The Sociomicrobiology of Antivirulence Drug Resistance: a Proof of Concept

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ABSTRACT **Antivirulence drugs disarm rather than kill pathogens and are thought to alleviate the problem of resistance, although there is no evidence to support this notion. Quorum sensing (QS) often controls cooperative virulence factor production and is therefore an attractive antivirulence target, for which inhibitors (QSI) have been developed. We designed a proof-of-principle experiment to investigate the impact of bacterial social interactions on the evolution of QSI resistance. We cocultured *Pseudomonas aeruginosa* QS-deficient mutants with small proportions of the QS-proficient wild type, which in the absence of QSI mimic QSI-sensitive and -resistant variants, respectively. We employed two different QS-dependent nutrients that are degraded by extracellular (public) and cell-associated (private) enzymes. QS mutants (QSI-sensitive mimics) behaved as social cheaters that delayed population growth and prevented enrichment of wild-type cooperators (QSI-resistant mimics) only when nutrient acquisition was public, suggesting that QSI resistance would not spread. This highlights the potential for antivirulence strategies that target cooperative behaviors and provides a conceptual framework for future studies.**

Infectious diseases are the second-leading cause of death worldwide and cause significant morbidity. A factor contributing to the prevalence of infectious disease has been the development and spread of resistance to current antibiotics (1). Despite this alarming trend, research into the discovery of new antibiotics by large pharmaceutical companies has dwindled (2). Traditionally, antibiotics have been classified by their ability to either kill bacteria (bacteriocidal) or inhibit bacterial growth (bacteriostatic) by targeting functions essential to bacterial viability. While historically effective, this approach imposes selective pressure that results in the evolution of resistant strains (1). An alternative approach is to develop "antivirulence" drugs that disarm pathogens within their host (1, 3). These new compounds would target specific factors essential for successful infection, such as toxin function, toxin delivery, virulence gene regulation, or cell adhesion. The benefits of this approach may be 2-fold: reduction in selective pressure for resistance and preservation of the host microflora.

Cell-cell communication or quorum sensing (QS) is one important target for antivirulence therapy because it controls virulence gene expression in many bacterial pathogens (4). In the opportunistic pathogen *Pseudomonas aeruginosa*, QS is mediated by diffusible acyl-homoserine lactone (acyl-HSL) signals (5). Two interconnected pairs of signal synthases and cognate receptors (LasI-LasR and RhlI-RhlR) control the transcription of more than 300 genes, many of which encode virulence factors, including extracellular enzymes, toxins, and secondary metabolites (5). A number of QS inhibitors (QSIs) have been developed with efficacy against *P. aeruginosa* QS *in vitro* and *in vivo* (6). In particular, receptor-targeting acyl-HSL analogs such as halogenated furanones have been studied in great detail. However, to this date, there have been no experimental data on the evolution of resistance to antivirulence drugs. Presumably, QSI resistance mechanisms would be similar to those conferring resistance to traditional antibiotics, namely, limited access, efflux, enzymatic inactivation, and target modification. A recent review by Defoirdt et al. suggested that QS proficiency and hence QSI resistance would be selected for *in vivo* during infection, whenever QS promotes colonization, systemic spread, or immune evasion (7). However, this opinion does not consider social interactions that take place during QS.

P. aeruginosa QS coordinates the production of many important extracellular factors that are cooperative "public goods" for the population (8). Mutants that do not produce these goods, but benefit from them, are considered social cheaters (8, 9). Under culture conditions that favor QS, such cheaters emerge in the form of receptor-negative, signal-blind *lasR* mutants (10). They invade wild-type populations with negative frequency dependence (11–13). As their proportion increases in a population, their relative fitness decreases as there are fewer cooperators to exploit. In *P. aeruginosa*, signal-blind mutants are favored over signal-negative mutants because common goods production is much more costly than signal production (13).

These social interactions have generally been investigated with low proportions of cheaters, but the situation is expected to be reversed in the emergence of QSI resistance: if strains evolved resistance and retained infectivity, they would likely become QS-proficient cells in a population of QS-deficient social cheaters. Based on a previous model (14), we predict that the exploitation of QSI-resistant clones by the QSI-sensitive majority would greatly slow the development of resistance and prevent the enrichment of a QSI-resistant subpopulation. We tested this prediction in the present study.

EXPERIMENTAL RATIONALE

As a proof of principle, we designed an experiment that simulates the development of a QSI-resistant subpopulation of *P. aeruginosa*. We employed strains that, in the absence of QSI, mimic the phenotypes of QSI-resistant and -sensitive cells. QSI-resistant variants are represented by the *P. aeruginosa* wild type, and QSI-

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sensitive variants are represented by signal-blind *lasR rhlR* double mutants. We reasoned that a desirable, potent QS inhibitor would completely block acyl-HSL signal reception in *P. aeruginosa*. We determined the fitness of these strains, individually and mixed, *in vitro* under growth conditions that require QS. In this respect, selective pressures are akin to those encountered by *P. aeruginosa* in QS-dependent infections. We used a minimal medium that contains either bovine serum albumin (BSA) or adenosine as the sole carbon (C) source. We considered BSA a "publicly acquired" and adenosine a "privately acquired" C source. While both C sources require QS-controlled enzymes, the former is degraded extracellularly by proteases (11), benefitting the entire population, and the latter is metabolized in the periplasm by a nucleoside hydrolase (15), benefitting only the individual producing cell. The importance of extracellular proteases such as elastase in *P. aeruginosa* pathogenesis is well established (16), whereas the significance of adenosine utilization and its regulation by QS is not clear. Regardless, the distinction between public and private goods allows us to make general inferences about the effect of antivirulence drugs that target extracellular (secreted) versus cell-associated virulence factors. We predict that social conflict should have a role only when QSIs target the production of public, extracellular factors.

COCULTURING EXPERIMENTS

To assess whether social conflict could affect the emergence of QSI-resistant variants, we initiated QS mutant cultures ("QSI-sensitive mimics") with various proportions (50, 10, and 1%) of wild-type cells ("QSI-resistant mimics") and measured the duration of growth to stationary phase. As controls, we grew resistant-mimic-only cultures containing the same resistant-mimic inoculum as that used for the cocultures. A decrease in the inoculum size of the resistant mimic itself delayed growth, presumably because cell numbers are reduced, and because acyl-HSL signals and extracellular enzymes present in the inoculum are diluted. Importantly, however, in BSA medium we observed a significant delay in the growth to stationary phase for the cocultures with 50 and 10% resistant-mimic inocula compared with the respective resistant-mimic-only controls (Fig. 1A). Thus, the presence of the QSI-sensitive mimic incurs a significant cost to population growth when nutrient acquisition is public. The resistant-mimic-only control for the 1% coculture failed to reach high density within the duration of the experiment (Fig. 1A). Not surprisingly, the corresponding mixed culture also did not reach saturation.

Growth characteristics were different when adenosine was the sole C source. While the sensitive mimic grew significantly slower than the resistant mimic in single culture, the presence of the sensitive mimic in coculture did not slow population growth (Fig. 1B). In fact, cocultures at 10 and 1% initial resistant-mimic frequency reached saturation somewhat faster (Fig. 1B). This result is likely due to the ability of the QS-deficient sensitive mimic to grow slowly on adenosine and contribute to total population growth in cocultures. As shown earlier, growth on adenosine is not as stringently controlled by QS as is growth on BSA (15).

To investigate the relative fitness of the QSI-resistant mimic in coculture, we quantified the proportion of cells of the resistant mimic at the beginning and the end of growth. In BSA medium, the resistant mimic showed no enrichment at either inoculation ratio (Fig. 1C). In fact, there was a statistically significant decrease in frequency when inocula were 50% and 10%. Consequently, the relative fitness of the resistant mimic is <1 (Fig. 1D; see Materials and Methods for the calculation of relative fitness). On the other hand, there is a significant increase in the frequency of the resistant mimic in adenosine coculture, and the relative fitness is >1 in all cases (Fig. 1C and 1D). We did not calculate the relative fitness for the 1% BSA coculture, which failed to reach saturation and therefore cannot be directly compared with the other cultures.

We were also able to discern trends about the relationship between inoculum proportion and relative fitness (commonly referred to as frequency-dependent relative fitness), although this is typically done with cultures grown for equal amounts of time (11, 13, 17). In our experiment, the duration of competition is different for each initial frequency, because cultures reached stationary phase at different times. The result may be frequency independence when social conflict predominates, as is the case in BSA medium: cultures with lower proportions of cooperating resistant mimics grow more slowly and are exploited by cheating sensitive mimics less efficiently, but cheaters also have more time to exploit cooperators. Indeed, there is no difference in the relative fitness of the resistant mimics with 50% and 10% initial frequencies (Fig. 1D). In adenosine medium, where social conflict is insignificant, relative fitness appears negative frequency dependent, presumably because resistant mimics, when inoculated at low frequency, have more time to grow and outcompete sensitive mimics.

IMPLICATIONS

Our findings provide an idea about how microbial social interactions might affect the evolution of antivirulence drug resistance during infection. With respect to QSI, the results obtained with BSA appear more clinically relevant than those obtained with adenosine, as it is the QS-controlled production of extracellular (public) virulence factors that contributes to infection. These data suggest that social cheating would play an important role in reducing the development of resistance to QSI and—perhaps even more significantly—also suggest that QSI-resistant mutants would be unable to enrich during infection. This would be completely different from the expansion of clones that are resistant to traditional antibiotics. What if QSI resistance was itself due to an extracellular product, presumably an enzyme that inactivates the inhibitor? Conceivably, resistant cells could convert sensitive neighbors into virulence factor-secreting cooperators but would still be at a selective disadvantage due to their production of expensive resistance proteins to the benefit of the entire group.

In terms of tangible implications for experimental research, our results suggest that QSI-resistant mutants are best identified in the absence of social conflict, e.g., in adenosine medium. More generally, a comparison of the effects of "public" and "private" nutrient acquisition on fitness indicates that using small-molecule inhibitors to target production of shared virulence factors, such as extracellular enzymes or toxins, may be more effective than targeting private virulence factors, such as cell-associated adhesins. However, we also note that the targeting of cooperative traits may bear certain epidemiological risks. It has been shown that QSI treatment increases the prevalence of virulent *P. aeruginosa* bacteria during infection as it disfavors the invasion of QS-proficient populations by QS-deficient cheaters (18).

In addition to the impact of social conflict, the role of the infective dose needs to be considered in the emergence of QSI resistance. QSI-resistant cells initially likely comprise a very small fraction of the infecting population, which may be so low that it does not constitute a quorum in the first place and may not be able to express virulence factors when treated with a QSI early. Although

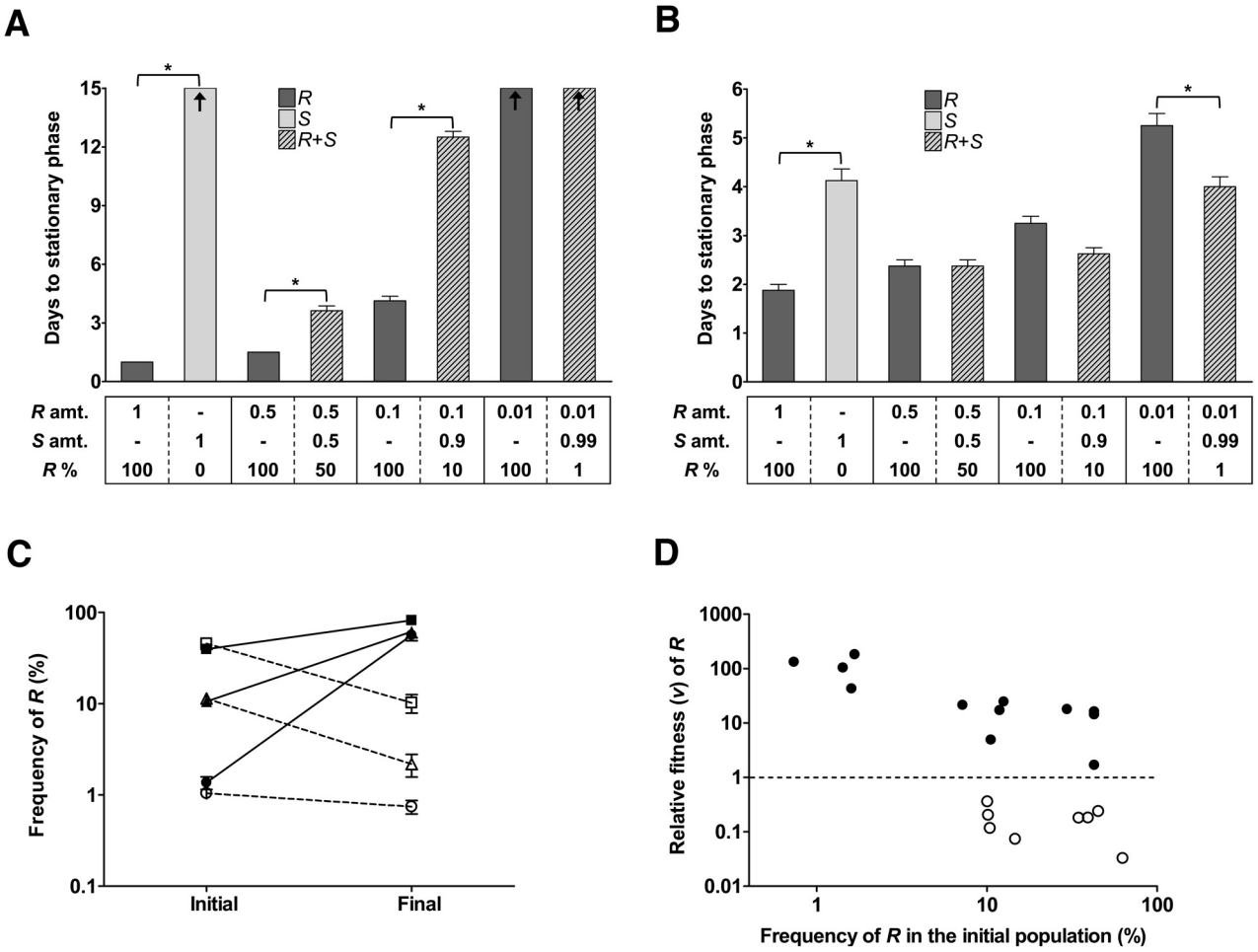

FIG 1 The role of social conflict in the emergence of QSI resistance revealed through coculturing. Assays were performed with a *P. aeruginosa lasR rhlR* mutant (QSI-sensitive mimic, *S*) and its wild-type parent (QSI-resistant mimic, *R*). (A and B) Duration of culture growth to stationary phase in minimal medium with BSA (A) or adenosine (B) as the sole C source. The relative amounts of *R* and *S* inocula (normalized for between-culture comparison) as well as the percentages of *R* in the respective single cultures and cocultures are indicated. Arrows indicate that the culture failed to reach stationary phase within the duration of the experiment (15 days). Error bars indicate standard deviations of the means of four replicates. Statistically significant differences between pairs (*R* and *S* alone; *R* + *S* coculture and the corresponding *R*-only culture) were determined using one-way analysis of variance and Bonferroni's *post hoc* multiple comparison. Brackets with asterisks indicate *P* values of <0.05. (C) Enrichment of the resistant mimic in coculture. Initial and final frequencies of *R* inoculated at approximately 1% (circles), 10% (triangles), and 50% (squares) in BSA and adenosine media (open and filled symbols, respectively) are shown. Error bars indicate standard deviations of the means of four replicates. Statistical significance was determined by a two-tailed unpaired *t* test. All changes in the mean *R* frequency were found to be significant ($P < 0.05$) except for the BSA coculture inoculated at 1%. (D) Relative fitness (v) of the resistant mimic in BSA (open circles) and adenosine (filled circles) cultures. Relative fitness was calculated as the comparison of the initial and final *R* frequencies. Differences in the mean relative fitness between BSA cultures inoculated at approximately 10% and 50% were not significant [$F(1,6) = 0.151$, $P > 0.05$], and differences in the mean relative fitness between all adenosine cultures were significant [$F(2,9) = 11.5$, $P < 0.05$], as determined by one-way analysis of variance.

not the primary focus of our experiment, decreasing inocula of the QSI-resistant mimic emulated this effect to a certain degree.

The fitness effects that we observed during *in vitro* culturing are likely less pronounced with real QSI-sensitive and -resistant strains during an actual infection. A QSI-resistant strain may be less intrinsically fit than the wild-type strain, whereas a QSI-sensitive strain would probably be more fit than a QS deletion mutant because the level of inhibition achievable with QSIs may never approach 100%. We also assumed that next-generation QSIs would effectively inhibit a range of acyl-HSL receptor homologs, including LasR and RhlR. Previous *in vitro* evolution experiments have shown that *lasR* single mutants can regain the ability to produce certain QS-controlled factors, including exoproteases, possibly through compensation by the *rhl* system (10, 19). These re-evolved cooperators appear to be resistant to exploitation by cheaters, similar to the "Phoenix" variant of the fruiting bacterium *Myxococcus xanthus* (20), but it is not known whether they are also virulent. If they retained full virulence, then a broad-spectrum QSI would be needed to avoid the rapid development of resistance. Finally, bacterial population structuring *in vivo* could reduce interactions between QSI-sensitive and -resistant cells compared with a well-mixed batch culture. Such limited dispersal would increase the fitness of QSI-resistant clones (11, 21) but is unlikely to completely eliminate social conflict, as *P. aeruginosa lasR* mutants are still able to exploit wild-type populations during experimental infection of mice (12).

One last unknown in the development of resistance to QSI is the contribution of the immune system during infection. QS has

been shown to protect *P. aeruginosa* from polymorphonuclear leukocytes (22). It is therefore conceivable that immune cells would preferentially target and remove the QS-deficient subpopulation, essentially enriching for QSI-resistant cells. Appropriately designed experiments with animal infection and cell culture models should be able to address these complexities. Our work provides a conceptual basis for such studies.

MATERIALS AND METHODS

Bacterial strains were the QSI-resistant mimic, a *P. aeruginosa* PAO1 wild-type strain chromosomally tagged with a trimethoprim resistance marker (13) from pUC18T-mini-Tn*7*TTp (23), and the QSI-sensitive mimic, a PAO1 Δ*lasR*::Tc^r Δ*rhlR*::Gm^r double mutant (24). As determined previously, the presence of the respective tag or antibiotic resistance cassette did not affect growth (13, 25). Bacteria were routinely cultured on Lennox LB agar plates or in Lennox LB broth buffered with 50 mM MOPS [3-(*N*-morpholino)-propanesulfonic acid, pH 7.0]. For QS growth experiments, bacteria were cultured in M9 minimal medium (10) containing either 1% BSA (Sigma) (11) or 0.1% adenosine (Sigma) (15). Growth experiments were carried out in an incubator/shaker at 37°C in glass culture tubes containing 4 ml of medium.

QS growth experiments were conducted in principle as described previously (10, 11, 13). Experimental cultures were started from overnight (18-h) LB-MOPS cultures that had been inoculated with a freshly grown single colony of the respective *P. aeruginosa* strain. M9-BSA and M9-adenosine cultures were inoculated to optical densities at 600 nm (OD_{600}) of 0.3 and 0.05, respectively, with resistant and/or sensitive mimics at specific frequencies based on OD_{600} (Fig. 1). Cells were not washed prior to inoculation, as washing greatly delayed growth and reduced reproducibility. To control for differences in growth caused solely by varying inoculum sizes, resistant-mimic-only cultures were inoculated with the same resistant-mimic inoculum as that used for the cocultures (Fig. 1). Appropriately diluted aliquots of these cultures were plated to determine the initial CFU/ml. Every 12 h, culture aliquots were removed to determine OD_{600}. Time span was recorded, and aliquots were removed for dilution plating when cultures reached a threshold density indicative of entry into stationary phase ($OD_{600} \geq 2.5$ for M9-BSA and $OD_{600} \geq 0.45$ for M9-adenosine). Given the different inocula, the numbers of generations needed to reach stationary phase are very similar in M9-BSA and M9-adenosine media. Cultures that failed to reach the respective threshold OD_{600} were plated after 15 days. In cocultures, resistant- and sensitive-mimic subpopulations were distinguished by plating on LB agar supplemented with either trimethoprim at a concentration of 100 μg/ml or tetracycline at a concentration of 50 μg/ml, respectively.

The relative fitness (ν) of the resistant mimic in each coculture was determined by comparing its initial and final frequencies during growth, with $\nu = x_1(1 - x_0)/x_0(1 - x_1)$, where x_0 and x_1 are the initial and final resistant-mimic frequencies, respectively (11, 13, 17). The value ν signifies whether the resistant-mimic population increases in frequency ($\nu > 1$), decreases in frequency ($\nu < 1$), or remains at the same frequency ($\nu = 1$) over the duration of the experiment.

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ADDENDUM IN PROOF

Upon acceptance of our paper, T. Maeda et al. (ISME J., 15 September 2011, posting date, doi:10.1038/ismej.2011.122) reported on the identification of *P. aeruginosa* mutants that are moderately resistant to a furanone QSI through overexpression of an efflux pump. These strains were enriched from a mutant pool in QSI-containing adenosine medium, supporting our prediction that antivirulence drug resistance can readily evolve when the trait under selection is non-cooperative.

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