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Quorum Sensing Protects *Pseudomonas aeruginosa* against Cheating by Other Species in a Laboratory Coculture Model

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ABSTRACT

Many species of bacteria use a cell-cell communication system called quorum sensing (QS) to coordinate group activities. QS systems frequently regulate the production of exoproducts. Some of these products, such as proteases, are "public goods" that are shared among the population and vulnerable to cheating by nonproducing members of the population. Because the QS system of the opportunistic pathogen *Pseudomonas aeruginosa* regulates several public goods, it can serve as a model for studying cooperation. Bacteria also commonly regulate antimicrobial production through QS. In this study, we focused on the hypothesis that QS-regulated antimicrobials may be important for *P. aeruginosa* to protect against cheating by another bacterial species, *Burkholderia multivorans*. We assessed laboratory cocultures of *P. aeruginosa* and *B. multivorans* and investigated the importance of three *P. aeruginosa* QS-regulated antimicrobials, hydrogen cyanide, rhamnolipids, and phenazines, for competition. We found that *P. aeruginosa* dominates cocultures with *B. multivorans* and that the three antimicrobials together promote *P. aeruginosa* competitiveness, with hydrogen cyanide contributing the greatest effect. We show that these QS-regulated antimicrobials are also critical for *P. aeruginosa* to prevent *B. multivorans* from cheating under nutrient conditions where both species require a *P. aeruginosa* quorum-regulated protease for growth. Together our results highlight the importance of antimicrobials in protecting cooperating populations from exploitation by other species that can act as cheaters.

IMPORTANCE

Cooperative behaviors are threatened by social cheating, wherein individuals do not produce but nonetheless benefit from shared public goods. Bacteria have been shown to use several genetic mechanisms to restrain the emergence of cheaters from within the population, but public goods might also be used by other bacterial species in the vicinity. We demonstrate that a public good produced by *Pseudomonas aeruginosa* can be used by another species, *Burkholderia multivorans*, to obtain carbon and energy. We also show that *P. aeruginosa* antimicrobials that are coregulated with the public good prevent invasion by the cheating species. Our results demonstrate that cross-species cheating can occur and that coregulation of public goods with antimicrobials may stabilize cooperative behavior in mixed microbial communities.

Many species of *Proteobacteria* use acyl-homoserine lactone (AHL) quorum sensing (QS) to regulate genes in a cell density-dependent manner (1, 2). In the opportunistic human pathogen *Pseudomonas aeruginosa* there are two complete AHL QS circuits, LasI-LasR and RhlI-RhlR. The LasI-LasR system is activated by *N*-3-oxo-dodecanoyl homoserine lactone ($3OC_{12}$-HSL) (3), and the RhlI-RhlR system is activated by butanoyl homoserine lactone (C_4-HSL) (4). LasR is required for RhlR activity. LasR and RhlR together regulate the production of dozens of genes in *P. aeruginosa* (5, 6), and many of the genes regulated by LasR and RhlR are involved in the production of exoproducts (6). Because they are extracellular, the exoproducts may constitute "public goods" that benefit all of the members of the population, regardless of which individuals are producing them (7–9). Shared public goods are susceptible to social cheating, or defection, by individuals that utilize the public goods while avoiding the cost of their production. A QS-controlled public good in *P. aeruginosa* is an extracellular protease, elastase (8). Under circumstances where elastase production is required to obtain carbon and energy, LasR mutant social cheaters can arise in the population (7, 8).

Many bacteria also use QS to regulate the production of antimicrobials. Examples include production of bactobolins by *Burkholderia thailandensis* and of violacein by *Chromobacterium violaceum*, among others (10, 11). In the case of *P. aeruginosa*, many antimicrobials are regulated by RhlR; these include peroxides, phenazines, rhamnolipids, and cyanide (6, 12, 13). The benefits of using QS to control antimicrobials are unknown, but it is thought that this regulation may be important for competition with other species. Several studies with laboratory cocultures have shown that QS controls antimicrobials that are important for competition (14–19).

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We are interested in understanding why QS commonly controls antimicrobials and how QS-regulated antimicrobials might be important for stabilizing public goods cooperation during interspecies competition. Specifically, our hypothesis is that QS-controlled antimicrobials may offer protection against cheating by other species. For this study, we focused on *P. aeruginosa* because of the increasing body of work describing the role of QS, and QS-controlled elastase, in cooperation (7, 8). We chose as a competitor the opportunistic pathogen, *Burkholderia multivorans*. Although *B. multivorans* and *P. aeruginosa* are saprophytic opportunistic pathogens that can coexist in soil and in lung infections of patients with the genetic disease cystic fibrosis, we focused on *B. multivorans* because the growth rate and yield of *B. multivorans* are similar to those of *P. aeruginosa* under our conditions. We also chose this strain of *B. multivorans* because it does not produce a protease similar to elastase, which would confound our interpretation of data. We show that in laboratory cocultures, *P. aeruginosa* QS-controlled antimicrobials can protect against public-goods cheating by *B. multivorans*. This may be one explanation for why cooperative public goods and antimicrobial production are coregulated by quorum sensing in *P. aeruginosa*.

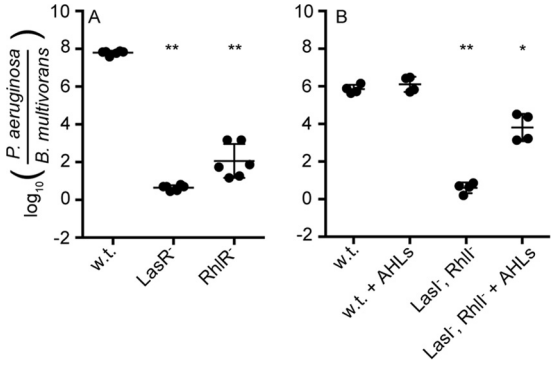

FIG 1 *B. multivorans* competition with wild-type (w.t.) *P. aeruginosa* and quorum-sensing mutants. Coculture experiments were carried out in Luria-Bertani (LB) broth. The relative fitness of each strain is shown as the ratio of *P. aeruginosa* to *B. multivorans* at 24 h, determined by selective plating and colony counts. (A) *B. multivorans* in competition with wild-type, LasR$^-$, and RhlR$^-$ *P. aeruginosa*. (B) Complementation of the quorum-null phenotype. The *P. aeruginosa* AHLs C_4-HSL and $3OC_{12}$-HSL (5 μM each) were added to culture tubes prior to inoculation. The solid lines represent means for each group. Each symbol represents the outcome of an individual experiment; there were at least four independent experiments for each condition. Statistical analysis by *t* test compared to wild type: *, $P \leq 0.002$; **, $P < 1 \times 10^{-7}$.

MATERIALS AND METHODS

Bacterial strains and media. Bacteria were grown in minimal medium with 1% (wt/vol) sodium caseinate as a carbon source (casein broth) (8) or in Luria-Bertani (LB) broth buffered to pH 7 with 50 mM morpholinepropanesulfonic acid (MOPS) (20). Exogenous elastase was added when required as previously described (7). When appropriate, the following antibiotics were used (per ml): gentamicin at 30 μg or 100 μg (for selection of *P. aeruginosa* transconjugants made from pUC18 miniTn*7* or pEXG2 derivatives, respectively) or 15 μg (for *Escherichia coli*), and for selection from coculture growth, trimethoprim at 100 μg (to select for *P. aeruginosa*) and 10 μg gentamicin with 17.3 μg polymyxin B (to select for *B. multivorans*).

The bacterial strains and plasmids used are listed in Table S1 in the supplemental material. We used wild-type and mutant derivatives of *P. aeruginosa* strain PAO1-UW (referred to as PAO1) (21) and a wild-type *B. multivorans* strain (strain AMT 0468-1) (J. Burns, unpublished data). PAO1-derived strains with transposon insertions in *phzA1*, *hcnC*, and *rhlB* were described previously (22), and double and triple mutants of these were constructed by sequentially transferring the genomic DNA containing the mutant allele into the strain of interest using previously described methods (23). In each case, Cre recombination was used to remove the antibiotic resistance marker (24) prior to introducing mutation-containing genomic DNA fragments. PAO1-derived strains with unmarked, in-frame deletions of *lasR*, *hcnC*, and *rhlR* were described previously (25), and we constructed unmarked, in-frame deletions of *rhlB* using similar methods. Briefly, PCR-amplified DNA fragments flanking *rhlB* were cloned into pEXG2, which was used to transform *Escherichia coli* S17-1, and then the pEXG2 derivatives were crossed into PAO1 by mating. Transconjugants were selected on *Pseudomonas* isolation agar containing gentamicin, and deletion mutants were selected with no-salt LB agar containing 10% (wt/vol) sucrose. The *hcnC rhlB* double mutant was constructed by introducing the pEXG2 plasmid containing the *hcnC* deletion fragments into PAO1 Δ*rhlB*. To complement the *hcnC* mutation, we introduced an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible *hcnC* into the neutral *att* site using the pUC18miniTn*7*-LAC plasmid as described previously (26). Mutant construction was confirmed in all cases by PCR.

Coculture experiments. Cocultures with *Pseudomonas aeruginosa* and *Burkholderia multivorans* were performed at 37°C. To inoculate cocultures in LB broth, pure cultures were grown to mid-logarithmic phase, subcultured to fresh LB broth to an optical density at 600 nm (OD_{600}) of 0.1, and grown to an OD_{600} of 1 before combining. The inoculum of each species in the coculture was 1×10^7 to 5×10^7 CFU/ml. Cocultures in LB broth were grown for 24 h before plating to enumerate each species. To inoculate cocultures in casein broth, late-logarithmic-phase cultures of *B. multivorans* (OD_{600} of 1.0) were washed twice with phosphate-buffered saline (PBS) and suspended in casein broth prior to combining at the indicated ratio with approximately 5×10^7 CFU/ml *P. aeruginosa*. The *P. aeruginosa* inoculum was from a logarithmic-phase culture grown in casein broth (wild type and the triple-antimicrobial mutant) or in LB broth and washed with PBS (LasR mutant). Cocultures were grown for 24 h and then diluted 1:100 into fresh casein broth for three consecutive days. All cocultures were grown in 3 ml of medium in 18-mm glass tubes and incubated with shaking at 250 rpm. The ratio or CFU of each species was determined on LB agar plates with antibiotic selection as described above.

RESULTS

***P. aeruginosa* relies on quorum-regulated hydrogen cyanide, rhamnolipids, and pyocyanin to compete with *B. multivorans*.** *P. aeruginosa* produces several QS-controlled antimicrobials that are important for coculture competition with other bacterial species, for example, *Agrobacterium tumefaciens* and *Staphylococcus aureus* (14, 16, 27). We sought to examine the role of *P. aeruginosa* QS during competition with *B. multivorans*. We assessed the competitiveness of the *P. aeruginosa* wild type or QS receptor mutants (LasR$^-$ or RhlR$^-$) in coculture competition with wild-type *B. multivorans*. We also assessed a *P. aeruginosa* AHL synthase mutant (LasI$^-$ RhlI$^-$) in competition. We found that wild-type *P. aeruginosa* outcompeted *B. multivorans*, and this advantage was lost in either the LasR$^-$ or RhlR$^-$ single mutant (Fig. 1). The AHL synthase-deficient LasI$^-$ RhlI$^-$ double mutant had a competitive defect similar to that of either of the single receptor mutants, and this defect could be rescued by supplying the *P. aeruginosa* AHLs $3OC_{12}$-HSL and C_4-HSL to the growth medium (Fig. 1A). Because RhlR is activated by LasR, these results suggest the possibility that LasR acts through RhlR to regulate factors that are important for *P. aeruginosa* to compete with *B. multivorans*.

Three known RhlR-controlled antimicrobials in *P. aeruginosa*

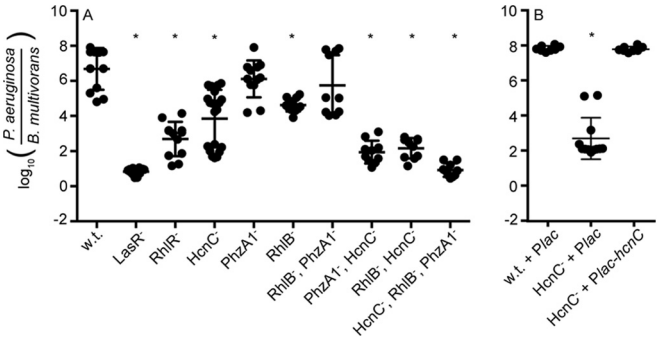

FIG 2 *B. multivorans* competition with wild-type *P. aeruginosa* and quorum-sensing and antimicrobial mutants. Coculture experiments were carried out in Luria-Bertani (LB) broth. (A) *P. aeruginosa* strains are wild type (PAO1) or mutants deficient for production of LasR (*lasR*), RhlR (*rhlR*), hydrogen cyanide (*hcnC*), pyocyanin (*phzA1*), and/or rhamnolipid (*rhlB*). The starting ratio of *P. aeruginosa* to *B. multivorans* was 1:1. The final (24-h) ratio of *P. aeruginosa* to *B. multivorans* was determined by selective plating and colony counts. (B) Hydrogen cyanide complementation restores the wild-type phenotype. IPTG (1 mM final concentration) was added prior to inoculation to induce hydrogen cyanide production from the P_{lac} promoter. The solid lines represent means for each group. Each symbol represents the outcome of an individual experiment; there were at least six independent experiments for each condition. Statistical analysis by *t* test compared to wild type: *, $P \leq 1 \times 10^{-5}$.

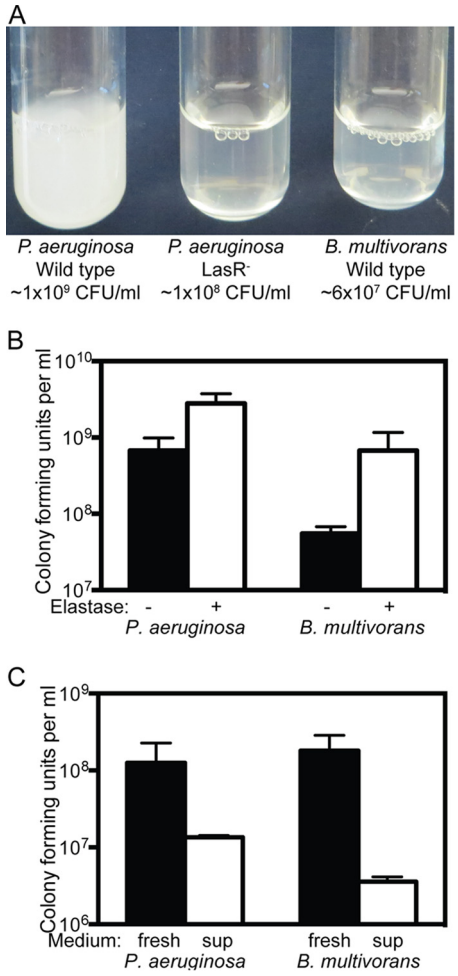

FIG 3 Bacterial growth with casein or Casamino Acids. (A) *P. aeruginosa* and *B. multivorans* cultures grown in casein medium. The turbidity and growth yields at 24 h are shown. (B) Final cell densities of *P. aeruginosa* and *B. multivorans* after 24 h of growth in casein medium (black bars) or casein medium with exogenously added elastase protease (0.095 U/ml) (white bars). (C) Final cell density of *B. multivorans* or *P. aeruginosa* after 24 h of growth in 0.01% Casamino Acids (black bars) or in filtered fluid from casein-grown stationary-phase cultures of the other species (white bars) (see the text). In each case, the final cell density was determined by dilution plating.

have been shown to promote competitiveness with other species: the phenazine pyocyanin, hydrogen cyanide, and rhamnolipids (14). We investigated the individual and combined roles of each of these in competition with *B. multivorans*. Individually, pyocyanin, hydrogen cyanide, and rhamnolipid production had a minimal contribution to competitiveness. However, in combination there was a significant effect. A mutant defective for all three of the antimicrobials was five orders of magnitude less competitive than the wild type, similar to the case for both the $LasR^-$ and $RhlR^-$ mutants (Fig. 2A). The results of competitions with *P. aeruginosa* double-antimicrobial mutants indicated that hydrogen cyanide had the greatest contribution to the competitive ability of *P. aeruginosa*. We were able to restore competitiveness of hydrogen cyanide mutants by expressing the hydrogen cyanide synthase gene *hcnC* from a neutral site in the chromosome (Fig. 2B). These results indicate that the RhlR QS regulator is important for *P. aeruginosa* to compete with *B. multivorans* due to production of RhlR-controlled hydrogen cyanide and, to a lesser extent, pyocyanin and rhamnolipids.

***B. multivorans* can cheat on the *P. aeruginosa* protease elastase.** Because LasR is required for the production of the protease elastase, and elastase is needed to proteolyze casein to liberate nutrients for growth, LasR mutants show poor or no growth on casein as the sole carbon and energy source (Fig. 3A). We found that our strain of *B. multivorans* also did not proteolyze casein (Fig. 3A) and demonstrated poor growth in casein medium compared to identically grown cultures with the elastase supplied exogenously (Fig. 3B). Thus, *B. multivorans* does not produce proteases that promote growth on casein but can benefit from exogenous elastase.

Our results are consistent with the idea that *B. multivorans* can utilize nutrients liberated by *P. aeruginosa* elastase. However, in cooperating systems, true cheaters must compete with the cooperators for the same resources. To test whether *B. multivorans* and *P. aeruginosa* are competing for carbon and energy in casein medium, we assessed growth of each species in a defined medium with limiting Casamino Acids (the breakdown product of casein). We grew each species in Casamino Acids, filter sterilized the culture fluid, and used the spent supernatant as a growth medium for the other species. When grown on Casamino Acids, each species increased from a starting density of 5×10^6 to about 10^8 cells per ml; however, when grown on spent filtrates, neither species increased in cell density (Fig. 3C). These results indicate that pregrowth in Casamino Acids depletes the medium of the nutrients required for growth of the other species. This is consistent with the idea that in casein medium, *B. multivorans* and *P. aeruginosa* compete for the nutrients liberated by elastase. Together, our results support the conclusion that *B. multivorans* is able to cheat on the *P. aeruginosa* public good elastase.

Quorum-controlled antimicrobials protect *P. aeruginosa* cooperators from *B. multivorans* invasion. Our results showed that *P. aeruginosa* QS-controlled antimicrobials are important for

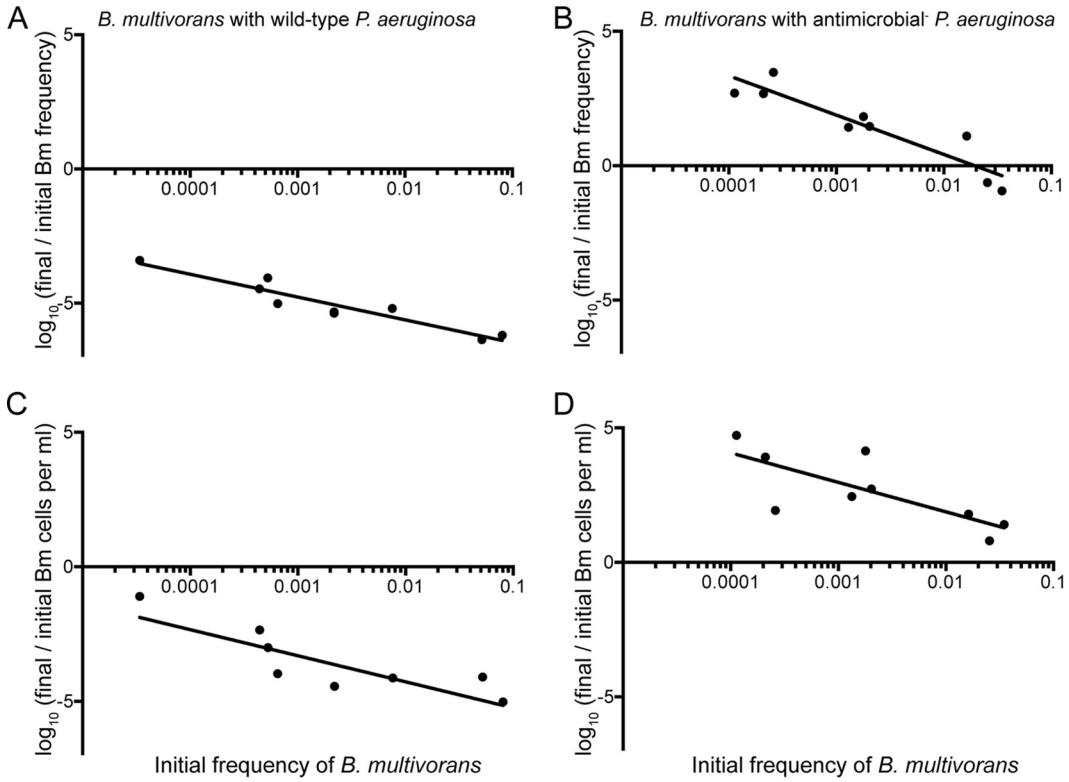

FIG 4 Competitions in casein medium. Wild-type *B. multivorans* was inoculated at various frequencies with either wild-type *P. aeruginosa* (A and C) or the antimicrobial-defective (*hcnC phzA1 rhlB*) mutant (B and D). Competitions were carried out in nutrient-limited casein medium as described in Materials and Methods. In panels A and B, the data are plotted as the frequency of *B. multivorans* relative to *P. aeruginosa* ($\log_{10}$ final/initial frequency). In panels C and D, the same data are plotted as the fitness of *B. multivorans* alone ($\log_{10}$ final/initial CFU). Some of these data are also shown in Table 1. Lines indicate a nonlinear regression fit using an ordinary fit model.

competitiveness with *B. multivorans* under nutrient-rich conditions (Fig. 2A). We also showed that *P. aeruginosa* QS-controlled elastase is a public good that can be cheatable by *B. multivorans* (Fig. 3). We hypothesized that during coculture growth, *B. multivorans* can avail itself of resources liberated by *P. aeruginosa* elastase and that the *P. aeruginosa* QS-controlled antimicrobials can protect against *B. multivorans* cheating. We tested this hypothesis by growing *B. multivorans* with either wild-type or triple-antimicrobial mutant (*hcnC rhlB phzA1*) *P. aeruginosa* in casein medium and varying the *B. multivorans* starting frequency with respect to *P. aeruginosa*. We found that regardless of the starting frequency, *B. multivorans* was essentially eliminated from the culture with wild-type *P. aeruginosa* after 48 h of passage (Fig. 4A and C and Table 1). However, the *P. aeruginosa* triple-antimicrobial mutant was unable to prevent *B. multivorans* from increasing in the population; with the triple-antimicrobial mutant, *B. multivorans* came to comprise 10% of the final population, regardless of the starting frequency (Fig. 4B and Table 1). Not surprisingly, because the *P. aeruginosa* LasR$^-$ mutant does not produce proteases needed for maximal growth in the casein medium, *B. multivorans* grew poorly with the LasR$^-$ mutant compared with the protease-producing triple-antimicrobial *P. aeruginosa* strain (Table 1).

The results demonstrate a negative frequency-dependent selection of *B. multivorans* when grown in coculture with the *P. aeruginosa* triple-antimicrobial mutant in casein medium (Fig. 4B and D). This finding is consistent with *B. multivorans* cheating on the *P. aeruginosa*-produced public good elastase (28) because the benefit of cheating decreases as the frequency and number of cooperators decline. That is, as cheaters increase in frequency, they "cheat" less effectively because there are fewer cooperators to provide the public good. There are other potential explanations for negative frequency dependence that would be consistent with the observed results, including niche construction (29). If the observed selection were not a consequence of a cooperator-cheater dynamic, we would expect negative frequency dependence under other conditions where *B. multivorans* and the antimicrobial-deficient *P. aeruginosa* are grown together. However, we observed that in LB, *B. multivorans* had no fitness benefit at any starting frequency (see Fig. S1 in the supplemental material). This further supports the idea that the invasion of *P. aeruginosa* by *B. multiv-*

TABLE 1 Final yields of *P. aeruginosa* and *B. multivorans* in casein coculture

P. aeruginosa strain cocultured with wild-type *B. multivorans*	Final growth yield (CFU/ml)[a]	
	P. aeruginosa	*B. multivorans*
Wild type	$9 (\pm 7) \times 10^9$	$<1 \times 10^2$
LasR$^-$ mutant	$1 (\pm 1) \times 10^8$	$5 (\pm 0.3) \times 10^7$
Antimicrobial mutant[b]	$5 (\pm 4) \times 10^9$	$4 (\pm 3) \times 10^8$

[a] The values are the means from at least three independent experiments, with ranges indicated in parentheses. The starting ratio of *B. multivorans* to *P. aeruginosa* was 1:1,000 (see Materials and Methods).
[b] The *P. aeruginosa* antimicrobial mutant has disruptions in three antimicrobial genes (*hcnC*, *phzA1*, and *rhlB*).

orans in casein medium reflects *B. multivorans* cheating on the public good elastase.

DISCUSSION

Social cheating poses a threat to cooperating populations because cheaters have the potential to overrun the population and cause the cooperative behavior to be lost (30, 31). We previously described mechanisms by which quorum-sensing mutants are restrained in populations (25, 30). However, in many environmental situations, cooperating populations of bacteria may coexist with other species that could also avail themselves of the products of public goods. This is a form of cheating by other species. Here we used a laboratory coculture model to provide evidence that supports the idea that coregulation of antimicrobials with public goods can prevent a competitor from cheating.

In this light, *B. multivorans* may be viewed as a type of cheater that can utilize the nutrients liberated by *P. aeruginosa* proteases without incurring any costs. By definition, a cheater must engage in rivalrous competition for public goods with other members of the population. Our evidence indicates that *P. aeruginosa* and *B. multivorans* may compete for the same nutrients (Fig. 3), demonstrating that *B. multivorans* can act as a cheater in cooperating populations of *P. aeruginosa*. Previous studies have shown that LasR mutants can also act as cheaters (7, 8). LasR mutants are more classical cheaters in that they arise from the wild type and therefore have metabolism and fitness similar to those of the wild type. However, the *B. multivorans* cheater is fundamentally different from the LasR mutant cheaters, and *B. multivorans* and *P. aeruginosa* are not equal competitors. For example, *P. aeruginosa* may inhibit growth of *B. multivorans* by using intoxicants, and some of these may be QS independent. This may explain why, in our experiment, we observed that *B. multivorans* invaded the population and reached 10% of the total population (Fig. 4), whereas LasR mutants reach 30% or more of the total population and can, under certain circumstances, cause the population to collapse (8, 25, 30).

Many antimicrobials have more than one function. Hydrogen cyanide, for example, is classically thought of as a poison that can promote virulence in a host or competitiveness with other species (32, 33). The latter is supported by results in the present study indicating that hydrogen cyanide, with several other antimicrobials, promotes the ability of *P. aeruginosa* to compete with *B. multivorans* (Fig. 2A). However, hydrogen cyanide is also important for controlling intraspecies cheating in *P. aeruginosa*. Hydrogen cyanide was recently shown to be important for policing cooperating *P. aeruginosa* populations against runaway cheating (25). This is because hydrogen cyanide produced by cooperators inhibits its growth of LasR mutant cheaters and prevents them from invading to a high frequency (25). Hydrogen cyanide production by natural populations of *P. aeruginosa* may have different roles dependent on environmental conditions.

Social cheating has generally been described as defection from cooperation by individuals within a population. Examples of social cheaters include QS mutants in the case of *P. aeruginosa* or tax avoiders in the case of humans (34). However, bacteria commonly live in environments where many species may be present. These other species represent a unique threat to cooperating populations because they are not necessarily amenable to species-specific restraints on cheating. Our laboratory coculture experiments describe how, by coproducing antimicrobials with cheatable public goods, cooperating populations are able to protect themselves against such interlopers. In this case, cheating is not defection from cooperation *per se* but the use of a public resource provided by another species to the detriment of the providing species (through resource consumption). It is difficult to speculate whether this type of cheating may occur in the context of a cooperating bacterial community; however, recent work on interspecies cooperation suggests that it might be possible (35–37). Our data also add to the growing body of evidence suggesting that the large size of the quorum-sensing regulon of *P. aeruginosa* reflects multiple environmental pressures on quorum sensing, including competition with other species.

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