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Bacterial Quorum Sensing and Metabolic Incentives to Cooperate

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Abstract

The opportunistic pathogen *Pseudomonas aeruginosa* uses a cell-cell communication system termed “quorum sensing” to control production of public goods, extracellular products that can be used by any community member. Not all individuals respond to quorum-sensing signals and synthesize public goods. Such social cheaters enjoy the benefits of the products secreted by cooperators. There are some *P. aeruginosa* cellular enzymes controlled by quorum sensing, and we show that quorum sensing–controlled expression of such private goods can put a metabolic constraint on social cheating and prevent a tragedy of the commons. Metabolic constraint of social cheating provides an explanation for private-goods regulation by a cooperative system and has general implications for population biology, infection control, and stabilization of quorum-sensing circuits in synthetic biology.

Acyl-homoserine lactone (AHL) quorum sensing is common among *Proteobacteria*. This is a form of cell-cell communication that allows bacteria to monitor their population density and activate specific sets of genes when populations have reached a threshold density (1–3). Quorum sensing controls the production of a variety of public goods, functions shared by all individuals in the group. Experiments with *P. aeruginosa* support the view that quorum sensing controls cooperativity between *P. aeruginosa* cells (4–6). AHL signaling in *P. aeruginosa* is complex and involves multiple signals and receptors (7). A key system involves LasI, which generates the diffusible AHL signal *N*-3-oxododecanoylhomoserine lactone (C12-HSL) and LasR, a C12-HSL-responsive transcription activator (8). Together C12-HSL and LasR directly activate dozens of genes (9), with a heavy overrepresentation of genes coding for extracellular factors or production of extracellular factors, although there are some LasR-activated genes coding for cellular enzymes. Cellular enzymes are not shared among individuals so we call them private goods (10). By contrast, LasR-activated extracellular proteases are known as public goods (5). Growth of *P. aeruginosa* with casein or bovine serum albumin as the sole source of carbon and energy requires these proteases and encourages the emergence of LasR-mutant social cheaters (4, 5), which do not incur the cost of expressing quorum-controlled functions but benefit from the public goods produced by other members of the group. These cheaters have the potential to cause a “tragedy of the

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commons" wherein the population will be overtaken by defectors and ultimately be unable to avail itself of the protein growth substrate (4, 11, 12). Quorum sensing also controls expression of a few private goods. Thus, we asked whether quorum control of such private goods might control social cheating in groups of cooperators.

Microarray analyses (9) suggest that quorum-sensing control of private goods is relatively rare. We used BIOLOG phenotype arrays to confirm this. We compared growth of a *P. aeruginosa* AHL synthesis mutant plus or minus added AHLs on 190 soluble carbon and energy sources and 380 soluble nitrogen sources. Growth was improved in the presence of signal with Glu-Tyr, His-Trp, Tyr-Gly-Gly, Tyr-Phe, Tyr-Trp, and Tyr-Tyr as nitrogen sources, and inosine and D-trehalose as carbon sources (tables S1 and S2). Quorum sensing–dependent growth on inosine was predicted from previous studies showing expression of the cytoplasmic enzyme nucleoside hydrolase gene (*nuh*) (9) and growth on adenosine was LasR-dependent (13, 14).

Because there is a literature on quorum sensing control of adenosine metabolism we focused our subsequent experiments on whether the obligate pairing of protease secretion (a public good) and adenosine metabolism (a private good) might restrict the ability of social cheaters to invade the population. As described previously (5), long-term growth achieved by daily culture transfer (about 100 generations at about 6 generations per day) with casein as the sole carbon source results in the emergence of a substantial population (about 40%) of putative LasR mutants (Fig. 1A); these individuals do not exhibit ex-tracellular protease activity and cannot grow on adenosine agar. We sequenced *lasR* in 13 protease-negative, adenosine growth–negative isolates from three independent experiments, and consistent with previous analyses (4, 5), we found LasR-null mutations in every case (C221T, G541A, and C683T, all of which have a LasR-null phenotype).

To test our hypothesis that LasR regulation of a private good constrains the emergence of social cheaters, we grew *P. aeruginosa* with varying concentrations of adenosine plus casein such that, together, the total available carbon source was 1% (w/v). When adenosine was more than 50% of the total, protease-negative, adenosine growth–negative mutants never exceeded 0.2% of the total population (Fig. 1A). Growth of wild-type *P. aeruginosa* on adenosine alone was slow (doubling time of 10 to 12 hours compared with 4 hours on casein) (fig. S1). At the start of an experiment, growth on 50% adenosine, where cheaters eventually emerge, is similar to that on 75% adenosine where cheaters are suppressed (Fig. 1B). Although growth rates vary by condition and day (fig. S1), by day 25, cultures grown with either 50 or 75% adenosine reached a higher density than at the beginning of the experiment (Fig. 1B). We determined the doubling times for cultures grown on 0.25% casein and 0.75% adenosine to be 4 hours at day 1 and 3 hours at day 25. Note that isolates from a day-25 culture grow well with adenosine as the sole carbon and energy source (fig. S1), and although they show normal protease production on skim milk agar plates, these isolates do not grow well in liquid medium with casein as the sole carbon and energy source. The cost of good growth on adenosine appears to be a loss of ability to grow on casein alone. We do not know the genetic basis or evolutionary significance of this finding.

Our hypothesis is that control of adenosine catabolism by quorum sensing serves as a means to suppress cheating. An alternate hypothesis consistent with the results shown in Fig. 1A is that metabolism of any private good can suppress cheating. To test this alternate hypothesis we replaced adenosine with D-glucose, which is metabolized by cellular enzymes of the hexose monophosphate pathway in a manner independent of quorum sensing. Even when glucose constituted 75% of the available carbon, cheaters emerged and reached a substantial proportion of the population (Fig. 1C), which shows that linkage of public and private goods through quorum sensing is required for cheater control.

One might argue that because casein levels were reduced as adenosine levels were increased in the experiments above, cheater suppression results from resource limitation. We addressed this concern in two ways. First, we showed that cheaters emerge in a medium with low levels of casein (0.25%) in the absence of adenosine (Fig. 1C), whereas they do not with 0.25% casein and 0.75% adenosine (Fig. 1A). Second, we asked whether the emergence of LasR mutants would be decreased when the concentration of casein was a constant 0.5% and the concentration of adenosine was the sole variable. We used three concentrations of adenosine: 0.5, 0.75, and 1.5%. As in the experiment described in Fig. 1A, cheater emergence was suppressed if adenosine constituted more than half the available carbon (Fig. 1D).

To ascertain whether suppression of social cheating in media with adenosine is a general characteristic of *P. aeruginosa*, we investigated a quorum sensing–competent isolate from a cystic fibrosis (CF) lung infection. As with the laboratory strain PA01, cheaters emerge after 15 to 20 days of growth on casein, and emergence is suppressed in media with sufficient adenosine (Fig. 1E). Furthermore, we tested *lasI, rhlI* mutants deficient in the quorum-sensing signal from six environmental and clinical *P. aeruginosa* isolates (15) and showed that, in all but one case, growth of the mutant on adenosine agar plates was greatly impaired compared with the parent (Fig. 1F). Thus quorum-sensing control of adenosine metabolism is common in *P. aeruginosa* isolates, and adenosine suppression of LasR social cheats is not restricted to PA01. We note that quorum-sensing control of adenosine catabolism is not universal, as the isolate BE177 did not require quorum sensing for growth on adenosine, but adenosine hydrolase is just one of the private goods controlled by quorum sensing in *P. aeruginosa*.

In the previous experiments, social cheaters emerged in populations forced to cooperate by restricting the carbon and energy source to casein. We next asked how rapidly cheaters can be purged from *P. aeruginosa* populations by switching from growth on casein alone to growth on casein plus adenosine. We transferred mixed populations of cooperators and cheaters from day 25 on casein alone to 0.1% casein and 0.9% adenosine. As judged by growth on adenosine agar plates and protease production on skim milk agar plates, there is a strong selection against social cheaters when adenosine is present (Fig. 2A). We also found that when mixed populations of cheaters and cooperators were transferred to Luria-Bertani (LB) broth, a rich growth medium with multiple soluble carbon and energy sources, there was no selection for or against cheaters (Fig. 2B). Thus, neither cheaters nor cooperators have a strong advantage under conditions where public goods are not needed and cooperation is not beneficial.

It seems likely that in many environments where *P. aeruginosa* thrives, multiple carbon and energy sources will be available to this metabolically versatile bacterium. Can adenosine-dependent cheater restraint occur when a solute metabolized by a quorum sensing–independent private good is available? To address this question, we performed an experiment with 0.1% casein, 0.9% adenosine, and 1% glucose, which in itself does not suppress the emergence of cheaters in casein medium (Fig. 1C). The inoculum was a mixed population of cooperators and cheaters that had developed after 25 days on casein alone. We found that the selection against cheaters was at least as strong as that with casein and adenosine only (Fig. 2C).

It is of interest that, in our experiments with casein and those published by others (5), a stable equilibrium is reached with coexistence of cooperators and cheaters. In these experiments, the cost of cooperation is relatively low (higher than in LB broth but not so high that an equilibrium cannot be reached), and the population of cheaters is limited to roughly 40% of the total. We sought to increase the cost of cooperation by making casein

not only the sole source of carbon but also the sole source of nitrogen (by eliminating NH_4Cl as a nitrogen source). We reasoned that by increasing the demand for protease, the cost of cooperation would increase, and our experiments bear this out. Without NH_4Cl, cheaters emerge, and by day 18 to 25, they constitute about 80% of the population. By several measures, public goods, including elastase and phenazines, produced by the cooperators from day 18 to 25 cultures grown in NH_4Cl-free medium are about 10-fold that of the parent (Fig. 3A). One obvious manifestation of the hyperactive quorum-sensing system in cooperators is that, after 18 to 20 days, cultures are visibly blue as a result of copious pyocyanin production (Fig. 3B). Production of this pigment is quorum-sensing regulated (16). We presume the increased cost of cooperation imposed by the nutritional conditions of this experiment leads to a greater relative benefit for cheaters. This results in a rapid emergence of a large cheater population followed by a population collapse, a tragedy of the commons (Fig. 3C).

Can private-goods regulation by quorum sensing protect the population from a tragedy of the commons when the cost of cooperation is relatively high? To address this, we included adenosine with casein in the absence of NH_4Cl. Within the 30-day duration of our experiment there was no population collapse. Cheaters emerged, but they never exceeded 5% (Fig. 3C).

A central tenet of cooperation is that for propagation of a cooperative trait, the group must be as or more successful than the individual, and it must also be (relatively) free of cheaters. Here, we provide an explanation for why bacteria might maintain one or several functions for metabolism by private goods under quorum-sensing control. This explanation is wholly consistent with the view that quorum sensing serves as a communication system for coordination of cooperative behaviors. We believe that co-regulation of functions required for utilization of public and private goods can provide an incentive against cheating. Quorum-sensing control of relatively poorly utilized substrates metabolized by private goods limits the cost of this strategy. LasR regulates private goods other than adenosine hydrolase (9), which should limit the benefit from liberating any single one of these goods from quorum regulation [as in the case of strain BE177 (Fig. 1F)]. It will be of interest to determine whether this type of cheater control is a broadly distributed biological phenomenon or perhaps isolated to *P. aeruginosa* and a few other bacterial species.

Our results provide one plausible explanation for why quorum-sensing mutant social cheaters are not abundant in free-living populations of *P. aeruginosa*. Of course, other mechanisms by which evolution of social cheaters might be restricted exist. Dispersal of individuals to new habitats where cooperative behavior is important would select against social cheats. Such a single-cell bottleneck has just been described in the social amoeba *Dictyostelium discoideum* (17); this organism also makes use of pleiotropic regulation by a differentiation factor to prevent cheating (18). We believe that an understanding of the selective pressures for maintenance of quorum sensing–controlled cooperation might lead to an ability to manage bacterial populations in a natural setting. We do not understand what forces might have selected quorum control of private goods together with public goods. Constraint on cheating is a consequence of this co-regulation but the co-regulation did not necessarily evolve for this purpose. Finally, social cheating will hamper the use of synthetic quorum-sensing circuits for production of secreted enzymes in biotechnological applications. Our results suggest one way in which synthetic systems could be engineered to suppress such phenomena.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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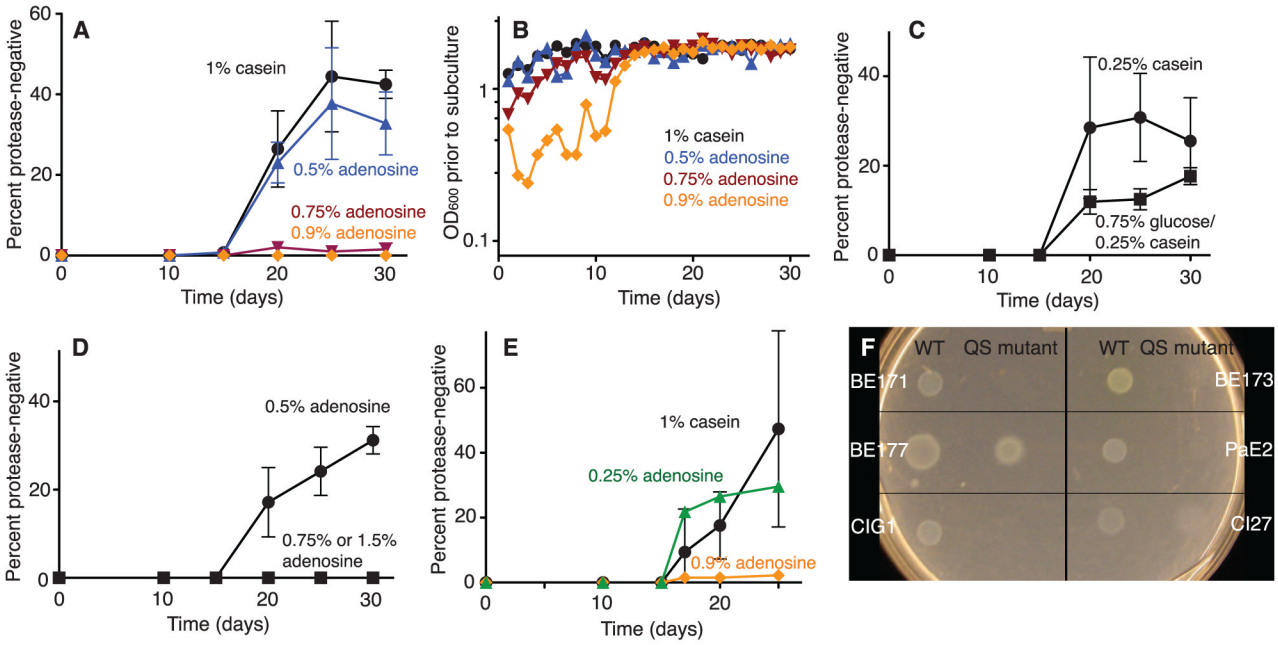

Fig 1.

Adenosine restrains emergence of social cheaters in casein medium. (**A**) *P. aeruginosa* PAO1 in minimal medium containing casein alone or casein and adenosine at increasing levels of adenosine (total of casein and adenosine is 1% w/v). Protease-negative cheater abundance was determined at 5-day intervals and was essentially identical to the cheater abundance determined by absence of growth on adenosine agar. (**B**) Growth yields at 24-hour intervals measured as optical density at 600 nm (OD_{600}). The cell densities (OD_{600}) after 30 days were 1.84 in casein, 1.93 in 0.75% casein with 0.25% adenosine, 1.99 in 0.5% casein with 0.5% adenosine, 1.81 in 0.25% casein with 0.75% adenosine, and 1.89 in 0.1% casein with 0.9% adenosine. The total number of generations in all the experimental groups was nearly equal after 30 days. As discussed in the text, variants with improved growth on adenosine emerged and were isolated from day 25 cultures. (**C**) The reduction in LasR mutants is not a consequence of resource denial or substitution. Cheaters emerge when strain PAO1 is grown on 0.25% casein with or without 0.75% glucose in place of adenosine. (**D**) Cheater emergence is restrained when casein is at a fixed concentration and adenosine concentration exceeds 0.5%. (**E**) The clinical isolate CI27 was grown in minimal medium containing casein or casein and adenosine [as in (A) for strain PAO1]. (**F**) Quorum sensing is required for growth of most, but not all, *P. aeruginosa* isolates tested. We grew six different *P. aeruginosa* strains (left) and their respective *lasI*, *rhlI* mutants (right) on adenosine agar as described in the methods. In all cases, the wild-type strains grew well. With the exception of BE177, the mutants did not show appreciable growth on adenosine. Except for panels (B) and (F), which are representative experiments, panels show means ± SEM for three independent experiments.

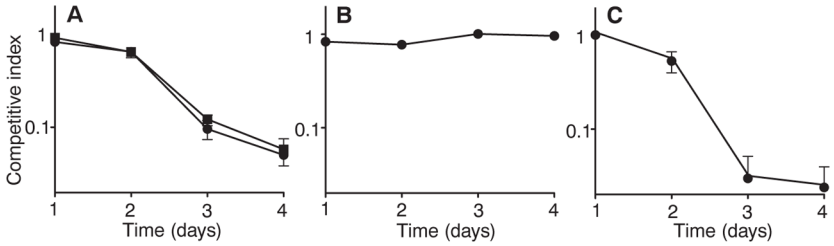

Fig 2.

Adenosine reduces the relative fitness of LasR mutants. (**A**) Cheaters are rapidly eliminated from adenosine-enriched medium. A mixture of cheaters and wild-type bacteria derived from either strain PAO1 (squares) or CI27 (circles) growing on casein alone was transferred to 0.9% adenosine and 0.1% casein and subcultured daily. The abundance of protease-negative bacteria was assessed on skim milk agar. (**B**) There is no selection against cheaters in LB broth. A mixture of cheaters and wild-type bacteria derived from strain PAO1 was grown in LB broth buffered with MOPS, pH 6.8, with daily subculture, and protease-negative bacteria were counted daily. (**C**) The addition of glucose, which is metabolized by a quorum sensing–independent pathway, does not prevent adenosine suppression of social cheaters. A wild-type and cheater mixture derived from strain PAO1 was placed into medium containing 1% glucose, 0.9% adenosine, and 0.1% casein, with daily subculture, and protease-negative bacteria were enumerated daily. In all panels, data are displayed as a competitive index or the end point cheater:wild type ratio compared with the initial ratio on a logarithmic scale. Data are shown as means ± SEM and reflect the combined results of three independent experiments.

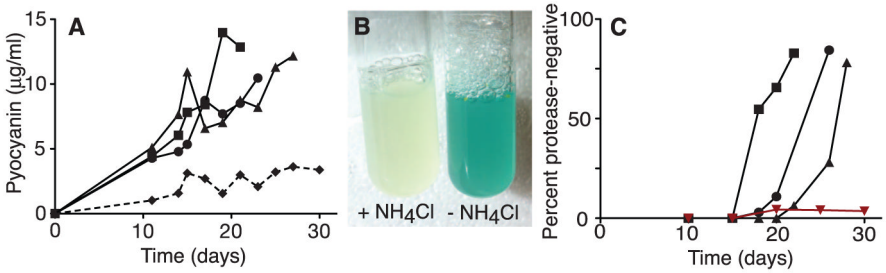

Fig 3.

Quorum-sensing regulation of a private good can prevent a tragedy of the commons. (**A**) Pyocyanin in cultures of *P. aeruginosa* PAO1 grown with casein as the sole nitrogen, carbon, and energy source (solid lines, three independent experiments). By day 10, there is three to four times as much pyocyanin at the time of transfer as pyocyanin in a culture grown with NH4Cl as a nitrogen source (dotted line). Pyocyanin was measured as described elsewhere (19). (**B**) Hyperproduction of the blue phenazine pigment pyocyanin is evident in NH_4Cl-free medium by day 20. (**C**) Overexpression of quorum sensing–controlled factors correlates with the emergence of LasR mutants, which reach about 80% of the total population, after which cultures do not grow upon transfer. The tragedy of the commons does not occur in a medium containing adenosine (0.75%) and casein (0.25%) (inverted triangles) although LasR mutants are detectable (mean of three experiments; SEM at all points is <3%).