Nitrogen Source Stabilization of Quorum Sensing in the *Pseudomonas aeruginosa* Bioaugmentation Strain SD-1

Mei-Zhen Wang,^{a,b} Bai-Min Lai,^a Ajai A. Dandekar,^c Yu-Sheng Yang,^a Na Li,^{a,b} Jun Yin,^{a,b} Dong-Sheng Shen^{a,b}

School of Environmental Science and Engineering, Zhejiang Gongshang University, Hangzhou, People's Republic of China^a; Zhejiang Provincial Key Laboratory of Solid Waste Treatment and Recycling, Hangzhou, People's Republic of China^b; Department of Medicine, University of Washington School of Medicine, Seattle, Washington, USA^c

ABSTRACT *Pseudomonas aeruginosa* SD-1 is efficient at degrading aromatic compounds and can therefore contribute to the bioremediation of wastewater. *P. aeruginosa* uses quorum sensing (QS) to regulate the production of numerous secreted “public goods.” In wastewater bioaugmentation applications, there are myriad nitrogen sources, and we queried whether various nitrogen sources impact the stabilities of both QS and the bacterial populations. In a laboratory strain of *P. aeruginosa*, PAO1, the absence of a nitrogen source has been shown to destabilize these populations through the emergence of QS mutant “cheaters.” We tested the ability of SD-1 to grow in casein broth, a condition that requires QS for growth, when the nitrogen source with either NH₄Cl, NaNO₃, or NaNO₂ or with no added nitrogen source. There was great variability in susceptibility to invasion by QS mutant cheaters and, by extension, the stability of the SD-1 population. When grown with NH₄Cl as an extra nitrogen source, no population collapse was observed; by contrast, two-thirds of cultures grown in the presence of NaNO₂ collapsed. In the populations that collapsed, the frequency of social cheaters exceeded 40%. NaNO₃ and NaNO₂ directly favor QS mutants of *P. aeruginosa* SD-1. Although the mechanism by which these nitrogen sources act is not clear, these data indicate that the metabolism of nitrogen can affect the stability of bacterial populations, an important observation for continuing industrial applications with this species.

IMPORTANCE Bioaugmentation as a method to help remediate wastewater pollutant streams holds significant potential to enhance traditional methods of treatment. Addition of microbes that can catabolize organic pollutants can be an effective method to remove several toxic compounds. Such bioaugmented strains of bacteria have been shown to be susceptible to competition from the microbiota that are present in wastewater streams, limiting their potential effectiveness. Here, we show that bioaugmentation strains of bacteria might also be susceptible to invasion by social cheaters and that the nitrogen sources available in the wastewater might influence the ability of cheaters to overtake the bioaugmentation strains. Our results imply that control over the nitrogen sources in a wastewater stream or selective addition of certain nitrogen sources could help stabilize bioaugmentation strains of bacteria.

KEYWORDS quorum sensing, social cheating, bioaugmentation, long-term stabilization, inorganic nitrogen

Bioaugmentation, the use of selected microbial strains in activated sludge to strengthen their abilities for pollutant removal (1), has significant advantages in the degradation of toxic and hazardous compounds. These advantages include cost and

Received 15 April 2017 Accepted 15 May 2017

Accepted manuscript posted online 9 June 2017

Citation Wang M.-Z., Lai B.-M., Dandekar AA, Yang Y.-S., Li N, Yin J, Shen D.-S. 2017. Nitrogen source stabilization of quorum sensing in the *Pseudomonas aeruginosa* bioaugmentation strain SD-1. Appl Environ Microbiol 83:e00870-17. <https://doi.org/10.1128/AEM.00870-17>.

Editor Shuang-Jiang Liu, Chinese Academy of Sciences

Copyright © 2017 American Society for Microbiology. All Rights Reserved.

Address correspondence to Dong-Sheng Shen, shends@zju.edu.cn.

efficiency (2). However, bioaugmented systems present several challenges, including a propensity of these systems to rapidly lose efficiency (3). A primary cause of this kind of deterioration is the lack of stability of inoculated bacterial populations.

Because of this potential instability, there has been substantial effort in determining the factors that are involved in the loss of the bioaugmented strain in cultures (4). Most of the prior work has focused on the competition between inoculated bacteria and indigenous bacterial communities. This competition can be very important to long-term outcomes (5). In several studies, the abundance of inoculated bacteria progressively decreased (6), consistent with the idea that the “native” bacteria are able to outcompete the bioaugmentation strains. For this reason, some have suggested using or enriching indigenous bacteria in lieu of inoculated strains as an approach to reduce competition (7, 8).

However, besides interspecies competition, there are reasons that might account for the long-term stabilization (or lack thereof) of a bioaugmented system; in particular, populations might be destabilized from within. In previous studies, we found that bacterial quorum sensing (QS) improved the biofilm formation of inoculated bacteria and accelerated their colonization in the system (2, 9, 10). *Pseudomonas aeruginosa* has two complete acyl-homoserine lactone (AHL) QS systems, LasIR and RhlIR. The signal synthase LasI produces the signal *N*-3-oxododecanoyl-homoserine lactone (3OC₁₂-HSL), and RhlI produces *N*-butanoyl-homoserine lactone (C₄-HSL). These signals bind to the LasR and RhlR transcription factors, respectively, which regulate the transcription of hundreds of genes (11).

Pseudomonas aeruginosa QS controls the secretion of a wide variety of secreted products (11). These secreted products include proteases and phenazines, which can be considered “public goods,” i.e., products shared by all individuals in the group (12). *P. aeruginosa* has been used as a model system to understand public goods-mediated cooperation and cheating. To grow on medium containing casein as the sole source of carbon and energy, *P. aeruginosa* must secrete the protease elastase, a public good that is regulated by both LasR and RhlR. Growth under this nutrient condition encourages the emergence of LasR[−] mutant social cheaters, which do not incur the cost of expressing QS-regulated functions but benefit from the public goods produced by other individuals (13, 14). These cheaters have the potential to cause a “tragedy of the commons,” wherein the population will be overtaken by cheaters and ultimately be unable to propagate (15). We speculated that intraspecies invasion by social cheaters could affect the long-term stabilization of a bioaugmented system.

Several environmental factors have been shown to affect quorum sensing-regulated traits (16). For example, pH directly affects the stability of QS signals in bioaugmented systems (17), which can affect biofilm formation (2). Alkaline pH has also been shown to favor LasR[−] mutants under some growth conditions (18). Likewise, if casein is the sole source of nitrogen and carbon, bacteria overexpress QS-regulated traits (13) and are more susceptible to invasion by cheaters. This apparent ability of the nitrogen source to modulate QS led us to query whether different sources of nitrogen, including NH₄Cl, NaNO₃, and NaNO₂, which are common inorganic nitrogen sources in wastewater, might have an effect on bacterial population stability (19).

In this study, we used *P. aeruginosa* strain SD-1, which is a strain that could potentially be used in a bioaugmentation system (see Table S1 in the supplemental material). We investigated the impacts of various potential nitrogen sources on the population stability of SD-1 in QS-dependent casein medium. We show that SD-1 is differentially susceptible to social cheating on the basis of the nitrogen source available, and this is directly attributable to the effect of certain nitrogen sources on either the wild type or the QS mutant. We report here intraspecies stabilization of a bioaugmentation strain.

RESULTS

Long-term stability of strain SD-1 depends on the nitrogen source. In the long-term operation of a bioaugmented system, the abundance of the bioaugmented

strain might decline, which means there is a decrease in the ability to degrade the pollutants (20). In our previous study, by improving the fitness of the strain in the wastewater system, the bioaugmented strain in the wastewater system became more stable, thereby enhancing the degradation of pollutants (2). The long-term stability of bioaugmented bacterial populations is important to wastewater treatments that employ microorganisms to carry out pollutant removal. We tested the stability of strain SD-1 under conditions that require quorum sensing to obtain carbon and energy. We grew strain SD-1 in minimal medium with casein, which was the sole source of carbon and nitrogen, as has previously been described for *P. aeruginosa* strain PAO1 (13). We monitored for the emergence of quorum-sensing mutant “cheaters” (LasR[−] variants) over time. Under the condition where casein provided the sole source of nitrogen and carbon, we observed that the population was generally stable. Although LasR[−] mutant cheaters could be detected by their lack of protease production, they did not increase in frequency to a point where the population could no longer be propagated (Fig. 1A). However, in one case, the frequency of LasR[−] mutants increased such that the number of cooperators was inadequate to sustain growth on casein. At this frequency of cheaters, the population cannot be propagated, representing a tragedy of the commons. We verified by DNA sequencing that the protease-negative cheaters we observed were in fact LasR[−] mutants. The frequency of cheaters observed in these experiments was generally lower than in reports with PAO1 (13, 14), which likely reflects the difference in the strain of *P. aeruginosa* used in these experiments.

Because wastewater provides a number of potential nitrogen sources, we added either NH₄Cl, NaNO₃, or NaNO₂ to the casein medium. Again, we detected LasR[−] mutant cheaters under all conditions (Fig. 1D, F, and H), but there was a dramatic difference between the three nitrogen sources. In cultures augmented with NH₄Cl, we observed that all could be transferred stably; the frequency of LasR[−] mutants remained less than 45% (Fig. 1D). This was similar to what occurred under the condition where casein was the sole nitrogen source (Fig. 1A and B).

Cultures of *P. aeruginosa* SD-1 grown in the presence of NaNO₃ or NaNO₂ as potential nitrogen sources showed substantially different outcomes. Where NaNO₃ and NaNO₂ were present, the frequency of cheaters was generally higher, and as a consequence, one-third and two-thirds of the cultures, respectively, eventually could not be propagated on account of a high frequency of cheaters (Fig. 1E to H; see Table 1).

As a complementary experiment, we cocultured the wild type and the LasR mutant social cheaters in various initial proportions. We found that cheaters grew to higher yields and relative frequencies when NaNO₃ or NaNO₂ was the added nitrogen source (Fig. 2). These results support the phenomenon that we observed in the evolution experiment.

We next asked what would be the result of a combination of all of the exogenous nitrogen sources, using a similar approach of inoculating cooperating populations with various initial cheater frequencies. Surprisingly, the NaNO₂/NaNO₃ effect of destabilizing the population was dominant; extra NH₄Cl did not significantly inhibit the growth of cheaters when these nitrogen sources were present. Different nitrogen sources appear to alter the propensity of cooperating populations to a tragedy of the commons.

Nitrogen utilization by *P. aeruginosa* SD-1. In the above-described experiments, we observed that different nitrogen sources in the QS-dependent medium result in different probabilities of population collapse. One simple explanation for this finding would be inhibition of the wild-type strain in casein medium. To address this possibility, we compared the levels of growth of strain SD-1 under different extra-nitrogen treatments in casein medium. In casein medium, SD-1 exhibits diauxic growth, because it initially grows on a small amount of nutrients carried over from the prior culture and then requires QS activation and subsequent breakdown of casein by elastase (Fig. 3). As expected, there were no significant differences among the four treatments (Fig. 3),

FIG 1 Different nitrogen sources alter the propensity of cooperating populations to a “tragedy of the commons” in casein medium. (A) *Pseudomonas aeruginosa* SD-1 in casein medium without extra nitrogen. In only one case was there a tragedy of the commons. (B) Emergence of social cheaters in casein medium without extra nitrogen. In the case where the population collapsed, the ratio of social cheaters is higher than 60%. Comparisons of growth yields and cheater burden in casein medium with NH_4Cl (C), NaNO_3 (E), or NaNO_2 (G) added. (D, F, and H) Social cheaters under the nitrogen-added conditions. (D) Populations were stable when NH_4Cl was added, but under either the NaNO_3 -added (F) or NaNO_2 -added (H) condition, a failure to propagate the culture was common.

FIG 2 Social cheaters grew to higher relative frequencies in those groups in which NaNO₃ or NaNO₂ was added as a nitrogen source. Cultures were inoculated with the wild type and cheaters at initial ratios of 10,000:1 (A), 1,000:1 (B), 100:1 (C), and 10:1 (D). The final cheater ratios as percentages are shown.

indicating that the additional nitrogen sources do not impair bacterial growth under these conditions.

Although we added one of three (NH₄Cl, NaNO₃, or NaNO₂) additional nitrogen sources to the medium in our passaging experiments, the most abundant potential source of nitrogen is the casein itself. We next asked whether the added nitrogen sources affected the overall supply of nitrogen (as NH₄⁺, NO₃⁻, or NO₂⁻) in our cultures. We measured the abundance of the three ions over 24 h of growth in casein medium. Presumably

FIG 3 Addition of nitrogen does not impair the growth of the *Pseudomonas aeruginosa* SD-1 wild type in casein medium. We grew SD-1 in the presence of 8 mM NH₄Cl (black), NaNO₃ (red), or NaNO₂ (blue) in casein medium. All growth curves were essentially identical to that in which no added nitrogen (pink) was present. The data points are the means with SDs from three independent replicates.

FIG 4 The added nitrogen sources affected the overall supply of nitrogen (as NH_4Cl , NaNO_3 , or NaNO_2) in casein medium. (A) Concentrations of NH_4^+ . $[\text{NH}_4^+]$ increased quickly under all conditions and then plateaued at 500 mg/liter under NaNO_3^- and NaNO_2^- -treated conditions and 600 mg/liter in the NH_4Cl -added group. (B) Concentration of NO_3^- . $[\text{NO}_3^-]$ decreased quickly and stabilized at 200 mg/liter in all groups. (C) Concentration of NO_2^- . $[\text{NO}_2^-]$ was present in meaningful concentrations only in the NaNO_2^- -added group and was exhausted within 16 h. The additional nitrogen sources are 8 mM NH_4Cl (black), NaNO_3 (red), and NaNO_2 (blue). Casein as the sole source of carbon and nitrogen (pink) is the control. The data points are the means with SDs from three independent replicates.

because of the ammonium ions liberated from casein, $[\text{NH}_4^+]$ increased quickly under all conditions and then plateaued at 500 mg/liter under NaNO_3^- and NaNO_2^- -treated conditions and at 600 mg/liter in the group with NH_4Cl added (Fig. 4A). Conversely, $[\text{NO}_3^-]$ decreased quickly and stabilized at 200 mg/liter in all groups (Fig. 4B). In contrast to the similar utilization of NH_4^+ and NO_3^- by all groups, NO_2^- was present in meaningful concentrations only in the group in which NaNO_2 was added to the culture. In this case, the measured $[\text{NO}_2^-]$ was exhausted within 16 h (Fig. 4C). Therefore, the ability of SD-1 to use NH_4^+ , NO_3^- and NO_2^- as potential nitrogen sources was disparate.

Because casein provides the bulk of the nitrogen in all cultures in the above-described experiments as NH_4^+ , regardless of the added nitrogen source, we next asked how well each of these supported growth as the sole nitrogen source. To do so, we grew cultures of SD-1 using glucose as the sole source of carbon and energy and added either NH_4Cl , NaNO_3 , or NaNO_2 as the nitrogen source. The difference in growth using the three nitrogen sources was dramatic (Fig. 5). *P. aeruginosa* SD-1 cultures with

FIG 5 Differential ability of *Pseudomonas aeruginosa* SD-1 to use NH_4Cl , NaNO_3 , and NaNO_2 as potential nitrogen sources. Both NH_4Cl (black) and NaNO_3 (red) could be used efficiently as a nitrogen source by SD-1. However, both the growth rate and the cell yield in the NaNO_3 group were less than those in the NH_4Cl group. NaNO_2 (blue) could not be efficiently used as a nitrogen source by SD-1, and its growth was similar to that of the nitrogen-free control (pink). These experiments were performed in glucose medium with the different nitrogen sources added.

NH_4Cl grew briskly to a final optical density at 600 nm (OD_{600}) of 2.2. NaNO_3 could also be used efficiently as a nitrogen source, although both the growth rate and yield were lower than in the presence of NH_4Cl . On the other hand, NaNO_2 could not be efficiently used as a nitrogen source by SD-1, and its growth was similar to that of the nitrogen-free control (Fig. 5), although it was exhausted in casein cultures (Fig. 4C). Altogether, these results suggested that the differences in nitrogen metabolism could affect QS and therefore population stability under our conditions.

Metabolic burden on wild-type strains due to nitrogen metabolism. To investigate the influence of the three different inorganic nitrogen sources on *P. aeruginosa* QS, we measured the concentrations of secreted protease and pyocyanin in cultures. These soluble products are public goods, shared products that benefit the population as a whole, and are regulated by the AHL QS transcription factors LasR and RhIR.

After overnight culture in casein medium, we measured protease and pyocyanin in cooperating populations of SD-1 after 1 day of growth, when no LasR^- mutant cheaters could be detected. NaNO_2 addition to the casein cultures resulted in significantly greater protease production (3.7 ± 0.5 mg/liter) than either of the other potential nitrogen sources. The protease production by the other three groups was roughly one-third of that observed under the NaNO_2 -treated condition (1.1 ± 0.2 mg/liter to 1.5 ± 0.2 mg/liter) (Fig. 6A).

We observed a different pattern in the production of pyocyanin, as the group with NaNO_3 added produced 2.98 ± 0.10 mg/liter, significantly greater than the other groups (2.22 ± 0.14 mg/liter to 2.40 ± 0.09 mg/liter) (Fig. 6B). These results indicated that the added nitrogen-containing compounds could have differential effects on the production of soluble QS-regulated products, although there is not a uniform effect. The overproduction of one or the other of these secreted products was associated with a failure to propagate the culture (Fig. 1).

An additional possibility is that the nitrogen compounds themselves favor LasR^- mutants, resulting in a fitness difference that might account for the tragedy of the commons that we previously observed with the addition of NaNO_3 or NaNO_2 to the casein cultures. Because LasR^- mutants do not grow in casein medium and Casamino Acids provide an alternate nitrogen source, we compared the growth of LasR^- mutants with that of the parent SD-1 strain in glucose medium with different nitrogen sources. Both the wild type and the LasR^- mutants grew equally well in glucose medium supplemented with NH_4Cl (Fig. 7A), suggesting that neither is able to use this nitrogen source more efficiently. This equal growth is consistent with the equilibrium seen previously in casein medium (Fig. 1C and D). Under NaNO_3 -treated conditions, cheaters grew better than the wild type in the first 12 h of growth, whereas the wild type caught up thereafter (Fig. 7B). The NaNO_2 -treated group demonstrated the most dramatic difference between the wild type and the LasR^- mutant. In these cultures, LasR^-

FIG 6 Secreted product overproduction occurs in populations fated for a tragedy of the commons. (A) We measured 3OC₁₂-HSL (black) and secreted protease (gray) under the various conditions. In casein medium with NaNO₂ added, protease secretion (gray) was double that of the control (Non), although signal levels (black) across all groups were similar. (B) We measured C₄-HSL (black) and pyocyanin (gray) under the various conditions. In medium with NaNO₃ added, levels were higher than those in the no extra nitrogen (Non) casein medium.

mutant cheaters could use NO₂[−] as a nitrogen source and were able to grow, while the wild type demonstrated essentially no growth (Fig. 7C). Neither wild-type SD-1 nor the LasR[−] mutant grew in the control medium that contained 0.5% glucose without a source of nitrogen (Fig. 7D). Altogether, these data suggested that QS mutants gain an additional advantage in addition to social cheating in medium containing either NaNO₃ or NaNO₂.

DISCUSSION

Biological denitrification is an indispensable part of wastewater treatment, and various forms of inorganic nitrogen salts are commonly present in wastewater treatment systems. In this study, we asked if the presence of various inorganic nitrogen salts could affect the population stability of a *Pseudomonas aeruginosa* bioaugmentation strain, SD-1. This strain uses quorum sensing to regulate the production of a battery of extracellular products. Bacterial quorum sensing plays an important role in biological wastewater treatment, especially in bioaugmented systems (21). In bioaugmented systems, QS accelerates colonization by highly efficient degrading bacteria and regulates biofilm formation (2).

We tested the ability of SD-1 to grow using casein as a source of carbon and energy, a QS-dependent growth condition. Although in wastewater treatment, SD-1 will tend to form biofilms, our approach using planktonic conditions allowed us to test whether QS stability is compromised by the mixture of nitrogen sources in a nutrient stream.

In casein medium, SD-1 produces the public good elastase. Public goods cooperation, as in this case, encourages the emergence of cheaters that do not produce the public good. When SD-1 is grown in casein medium, these cheaters take the form of LasR[−] mutants (Fig. 1B and Table 1), and these LasR[−] mutant cheaters rise in frequency until they come to an equilibrium with the wild type. This equilibrium is mediated by

FIG 7 Metabolic burden on wild-type strains (solid lines) due to nitrogen metabolism and comparatively higher fitness of LasR⁻ mutants (dashed lines). (A) The wild type and the LasR⁻ mutants grew equally well in glucose medium supplemented with NH₄Cl. (B) LasR⁻ mutants have a growth advantage over the wild type in the first 12 h of growth in medium supplemented with NaNO₃. (C) Only LasR⁻ mutants, not the wild type, could use NO₂⁻ as a nitrogen source. (D) In the absence of a nitrogen source, neither wild-type SD-1 nor the LasR⁻ mutants can grow.

hydrogen cyanide (15) but under some circumstances can be destabilized and result in a tragedy of the commons (13, 15). This potential loss of a quorum-sensing population has significant implications for both the stability and the effectiveness of bioaugmented strains (10).

In this study, we compared the effects of three different nitrogen sources on the long-term population stabilization of strain SD-1 in casein medium. In control cultures or cultures supplemented with NH₄Cl, only one time did the population destabilize (indicated by the failure of the culture to grow after passage). However, in NaNO₃- or NaNO₂-augmented cultures, between one-third and two-thirds of cultures experienced a tragedy of the commons (Table 1). In the populations that collapsed, the ratio of cheaters was high and the production of the secreted proteases was significantly decreased compared with those of the stable cultures (see Fig. S1 in the supplemental material). These results support the observations from previous studies (13, 22) that, unlike RhlR-mediated QS (23), LasR-mediated QS is subject to social cheating in times of metabolic stress and therefore is not “metabolically prudent.”

Our results pointed to a differential effect of the various nitrogen sources on populations of SD-1. Under all casein growth conditions, regardless of whether an exogenous nitrogen source was added, NH₄⁺ was present in quantities in excess of that needed by strain SD-1. NO₂⁻ ions were only present when added to the culture and

TABLE 1 Tragedy of the commons probability with different nitrogen treatments

Extra nitrogen source	No. of expts	No. of tragedies of the commons observed	Probability
None	9	1	1/9
NH ₄ Cl	9	0	0
NaNO ₃	9	3	1/3
NaNO ₂	9	6	2/3

were poorly utilized by SD-1, while NO_3^- was used as a nitrogen source but less efficiently than NH_4^+ (Fig. 4 and 5). Intriguingly, there was differential production of certain secreted products between the three groups. The NaNO_2 -treated group produced substantially more protease, while the NaNO_3 -treated group produced much more pyocyanin than the other groups (Fig. 6).

One intriguing finding in the NaNO_2 -treated and NaNO_3 -treated groups is a growth distinction between the wild type and the LasR^- mutants. Under glucose growth conditions, in which either NO_3^- or NO_2^- is the sole nitrogen source, a LasR^- mutant has a metabolic advantage over the wild type. Under NaNO_3 -treated conditions, cheaters grew better than the wild type in the first 12 h of growth, while the wild type caught up thereafter (Fig. 7B). LasR^- mutant cheaters could use NO_2^- as a nitrogen source and were able to grow, while the wild type demonstrated essentially no growth (Fig. 7C). This suggests that the metabolic advantage could be conferred by a mutation of LasR . Although it seems unlikely that the mechanism is related to their potential role as a nitrogen source, both NO_3^- and NO_2^- might be utilized by strain SD-1 as electron acceptors (24).

In this context, the ability of *P. aeruginosa* to use NO_3^- and NO_2^- as part of electron transport is relevant (24). Either NO_3^- or NO_2^- can be used as an electron acceptor and in some conditions, particularly anaerobic ones, as a key metabolizing energy source (25), and quorum sensing has been reported to inhibit denitrification. Toyofuku et al. (26) reported that *P. aeruginosa* quorum-sensing regulators LasR and RhIR could inhibit denitrification. Similarly, *Nitrobacter winogradskyi* significantly increased the expression of *nirK* and *nirK* cluster genes (genes involved in denitrification) after quorum quenching (27). In our study, the addition of NaNO_3 or NaNO_2 changed the rate of denitrification (Fig. 4B) and also changed the QS phenotypes in culture (Fig. 6), suggesting some kind of reciprocal regulation phenomenon. This observation is consistent with the idea that quorum sensing has a role in integrating biological information for bacteria (28, 29).

To our knowledge, no prior study has evaluated the importance of intraspecies invasion in a bioaugmented system. The experiments in our study showed that the inoculated strain, SD-1, a highly efficient degrading strain in bioaugmented systems, mutates its quorum-sensing system, and in some circumstances, these social cheaters could induce the collapse of the population. Our studies used a pure culture of SD-1 strain to examine the intraspecies invasion and the fate of the population, which is different from its long-term evolution in wastewater. In wastewater treatment systems, other factors are important, including competition with other species (30). However, the general idea still applies, as secreted public goods might be shared with other species and other species might contribute to the destabilization of the bioaugmented population (31). Our findings also demonstrated that the metabolism of nitrogen can affect the stability of bacterial populations, an important observation for continuing industrial applications with this species.

MATERIALS AND METHODS

Bacterial strain and culture conditions. *Pseudomonas aeruginosa* SD-1 (CGMCC 1.2421), a bioaugmentation strain that efficiently degrades aromatic compounds, especially, 4-fluoronitrobenzene (see Table S1 in the supplemental material), was cultured at 37°C with shaking at 250 rpm in LB or in casein medium.

Casein medium, based on photosynthesis medium (32), was prepared as previously described (13), comprising 10 g/liter casein, 5 mM Na_2HPO_4 , 5 mM KH_2PO_4 , a series of trace elements, 10.95 mg/liter $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 6.98 mg/liter $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 66.7 mg/liter $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.54 mg/liter $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.392 mg/liter $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.25 mg/liter $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, 0.177 mg/liter $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 0.185 mg/liter $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 0.2 g/liter $\text{N}(\text{CH}_2\text{COOH})_3$, and 0.289 g/liter MgSO_4 . This base casein medium lacks an additional nitrogen source. To augment the casein medium with nitrogen, we added either 8 mM NH_4Cl , 8 mM NaNO_3 , or 8 mM NaNO_2 . Casein medium without added nitrogen was used as the control condition. All media were filter (0.22 μm) sterilized. In some experiments, we replaced casein as the carbon source with 5 g/liter glucose.

Initial inocula for long-term culture experiments in casein medium were from overnight LB cultures. The initial optical density at 600 nm was ~ 0.05 . Cultures were maintained for 24 h at 37°C with shaking

at 250 rpm; subsequently, we transferred 1% (vol/vol) culture to fresh medium at 24 h intervals for 30 days.

Quantification of LasR[−] mutant cheaters. We measured bacterial counts by dilution plating. We isolated individual colonies and patched them onto skim milk agar to enumerate LasR[−] mutant cheaters in the population. Wild-type bacteria produce elastase and therefore a zone of clearance on skim milk agar, while LasR[−] mutants do not (14). For each time point, we determined the phenotype of at least 100 individual colonies.

Analyses of nitrogen, quorum-sensing signals, protease, and pyocyanin. We determined concentrations of NH₄⁺, NO₃[−], and NO₂[−] according to standard methods described by the American Public Health Association (33). Secreted protease was detected with a Pierce fluorescent protease assay kit (Thermo).

To measure QS signals, we extracted 2 ml of culture with an equivalent volume of ethyl acetate (with 0.1% acetic acid) three times. The extracts were dried in a nitrogen evaporator. Finally, 0.5 ml of acidic ethyl acetate was used to redissolve signal molecules. We quantified *N*-3-oxododecanoyl-homoserine lactone (3OC₁₂-HSL) and *N*-butanoyl-homoserine lactone (C₄-HSL) using a bioassay strain as previously described (34, 35).

Pyocyanin was measured as described previously (36). Briefly, 1.5 ml of chloroform was used to extract 2.5 ml of supernatant. The pyocyanin was reextracted from the chloroform with 1 ml of 0.2 M hydrochloric acid. Finally, the absorbance at 520 nm of the supernatant was measured. The concentration of pyocyanin was equal to the absorbance multiplied by 12.8 mg/liter.

Statistical analysis. All data are presented as averages ± standard deviations (SDs). Differences among groups were identified by analysis of variance using SPSS (version 22). Differences were considered significant when *P* was <0.05.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.00870-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

ACKNOWLEDGMENTS

This study was supported by the Natural Science Foundation of Zhejiang Province (grant no. LY15C010001) and the National Natural Science Foundation of China (grant no. 31570490).

REFERENCES

- Herrero M, Stuckey DC. 2015. Bioaugmentation and its application in wastewater treatment: a review. *Chemosphere* 140:119–128. <https://doi.org/10.1016/j.chemosphere.2014.10.033>.
- Wang MZ, Zheng X, Zhang K, Ding YC, He HZ, Shen DS, Feng HJ. 2014. A new method for rapid construction of a *Pseudomonas* sp. HF-1 bioaugmented system: accelerating acylated homoserine lactones secretion by pH regulation. *Bioresour Technol* 169:229–235. <https://doi.org/10.1016/j.biortech.2014.06.098>.
- El Fantroussi S, Agathos SN. 2005. Is bioaugmentation a feasible strategy for pollutant removal and site remediation? *Curr Opin Microbiol* 8:268–275. <https://doi.org/10.1016/j.mib.2005.04.011>.
- Wang M, Yang G, Min H, Lv Z, Jia X. 2009. Bioaugmentation with the nicotine-degrading bacterium *Pseudomonas* sp. HF-1 in a sequencing batch reactor treating tobacco wastewater: degradation study and analysis of its mechanisms. *Water Res* 43:4187–4196. <https://doi.org/10.1016/j.watres.2009.07.012>.
- Thompson IP, van der Gast CJ, Ciric L, Singer AC. 2005. Bioaugmentation for bioremediation: the challenge of strain selection. *Environ Microbiol* 7:909–915. <https://doi.org/10.1111/j.1462-2920.2005.00804.x>.
- Yu FB, Ali SW, Guan LB, Li SP, Zhou S. 2010. Bioaugmentation of a sequencing batch reactor with *Pseudomonas putida* ONBA-17, and its impact on reactor bacterial communities. *J Hazard Mater* 176:20–26. <https://doi.org/10.1016/j.jhazmat.2009.06.006>.
- Yang Z, Guo R, Shi X, He S, Wang L, Dai M, Qiu Y, Dang X. 2016. Bioaugmentation of *Hydrogenispora ethanolica* LX-B affects hydrogen production through altering indigenous bacterial community structure. *Bioresour Technol* 211:319–326. <https://doi.org/10.1016/j.biortech.2016.03.097>.
- Li Q, Wang M, Feng J, Zhang W, Wang Y, Gu Y, Song C, Wang S. 2013. Treatment of high-salinity chemical wastewater by indigenous bacteria–bioaugmented contact oxidation. *Bioresour Technol* 144:380–386. <https://doi.org/10.1016/j.biortech.2013.07.004>.
- Zhang K, Zheng X, Shen DS, Wang MZ, Feng HJ, He HZ, Wang S, Wang JH. 2015. Evidence for existence of quorum sensing in a bioaugmented system by acylated homoserine lactone-dependent quorum quenching. *Environ Sci Pollut Res Int* 22:6050–6056. <https://doi.org/10.1007/s11356-014-3795-6>.
- Wang MZ, Zheng X, He HZ, Shen DS, Feng HJ. 2012. Ecological roles and release patterns of acylated homoserine lactones in *Pseudomonas* sp. HF-1 and their implications in bacterial bioaugmentation. *Bioresour Technol* 125:119–126. <https://doi.org/10.1016/j.biortech.2012.08.116>.
- Schuster M, Sexton DJ, Diggle SP, Greenberg EP. 2013. Acyl-homoserine lactone quorum sensing: from evolution to application. *Annu Rev Microbiol* 67:43–63. <https://doi.org/10.1146/annurev-micro-092412-155635>.
- Diggle SP, Griffin AS, Campbell GS, West SA. 2007. Cooperation and conflict in quorum-sensing bacterial populations. *Nature* 450:411–414. <https://doi.org/10.1038/nature06279>.
- Dandekar AA, Chugani S, Greenberg EP. 2012. Bacterial quorum sensing and metabolic incentives to cooperate. *Science* 338:264–266. <https://doi.org/10.1126/science.1227289>.
- Sandoz KM, Schuster M. 2007. Social cheating in *Pseudomonas aeruginosa* quorum sensing. *Proc Natl Acad Sci U S A* 104:15876–15881. <https://doi.org/10.1073/pnas.0705653104>.
- Wang M, Schaefer AL, Dandekar AA, Greenberg EP. 2015. Quorum sensing and policing of *Pseudomonas aeruginosa* social cheaters. *Proc Natl Acad Sci U S A* 112:2187–2191. <https://doi.org/10.1073/pnas.1500704112>.
- Lee J, Zhang L. 2015. The hierarchy quorum sensing network in *Pseudomonas aeruginosa*. *Protein Cell* 6:26–41. <https://doi.org/10.1007/s13238-014-0100-x>.
- Yates EA, Philipp B, Buckley C, Atkinson S, Chhabra SR, Sockett RE, Goldner M, Dessaux Y, Cámara M, Smith H. 2002. *N*-Acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of *Yersinia pseudotuberculosis*

- and *Pseudomonas aeruginosa*. Infect Immun 70:5635–5646. <https://doi.org/10.1128/IAI.70.10.5635-5646.2002>.
18. Heurlier K, Dénervaud V, Haenni M, Guy L, Krishnapillai V, Haas D. 2005. Quorum-sensing-negative (*lasR*) mutants of *Pseudomonas aeruginosa* avoid cell lysis and death. J Bacteriol 187:4875–4883. <https://doi.org/10.1128/JB.187.14.4875-4883.2005>.
 19. Lopes CL, Mees JBR, Sene L, Carvalho KQD, Christ D, Gomes SD. 2015. Effect of cycle time and airflow in biological nitrogen removal from poultry slaughterhouse wastewater using sequencing batch reactor. Engenharia Agrícola 35:567–577. <https://doi.org/10.1590/1809-4430-Eng.Agric.v35n3p567-577/2015>.
 20. Boon N, Goris J, Vos PD, Verstraete W, Top EM. 2000. Bioaugmentation of activated sludge by an indigenous 3-chloroaniline-degrading *Comamonas testosteroni* strain, I2gfp. Appl Environ Microbiol 66:2906–2913. <https://doi.org/10.1128/AEM.66.7.2906-2913.2000>.
 21. Shrout JD, Nerenberg R. 2012. Monitoring bacterial twitter: does quorum sensing determine the behavior of water and wastewater treatment biofilms? Environ Sci Technol 46:1995–2005. <https://doi.org/10.1021/es203933h>.
 22. van Delden C, Köhler T, Brunner-Ferber F, François B, Carlet J, Pechère JC. 2012. Azithromycin to prevent *Pseudomonas aeruginosa* ventilator-associated pneumonia by inhibition of quorum sensing: a randomized controlled trial. Intensive Care Med 38:1118–1125. <https://doi.org/10.1007/s00134-012-2559-3>.
 23. Xavier JB, Kim W, Foster KR. 2011. A molecular mechanism that stabilizes cooperative secretions in *Pseudomonas aeruginosa*. Mol Microbiol 79:166–179. <https://doi.org/10.1111/j.1365-2958.2010.07436.x>.
 24. Bock E, Schmidt I, Stüven R, Zart D. 1995. Nitrogen loss caused by denitrifying *Nitrosomonas* cells using ammonium or hydrogen as electron donors and nitrite as electron acceptor. Arch Microbiol 163:16–20. <https://doi.org/10.1007/BF00262198>.
 25. Lovley DR, Phillips EJ. 1988. Novel mode of microbial energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. Appl Environ Microbiol 54:1472–1480.
 26. Toyofuku M, Nomura N, Fujii T, Takaya N, Maseda H, Sawada I, Nakajima T, Uchiyama H. 2007. Quorum sensing regulates denitrification in *Pseudomonas aeruginosa* PAO1. J Bacteriol 189:4969–4972. <https://doi.org/10.1128/JB.00289-07>.
 27. Mellbye BL, Giguere AT, Bottomley PJ, Sayavedra-Soto LA. 2016. Quorum quenching of *Nitrobacter winogradskyi* suggests that quorum sensing regulates fluxes of nitrogen oxide(s) during nitrification. mBio 7:e01753-16. <https://doi.org/10.1128/mBio.01753-16>.
 28. Waters CM, Bassler BL. 2005. Quorum sensing: cell-to-cell communication in bacteria. Annu Rev Cell Dev Biol 21:319–346. <https://doi.org/10.1146/annurev.cellbio.21.012704.131001>.
 29. Miller MB, Bassler BL. 2001. Quorum sensing in bacteria. Annu Rev Microbiol 55:165–199. <https://doi.org/10.1146/annurev.micro.55.1.165>.
 30. Friman VP, Diggle SP, Buckling A. 2013. Protist predation can favour cooperation within bacterial species. Biol Lett 9:20130548. <https://doi.org/10.1098/rsbl.2013.0548>.
 31. Smalley NE, An D, Parsek MR, Chandler JR, Dandekar AA. 2015. Quorum sensing protects *Pseudomonas aeruginosa* against cheating by other species in a laboratory coculture model. J Bacteriol 197:3154–3159. <https://doi.org/10.1128/JB.00482-15>.
 32. Kim MK, Harwood CS. 1991. Regulation of benzoate-CoA ligase in *Rhodospseudomonas palustris*. FEMS Microbiol Lett 83:199–203. <https://doi.org/10.1111/j.1574-6968.1991.tb04440.x>.
 33. Clesceri LS, Greenberg AE, Eaton AD (ed). 1998. Standard methods for the examination of water and wastewater, 20th ed. American Public Health Association, Washington, DC.
 34. Pearson JP, Passador L, Iglewski BH, Greenberg EP. 1995. A second *N*-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A 92:1490–1494. <https://doi.org/10.1073/pnas.92.5.1490>.
 35. Pearson JP, Gray KM, Passador L, Tucker KD, Eberhard A, Iglewski BH, Greenberg EP. 1994. Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. Proc Natl Acad Sci U S A 91:197–201. <https://doi.org/10.1073/pnas.91.1.197>.
 36. Essar D, Eberly L, Hadero A, Crawford I. 1990. Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: interchangeability of the two anthranilate synthases and evolutionary implications. J Bacteriol 172:884–900. <https://doi.org/10.1128/jb.172.2.884-900.1990>.