, yet the benefits will be shared with the drug-sensitive wild-type population, thus selecting against the resistant mutants (Mellbye and Schuster 2011; Allen et al. 2014). If, however, the resistant and

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sensitive lineages are growing in a sufficiently spatially structured manner, the direction of selection can reverse if mutant cooperator cells are able to benefit preferentially neighboring mutant cooperators (Figure 7.3c; Allen et al. 2014).

A Candidate Evolutionarily Robust Drug with a "Regulatory Trap"

Here I will present evidence for evolutionarily robust control of *P. aeruginosa*, by targeting the collective virulence trait of siderophore production. As discussed above, siderophores are secreted iron-scavenging molecules that bind to and recover insoluble and sequestered iron. Ross-Gillespie et al. (2014) demonstrated that this iron-scavenging ability can be blocked via the addition of low doses of gallium(III) salts. Gallium, like iron, is a transition metal and binds with even greater affinity to extracellular siderophores. We were thus able to use low (sub-cytotoxic) doses of gallium to titrate out the functionality of secreted siderophores and attenuate *P. aeruginosa* infections *in vivo* (Ross-Gillespie et al. 2014). Using a wax moth larvae infection system, we compared survival curves following infection with wild-type *P. aeruginosa,* a siderophore knockout mutant, and wild-type plus chemical (gallium) suppression of siderophore functioning. Strikingly we found that the antivirulence drug is even more effective than the genetic manipulation in reducing mortality rat**e**. In other experiments, we have outlined a likely mechanism for this effect, based on the regulatory response to iron starvation (Ross-Gillespie et al. 2014). The genetic mutant is unable to use siderophores to scavenge for iron and is also relieved of the costs of siderophore production. The gallium-treated bacteria are, however, limited in their ability to scavenge iron. In addition, they pay the costs of continued siderophore production. What is more, under intermediate gallium dosing, bacteria respond to growing iron limitation by increasing their production of siderophores, and thus increasing their costs to a debilitating extent.

The *in vivo* treatment results illustrate that the efficacy of gallium treatment can exceed that of the genetic knockout due to a regulatory trap imposing additional expression costs on the wild type (Ross-Gillespie et al. 2014). How robust is this treatment to evolutionary responses to the drug? The models underlying Figure 7.3 predict that mutants which can restore collective iron scavenging will be selected against if the environment is sufficiently well mixed. We tested this prediction under iron-limited *in vitro* conditions, using a simple serial transfer experimental evolution design, and found support for gallium being evolutionarily robust. Specifically, we found that the degree of growth control imposed by gallium treatment did not significantly decline across the course of 12 days of evolution. In contrast, a range of antibiotic treatments exerting similar degrees of control at day one all displayed significant failure within 12 days, including multidrug treatment (Ross-Gillespie et al. 2014).

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Perspectives

The infection-attenuating impacts of genetic and chemically induced cheats suggest that there is some hope of a positive answer to the question posed in this chapter's title: Does social exploitation within pathogen populations pose an opportunity for novel therapeutic approaches? The relatively brief and *in vitro* experimental evolution conducted by Ross-Gillespie et al. (2014) offers further hope that targeting cooperative bacterial traits can lessen or even reverse selection for resistance to these novel therapeutics (Figure 7.3). While these results are encouraging and definitely merit further investigation, a number of concerns remain to be explored.

In terms of efficacy and combination therapy, the first requirement for any new anti-infective agent is that it works at least as well as current treatments. In the case of improvements on antibiotics, most antivirulence drugs are currently stuck in something of a gray area: they are considerably better than antibiotics at treating antibiotic-resistant infections but fall short of the levels of efficacy of antibiotics which treat sensitive bacteria. In the gallium treatment example discussed above, this lack of impressive treatment is apparent (i.e., no lives of wax-moth larvae were saved), although it should be noted that this was a model of an acute infection following injection of *P. aeruginosa* into the hemolymph, and clearance is challenging in this context.

One potential route to improve the efficacy of antivirulence drugs is to couple them with antibiotics in multidrug treatments, because in some scenarios, synergies have been observed (Hentzer et al. 2003). However, by improving clearance, the use of combination therapies will also potentially increase selection for resistance to one or both of the constituent ingredients (Allen et al. 2014).

In closing, in this chapter I have focused on the potential risk of the evolution of resistance and made the claim that in some scenarios this risk will be small. This does not imply, however, that treatment is therefore safe and risk free. Two other classes of ecological and evolutionary risk must also be considered: the epidemiological risk of increasing prevalence following treatment and the evolutionary risk of increased intrinsic virulence. For further discussion of these additional risks, see Vale et al. (2014) and Allen et al. (2014).

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