Denitrification is a well-studied respiratory system that is also important in the biogeochemical nitrogen cycle. Environmental signals such as oxygen and N-oxides have been demonstrated to regulate denitrification, though how denitrification is regulated in a bacterial community remains obscure. *Pseudomonas aeruginosa* is a ubiquitous bacterium that controls numerous genes through cell-to-cell signals. The bacterium possesses at least two N-acyl-l-homoserine lactone (AHL) signals. In our previous study, these quorum-sensing signals controlled denitrification in *P. aeruginosa*. In addition to the AHL signals, a third cell-to-cell communication signal, 2-heptyl-3-hydroxy-4-quinolone, referred to as the *Pseudomonas* quinolone signal (PQS), has been characterized. In this study, we examined the effect of PQS on denitrification to obtain more insight into the respiratory regulation in a bacterial community. Denitrification in *P. aeruginosa* was repressed by PQS, which was partially mediated by PqsR and PqsE. Measuring the denitrifying enzyme activities indicated that nitrite reductase activity was increased by PQS, whereas PQS inhibited nitric oxide reductase and the nitrate-respiratory chain activities. This is the first report to demonstrate that PQS influences enzyme activities, suggesting this effect is not specific to *P. aeruginosa*. Furthermore, when iron was supplied to the PQS-added medium, denitrifying activity was almost restored, indicating that the iron chelating property of PQS affected denitrification. Thus, our data indicate that PQS regulates denitrification primarily through iron chelation. The PQS effect on denitrification was relevant in a condition where oxygen was limited and denitrification was induced, suggesting its role in controlling denitrification where oxygen is present.
genic bacteria besides *P. aeruginosa*, suggesting a role in inter-species communication (17).

To obtain more insight into how energy production is regulated in a bacterial community, we examined the effect of PQS on denitrification. Based on our results, PQS affects denitrifying enzyme activities primarily due to the chelating activity of PQS. These results suggest that PQS not only affects respiration in *P. aeruginosa* but may also affect respiration in other species of bacteria. The low production of PQS in anaerobic conditions points out that this effect is relevant in interfaces of oxygen respiration and denitrification.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** The strains and plasmids used in this study are listed in Table 1. Bacterial strains were routinely grown at 37°C in Luria-Bertani (LB) medium or on LB agar plates. When necessary, gentamicin was added at the concentration of 10 μg/ml for *Escherichia coli* and 80 μg/ml for *P. aeruginosa*. PQS and N-(3-oxodecanoyl)-l-homoserine lactone (3-oxo-C12-HSL) were synthesized and purchased from NARD institute, Ltd. (Hyogo, Japan). A total of 20 mM PQS and 400 μM 3-oxo-C12-HSL stock solution in dimethyl sulfoxide was prepared and added to the culture as a final concentration of 50 μM or 1 μM, respectively. An equivalent amount of dimethyl sulfoxide was added to control cultures. Before beginning experimental cultures, *P. aeruginosa* was grown aerobically in 24-ml test tubes containing 4 ml of LB medium and was used to inoculate cultures at a starting optical density of 600 nm (OD600) of 0.01. The culture that examined the effect of 3-oxo-C12-HSL on denitrification was inoculated at a starting OD600 of 0.1. For anaerobic cultures, the air of butyl-rubber-sealed Hungate tubes or Erlenmeyer flasks was replaced with argon by flushing through a needle. Anaerobic growth, denitrification activity, and transcriptional activity were measured using 17-ml Hungate tubes containing 5 ml of LB medium (LB medium supplemented with 100 mM KNO3) incubated at 37°C with shaking at 200 rpm. Oxygen-limiting experiments were carried out by using butyl-rubber-sealed 17-ml Hungate tubes containing 2 ml LB medium incubated at 37°C with shaking at 170 rpm. Cells used to determine denitrifying enzyme activities were anaerobically cultured in a 500-ml Erlenmeyer flask containing 80 ml of LB medium at 37°C and 200 rpm. The *pqsA* transcriptional fusion plasmid was constructed by cloning the promoter region of *pqsA* (59), using *pmppqA* and *pmppqAR* PCR primers (Table 1), into the multicloning site of pME9 reporter plasmid. Plasmids were transformed into *P. aeruginosa* strain PA01 by electroporation (20). Complementation plasmids carrying *pqsR* or *pqsH* were constructed by cloning each gene into the multicloning site of *pUCP24*, *cppFRcppRR* or *cppEFcppE* primer pairs (Table 1) were used to amplify *pqsR* and *pqsH*, respectively.

**Construction of *P. aeruginosa* mutants.** The primers used in this study are listed in Table 1. *pqsA* was replaced with N2 and which contained 4 mM NADH, 0.2 mM phenazine methosulfate, and the membrane fraction of anaerobically cultured *P. aeruginosa* strains were assayed by using benzylviologen instead of oxygen as an electron donor (43). Five-hundred-microliter reaction mixtures for NADH-requiring activities contained 10 mM NaNO3 and the membrane fraction of *P. aeruginosa* strains was assayed by using benzylviologen instead of oxygen as an electron donor (43). Five-hundred-microliter reaction mixtures for NADH-requiring activities contained 10 mM NaNO3 and the membrane fraction of anaerobically cultured *P. aeruginosa*. The reactions were carried out at 30°C for 10 min.

**RESULTS**

**Anaerobic growth is repressed by PQS.** First, we examined the effect of PQS on the growth of *P. aeruginosa*. When grown aerobically in LB medium or anaerobically in LB medium, no change in growth was observed between the wild-type (WT) strain and the *Δpqsa* or *Δpqsh* mutants (Fig. 1). However, when PQS was added to the medium, a lag phase was observed in the aerobic culture, as had been observed previously (19) (Fig. 1A). This may be due to the protonid effect of PQS, as it was demonstrated in a recent paper (24). When PQS was added to anaerobic cultures (Fig. 1B), it resulted in a lower stationary-phase OD than that of the growth in LB medium alone. To investigate the mechanism of PQS suppres-
sion of anaerobic growth, we further examined the effect of PQS on denitrification.

PQS affects denitrifying enzyme activities. Denitrification is a process known to reduce $\text{NO}_3^-$ to $\text{N}_2\text{O}$ or $\text{N}_2$ gases and is physiologically important for acquiring energy under anaerobic conditions (61). During the denitrification process, $\text{NO}_3^-$ reductase (NAR), $\text{NO}_2^-$ reductase (NIR), NO reductase (NOR), and $\text{N}_2\text{O}$ reductase (NOS) are involved (61). Each denitrifying enzyme is

### TABLE 1. Bacterial strains, plasmids, and primers

| Strains, plasmids, and primers | Relevant characteristics | Source or reference |
|-------------------------------|--------------------------|---------------------|
| **Strains**                   |                          |                     |
| E. coli                       |                          | TaKaRa              |
| JM109                         | E. coli strain for transformation | TaKaRa              |
| DH5a                          | E. coli strain for transformation | TaKaRa              |
| S17-1                         | Mobilizer strain         | 48                  |
| **P. aeruginosa**             |                          |                     |
| PAO1                          | WT                       | 27                  |
| $\Delta$psa                   | PAO1 mutant with a deletion in the $\text{psa}A$ gene | This study          |
| $\Delta$psH                   | PAO1 mutant with a deletion in the $\text{psH}$ gene | This study          |
| $\Delta$psR                   | PAO1 mutant with a deletion in the $\text{psR}$ gene | This study          |
| $\Delta$lasI                  | PAO1 mutant with a deletion in the $\text{las}$I gene | 52                  |
| $\Delta$ril                   | PAO1 mutant with a deletion in the $\text{ril}$ gene | 52                  |
| $\Delta$psa $\Delta$psR       | PAO1 mutant with deletions in the $\text{psa}$ and $\text{psR}$ genes | This study          |
| $\Delta$psa $\Delta$psE       | PAO1 mutant with deletions in the $\text{psa}$ and $\text{psE}$ genes | This study          |
| $\Delta$psa $\Delta$lasI      | PAO1 mutant with deletions in the $\text{psa}$ and $\text{las}$I genes | This study          |
| $\Delta$psa $\Delta$ril       | PAO1 mutant with deletions in the $\text{psa}$ and $\text{ril}$ genes | This study          |
| **Plasmids**                  |                          |                     |
| pHSG398                       | Cloning vector; Cp'       | TaKaRa              |
| pG19II                        | pK19mobsac derived suicide vector; sacB Gm' | 36                  |
| pG19pqsA                      | $\text{psa}$ deletion cassette in pG19II | This study          |
| pG19pqsR                      | $\text{psR}$ deletion cassette in pG19II | This study          |
| pG19pqsE                      | $\text{psE}$ deletion cassette in pG19II | This study          |
| pG19pqsH                      | $\text{psH}$ deletion cassette in pG19II | This study          |
| pUCP24                        | Broad-host-range vector; Gm' | 55                  |
| pUCPpqsR                      | $\text{psR}$ in pUCP24    | This study          |
| pUCPpqsE                      | $\text{psE}$ in pUCP24    | This study          |
| pME9                          | pME4510 derived promoter-probe vector; $xylE$ Gm' | 52                  |
| pMEXpqsA                      | $\text{psa}$ promoter region in pME9 | This study          |
| pMEXnarK                      | $\text{nark}$ promoter region in pME9 | 52                  |
| pMEXnrS                       | $\text{nr}$S promoter region in pME9 | 52                  |
| pMEXNorC                      | $\text{nor}$C promoter region in pME9 | 52                  |
| pMEXNosR                      | $\text{nos}$R promoter region in pME9 | 52                  |
| **Primers**                   |                          |                     |
| pmpqAF3                       | 5'-GGAAATTCGGGGCGCAATGTTAGGTGTCCTTCTTTCCGG-3' |                     |
| pmpqAR                        | 5'-CCCAAGCTTTGGCCGGATGGAAGTGATGATCAGC-3' |                     |
| $\Delta$psAF1                 | 5'-GGCTCTAGAGCCAAAGGCTGCAACACATTGGACAGTGG-3' |                     |
| $\Delta$psAR1                 | 5'-GACTAGTGACAGACGTTCCTTCTTTCCGG-3' |                     |
| $\Delta$psAF2                 | 5'-CAGCGCCATGGAACGCTTCTGATGATGATCAGC-3' |                     |
| $\Delta$psAR2                 | 5'-GCCAAGCTTTGGCAAGGCTGCAACACATTGGACAGTGG-3' |                     |
| $\Delta$psHR1                 | 5'-GAGATTTACCTTGCTATCGCTGATCGCTG-3' |                     |
| $\Delta$psHF2                 | 5'-GGAAATTCCTTACCCGGTGGTGGATTAGTGG-3' |                     |
| $\Delta$psHR2                 | 5'-GAGATTTACCTTACCCGGTGGTGGATTAGTGG-3' |                     |
| $\Delta$psRF1                 | 5'-CCCAAGCTTTGGCCGGATGGAAGTGATGATCAGC-3' |                     |
| $\Delta$psRR1                 | 5'-GAGATTTACCTTCTGATGGAAGTGATGATCAGC-3' |                     |
| $\Delta$psRF2                 | 5'-GAGATTTACCTTCTGATGGAAGTGATGATCAGC-3' |                     |
| $\Delta$psRF2                 | 5'-GACTAGTGACAGACGTTCCTTCTTTCCGG-3' |                     |
| $\Delta$psEF1                 | 5'-CCCAAGCTTTGGAGGGGCAATCCATACGTGGTGG-3' |                     |
| $\Delta$psER1                 | 5'-GAGATTTACCTTGCTATCGCTGATCGCTG-3' |                     |
| $\Delta$psEF2                 | 5'-GAGATTTACCTTACCCGGTGGTGGATTAGTGG-3' |                     |
| $\Delta$psER2                 | 5'-GACTAGTGACAGACGTTCCTTCTTTCCGG-3' |                     |
| cppqRF                        | 5'-GCCAAGCTTTGCAAGGCTGCAACACATTGGACAGTGG-3' |                     |
| cppqRR                        | 5'-CCCAAGCTTTGCAAGGCTGCAACACATTGGACAGTGG-3' |                     |
| cppqER                        | 5'-GCCAAGCTTTGCAAGGCTGCAACACATTGGACAGTGG-3' |                     |
encoded by genes that are organized into distinct operons (2, 3, 29, 47). In order to examine the effect of PQS on transcription of each denitrifying enzyme, promoter fusion plasmids (52) with the xylE gene fused with promoter regions of narK1, nirS, norC, and nosR denitrifying genes were each transferred to a ΔpqsA mutant. As a control, the basal C23O activity was measured in a ΔpqsA mutant transformed with a pMEX9 plasmid with or without added PQS. C23O activity was measured during the mid-logarithmic phase (a 6-h incubation). The promoter-dependent C23O activity was determined by dividing its C23O activity by the basal C23O activity. When PQS was added to the culture of each strain at a final concentration of 50 μM, no significant effect on the promoter activity could be observed (Fig. 2). This result indicates that the denitrification regulation by PQS is not due to transcriptional regulation but is caused by other posttranscriptional mechanisms. To examine whether denitrification is regulated in posttranscriptional levels, denitrifying enzyme activities were measured during the mid-logarithmic phase. A ΔpqsA mutant was cultured with or without added PQS, and then NAR, NIR, and NOR denitrifying enzyme activities were measured. As a result, NAR activity was suppressed (66% activity compared to the NAR activity of a ΔpqsA mutant cultured without added PQS) in the culture incubated with PQS (Fig. 3A). Interestingly, NIR activity increased 1.8-fold in the culture incubated with PQS (Fig. 3B). NOR activity was suppressed to 64% when PQS was added to the culture (Fig. 3C). The changes in these denitrifying enzyme activities may be due to posttranscriptional regulation, or PQS may have affected enzyme activities. To further examine whether PQS affects denitrifying enzyme activities, an assay was carried out by adding PQS to each enzyme assay reaction mixture with ΔpqsA mutant cell fractions collected from cells cultured without PQS. As shown in Fig. 3C, NOR activity was inhibited by the PQS addition in vitro. On the other hand, NAR and NIR activity was not affected by the addition of PQS in vitro (Fig. 3A and B). Taken with the result that transcription of denitrifying genes was not regulated by PQS, our results indicate that NAR and NIR activities are regulated posttranscriptionally, requiring growth with PQS, and NOR activity is inhibited by PQS.

**PQS inhibits the NO₃⁻ respiratory chain.** Denitrifying enzymes are associated with the respiratory chain in which energy is generated. During NO₃⁻ respiration, the electron donated from NADH is transferred through the respiratory chain and finally accepted by NO₃⁻. The final step is mediated by NAR (61). In the NAR activity assay (Fig. 3A), an electron donor (benzylviologen) that donates electrons directly to NAR was used, and the activity of the NO₃⁻ respiratory chain was not taken into account. In order to examine whether the NO₃⁻ respiratory chain is affected by PQS, a NADH-derived NAR activity assay was carried out. When NADH was used as an electron donor, NADH-NAR activity was repressed in the

![FIG. 1. PQS effect on aerobic growth and anaerobic growth.](image1)

**FIG. 1. PQS effect on aerobic growth and anaerobic growth.** Aerobic growth (A) and anaerobic growth (B) of *P. aeruginosa* with or without exogenous PQS. PQS was added at a final concentration of 50 μM in all experiments. Three independent experiments were carried out, and representative data are shown.

![FIG. 2. PQS effect on denitrifying gene expression.](image2)

**FIG. 2. PQS effect on denitrifying gene expression.** C23O activity was measured in cells cultured for 6 h (mid-logarithmic phase). C23O activity (nanomoles of product/minute/milligram of protein) of denitrifying gene promoters was measured and was normalized by the C23O activity of promoterless control plasmids. +PQS, cultured with exogenous PQS; −PQS, cultured without exogenous PQS. The data displayed are the means ± standard deviations of more than three independent experiments.

![FIG. 3. PQS effect on denitrifying enzyme activities and NO₃⁻ respiratory chain.](image3)

**FIG. 3. PQS effect on denitrifying enzyme activities and NO₃⁻ respiratory chain.** NAR (A), NIR (B), NOR (C), and NADH-dependent NAR (D) activities were measured in cells cultured for 6 h (mid-logarithmic phase). PQS was added at a final concentration of 50 μM for the culture medium or the reaction mixture. Bar 1, PAO1; bar 2, ΔpqsA mutant cultured without added PQS; bar 3, ΔpqsA mutant cultured with PQS; bar 4, PQS added to the reaction mixture with cell fractions collected from a ΔpqsA mutant cultured without added PQS. More than three independent experiments were carried out, and the data represented are means ± standard deviations of triplicate assays.
culture with added PQS and was also inhibited in vitro when PQS was added to the reaction mixture (Fig. 3D). These results indicate that NO$_3^-$ respiration is inhibited by PQS. The fact that PQS inhibited NADH-derived NAR activity (Fig. 3D), but not benzylviologen-derived NAR activity (Fig. 3A), indicates that PQS’s inhibition occurs somewhere in the electron transfer from NADH to NO$_3$.

Iron-chelating property of PQS affects denitrification. In this study, our results addressed that PQS influences denitrification by affecting the denitrifying enzyme activities without regulating their transcription. PQS has been reported not only to regulate transcription in P. aeruginosa but also to chelate iron (7, 18). We considered that this chelating effect on iron may affect the denitrification activity. To test this hypothesis, FeCl$_3$ was added to the medium in addition to PQS. The iron addition fully restored suppression of NO$_3^-$ reduction and N$_2$O production in the culture with exogenous PQS (Fig. 4A and B), supporting our suggestion that NO$_3^-$ reduction and N$_2$O production were repressed by PQS even in the absence of PQS; NO$_3^-$ reduction and N$_2$O production were then restored by supplementing FeCl$_3$ (Fig. 4A and B). Interestingly, N$_2$ production was restored only partially by FeCl$_3$ in the PQS-added culture, and 2,2′-bipyridyl did not repress N$_2$ production (Fig. 4C). These results suggest that N$_2$O reduction to N$_2$, which is catalyzed by NOS, is regulated by mechanisms other than iron chelation by PQS.

PQS regulates N$_2$ production through PqsR and PqsE. A number of PQS-regulated genes require the cognate response regulator PqsR (11, 15, 54) or a functionally unknown PqsE, which is known to facilitate the response to PQS (19, 22). To further examine if the N$_2$O reduction to N$_2$ is regulated by this pathway, ΔpqsA ΔpqsR and ΔpqsA ΔpqsE double mutants were constructed. PQS was added to the mutants, and the amounts of NO$_3^-$ that was reduced and N$_2$O and N$_2$ that were produced during a 12-h incubation were measured. The denitrifying activities of ΔpqsA ΔpqsR and ΔpqsA ΔpqsE mutants (Fig. 5) were identical to that of ΔpqsA (Fig. 4). As shown in Fig. 5C, the repression of N$_2$ production by PQS was not observed in either the ΔpqsA ΔpqsR or the ΔpqsA ΔpqsE mutants, while PQS repressed N$_2$ production in ΔpqsA (Fig. 4C), indicating that N$_2$ production is regulated by PQS through PqsR and PqsE transcriptional regulator. Together with the result that N$_2$ production was not affected by an iron chelator (Fig. 4), our data indicate that PQS regulates N$_2$ production by a pathway that mediates PqsR and PqsE and does not depend on the iron chelation property of PQS. This regulation is likely to be involved in the transcriptional regulation of factors involved in N$_2$ production other than the nos operon, since PQS did not regulate nosR transcription (Fig. 2).

FIG. 4. Effect of iron chelation on denitrification. NO$_3^-$ reduction (A), N$_2$O production (B), and N$_2$ production (C) of ΔpqsA mutant. PQS, 2,2′-bipyridyl, and FeCl$_3$ were added at final concentrations of 50 μM, 120 μM, and 100 μM, respectively. Data displayed are means ± standard deviations of three independent experiments.
Relation of AHLs and PQS in denitrification regulation. In our previous study, AHLs regulated transcriptions of denitrifying genes (52). PQS is known to be incorporated in the AHL signaling pathway where the las quorum-sensing system regulates the PQS system and the PQS system regulates the rhl quorum-sensing system (38). To further investigate the relationship between AHL and PQS signaling systems in denitrification regulation, we first examined whether the las quorum-sensing requires PQS for denitrification regulation. When 3-oxo-C12-HSL was added to the culture, 3-oxo-C12-HSL regulated denitrifying activity at the same extent in a ΔlasI mutant and a ΔpqsA ΔlasI double mutant (Fig. 6A to C), indicating that PQS is not required for the 3-oxo-C12-HSL denitrification regulations. This result correlates with the fact that PQS is lacking under anaerobic conditions (later discussed in Fig. 7), suggesting that PQS has less effect on las quorum-sensing regulated phenotypes under anaerobic conditions. The N2O accumulation induced by 3-oxo-C12-HSL may be due to the imbalance of NOR and NOS activity, in which NOS activity may have been repressed by 3-oxo-C12-HSL to a greater extent than NOR activity, as is the case of NO accumulation caused by rhlR deletion (23). Furthermore, it was examined whether the rhl quorum-sensing system is incorporated in the effect of PQS on denitrification. When PQS was added to a ΔpqsA ΔrhlI double mutant, NO3− reduction to N2O was repressed, as had been observed in a ΔpqsA mutant (Fig. 6D and E). However, N2 production was repressed only partially by the PQS addition in the ΔpqsA ΔrhlI double mutant compared to the ΔpqsA mutant (Fig. 6F), indicating that the rhl quorum-sensing system is partially required for the repression of N2 production by PQS. Since N2 production was not fully repressed by the PQS addition to a ΔpqsA pqsR mutant, these data suggest that a transcriptional regulation mediated by PqsR and the rhl quorum-sensing system is involved in N2 production. According to our previous study (52), the rhl quorum-sensing system repressed nosR transcription; therefore, it may simply be considered that PQS induced the rhl quorum-sensing system through PqsR, leading to the repression of nosR transcription. However, repression of nosR transcription by PQS was not observed in this study (Fig. 2). The effect of PQS on nosR transcription may...
have been too little to be detected by the transcriptional assay; otherwise, there may be a complicated regulatory system in N\textsubscript{2} production. The result that the N\textsubscript{2} production in the ΔpqsA ΔpqsH strain was not repressed by PQS, while N\textsubscript{2} production in the ΔpqsA ΔrhlI strain was partially repressed by PQS, indicates that there are other factors regulated by PqsR that are involved in the N\textsubscript{2} production in addition to the rhl quorum-sensing system.

PQS signaling under anaerobic conditions. In order to reveal the PQS effect on denitrification, our experiments were carried out under anaerobic conditions. Interestingly, although PQS suppressed denitrification, no changes in growth between the WT and the ΔpqsA ΔpqsH mutants were observed (Fig. 1), and no differences in denitrification activity were observed between WT and non-PQS-producing strains (data not shown). To examine whether PQS is produced under anaerobic conditions, PQS was extracted from culture supernatants and was detected by TLC. When grown aerobically, PQS was detected but it could not be detected under anaerobic conditions (Fig. 7A and B). Although PQS was not produced under anaerobic conditions, the result that the addition of PQS to a ΔpqsR mutant did not repress N\textsubscript{2} production (Fig. 5C) suggests that PQS can regulate gene transcriptions in a PqsR-dependent manner under anaerobic conditions. To examine whether the PQS signaling system mediated by PqsR could be activated under anaerobic conditions, pqsA expression, which is known to be regulated by PQS through PqsR, was measured in the WT P. aeruginosa strain PAO1 and a ΔpqsA mutant. When PQS was added to PAO1, pqsA was induced, while it was not induced in the absence of PqsR (Fig. 7C). These results, along with results demonstrating the lack of PQS under anaerobic conditions (Fig. 7), indicate that cells under anaerobic conditions can respond to PQS through PqsR but cannot produce PQS. The reason why PQS is lacking under anaerobic conditions could be due to the final step of PQS synthesis, which is predicted to require diatomic oxygen (22).

Effect of PQS on denitrification is relevant under oxygen-limiting conditions. When considering the environment, oxygen concentration is not always constant and changes according to several factors, including cell population. The PQS effect on denitrification may be relevant under such conditions. To examine whether PQS affects denitrification under conditions where oxygen is gradually depleted and denitrification is induced, we cultured P. aeruginosa in a Hungate tube with a rubber cap. The oxygen concentration was 7.5 ppm at the start of the culture and became less than 1.7 ppm at the end of the culture (16 h). Under these conditions, PQS was detected in the supernatant (Fig. 7A and B), and the ΔpqsA, ΔpqsH, and ΔpqsR mutants reduced more NO\textsubscript{3}\textsuperscript{-} and produced more N\textsubscript{2}O than PAO1 (Fig. 8). These results indicate that denitrification is repressed by PQS when PQS is produced. Interestingly, the PQS production was lower compared to the aerobic conditions (Fig. 7A and B), suggesting that oxygen or N-oxides related to denitrification regulate PQS production. When PQS was added to the medium, PQS repressed NO\textsubscript{3}\textsuperscript{-} production and N\textsubscript{2}O production in PAO1 and the ΔpqsH mutant to a greater extent than in the ΔpqsA and ΔpqsR mutants (Fig. 8). The transcriptional regulator PqsR is known to regulate the pqsABCDE operon (54). Therefore, under oxygen-limiting conditions, other 2-alkyl-4-quinolones (AHQs), such as 2-heptyl-4-quinolone N-oxide (HONO) or HHQ, which are produced as a result of the transcription of the pqsABCDE operon (16), may be involved in denitrification regulation. The denitrification repression by PQS in the ΔpqsR mutant indicates that PQS represses denitrification without mediating PqsR, as had been observed under anaerobic conditions. To confirm if this denitrification suppression by PQS was a result of the iron-chelating activity, FeCl\textsubscript{3} was added to the medium. FeCl\textsubscript{3} addition increased denitrifying activity in PAO1 to the level of the non-PQS-producing mutants and restored denitrifying activity in the PQS-added ΔpqsA and ΔpqsH mutants (Fig. 8).

Taken together, PQS affects denitrification by chelating iron under oxygen-limiting environments where denitrification is induced and a sufficient amount of PQS is produced to influence denitrification.

**DISCUSSION**

Bacterial energy production is well known to be regulated by the environment. On the other hand, the environment surrounding the bacteria changes due to the bacterial population or community. However, the impact of cell-to-cell communication on regulating energy production has not been well documented. This research was carried out to gain more insight into respiration regulation in a bacterial population. Some studies in *Rhizobium* species have reported that cell-to-cell communication affects the growth rate, although the mechanisms remain obscure (14, 25, 46, 56). Another study demonstrated that butanediol fermentation in *Serratia* species is affected by quorum-sensing as a result of changes in acidic end
products (53). We have demonstrated previously that AHL signal molecules (C_2-HSL and 3-oxo-C_2-HSL) repress denitrification in *P. aeruginosa*, which was mediated by regulatory proteins RhlR or LasR exerting denitrifying gene expression (52). In contrast to the AHL regulation of denitrification in *P. aeruginosa*, the PQS effect on denitrification does not necessarily require the regulatory protein PqsR but depends primarily on its iron-chelating property and has a potential to inhibit denitrification in other species. PQS may have depleted iron from the medium, and besides, results from our in vitro assay adding PQS to the cell lysates (Fig. 3) suggest that PQS can affect enzyme activities, presumably by chelating iron. According to our knowledge, this is the first report to demonstrate that PQS affects enzyme activities. Thus, PQS is the third cell-cell communication molecule to be reported to control denitrification in *P. aeruginosa*, and this receptor-independent effect on denitrification by PQS may have an impact on *P. aeruginosa* interactions with other bacteria species.

Another fact suggesting the role of PQS in interspecies interactions is the presence of HHQ producers, which could induce PQS production. HHQ has been reported to be produced in the supernatant of some human pathogens (17). Once HHQ is produced in the supernatant, it can be converted to PQS by *P. aeruginosa* (16). The fact that PQS can affect denitrification by chelating iron, and that PQS production could be induced by other HHQ-producing bacteria, leads us to postulate that PQS may have an impact on a bacterial community of HHQ producers, and further studies in this area are expected.

The biosynthetic pathway that produces PQS also generates diverse AHQs besides HHQ (16). HQNO is one of the AHQs, known as a classical respiratory inhibitor that suppresses growth in gram-positive bacteria but not in gram-negative bacteria (34). The spectrum of HQNO is broader in vitro, where it was demonstrated to bind to a nitrate reductase subunit (NarI) of *Escherichia coli* (35). Although PQS and HQNO inhibit respiration, their mechanisms seem to differ. HQNO inhibits respiration by binding to quinone-reacting cytochromes and inhibits respiratory electron transfer from quinone to cytochromes (35), while PQS depends on iron chelating. According to the mechanism, PQS may inhibit respiration in other species, and it will be interesting to examine the difference in spectrum of HQNO in future research.

In this study, PQS did not decrease aerobic growth, but it did decrease anaerobic growth (Fig. 1). The amount of iron required for denitrification may be greater than that for aerobic respiration. Supporting this hypothesis, Diggle et al. (18) have demonstrated that PQS would decrease aerobic growth under iron-deficient conditions. In *P. aeruginosa*, the aerobic respiratory chain is predicted to be well branched, possessing five putative terminal oxidases (13), which may be another reason why PQS had less effect on aerobic growth than on anaerobic growth. Still, the regulation and the role of these terminal oxidases are poorly understood, and it will be interesting to examine whether these terminal oxidases are affected by PQS and to investigate the role of PQS in the aerobic respiratory chain.

The suppression of NO_3^- reduction and N_2O production by PQS iron chelation (Fig. 4), along with PQS's inhibition of NO_3^- respiration and NOR activities (Fig. 3), suggests that PQS inhibits NO_3^- respiration and NOR activity by chelating iron. Iron is related to the NO_3^- respiratory chain during the transfer of electrons from NADH to NO_3^- (21, 61). The NOR enzyme in *P. aeruginosa* is a cytochrome bc-type enzyme consisting of cytochrome c and cytochrome b (1, 61). Therefore, it will be reasonable to consider that PQS inhibits their activities by chelating iron. Conversely, it was surprising to us that NIR activity was not inhibited by PQS (Fig. 3B), because the *P. aeruginosa* NIR enzyme is a cytochrome cd1-type enzyme (60) that requires iron for its activity. There is a considerable difference in the hydrophobicity between the enzymes inhibited by PQS and the noninhibited enzymes. The PQS-affected NO_3^- respiration and NOR enzyme are associated with the cell membrane, while the NIR enzyme is soluble and located in the periplasm (61). Together with the fact that PQS is a highly hydrophobic molecule (10), it can be assumed that the protein hydrophobicity is one of the factors that determine whether PQS affects the activity or not. The result that PQS is associated with the cell membrane (33) could also support our study that PQS inhibits NO_3^- respiration and NOR enzyme activities. It will be interesting to further investigate whether the PQS effect on NO_3^- respiration and the NOR enzyme activities is due to direct interactions of PQS with the enzymes.

Denitrification regulation by PQS was due not only to the iron-chelating property of PQS but was also partially regulated through PqsE and PqsR regulatory proteins. The result that an iron chelator, 2,2'-bipyridyl, did not suppress N_2 production, while NO_3^- reduction and N_2O were suppressed (Fig. 4), suggests that N_2 production is regulated by PQS but by mechanisms other than iron chelation. This suggestion was confirmed by the result that PqsE and PqsR were required for PQS regulation of N_2 suppression (Fig. 5C). Results from the experiment in which PQS was added to a ΔpqsA ΔrhlI mutant indicate that several factors, including the rhl quorum-sensing system, are incorporated in this regulation (Fig. 6D to F). Collectively, our study demonstrates that denitrification is regulated by PQS by at least two pathways; one pathway is dependent on the iron-chelating property of PQS, and the other is dependent on transcriptional regulation by PQS mediated through PqsE and PqsR (Fig. 9). The fact that iron chelators inhibit N_2O production without inhibiting N_2 production may...
serve as a basis for development of a more efficient water treatment system, since N₂O emission during wastewater treatment is known to contribute to global warming and has become a problem. Previously, microarray data by Déziel et al. (15) comparing transcriptional profiles of WT with a ΔpqsR (pqsR) mutant suggested that denitrifying genes are controlled by PqsR. When pqsR was expressed on a plasmid in our experiment, denitrification activity was repressed in a ΔpqsA ΔpqsR mutant background that does not produce AHOs, including PQS (data not shown). Along with the result that PQS did not affect transcription of denitrifying genes (Fig. 2), these results suggest that PqsR regulates denitrifying genes alone without PQS when it is expressed in a sufficient amount. Still, the experiments of Déziel et al. (15) were carried out under aerobic conditions without NO₃⁻ added, and it will not be that simple to compare their results with ours using anaerobic conditions.

Our data indicate that the effect of PQS on denitrification is relevant where oxygen is present or was present and denitrification is induced. The regulation may be important in the transition from aerobic respiration to denitrification. Also, the regulation may be important in an environment where oxygen is localized, such as in biofilms, where oxygen is consumed at the surface and conditions become anaerobic inside (5). Our in vitro results (Fig. 3) suggest that PQS promotes NO accumulation, since NIR activity (which reduces NO₂⁻ to NO) was elevated, while NOR activity (which further reduces NO to N₂O) was suppressed after the addition of PQS into the medium. These results may explain the mechanism of NO accumulation in biofilms, which in turn regulates biofilm formation by upregulating bacterial motility (4). PQS has also been detected in the lungs of P. aeruginosa-infected individuals with cystic fibrosis, conditions which are considered to be O₂ limited (57) and where NO₂⁻ levels are sufficient for anaerobic growth (40). In addition, P. aeruginosa is known to denitrify even in the presence of oxygen (12, 51). Although the ecological role of aerobic denitrification is still uncertain, PQS denitrification regulation may be involved.

Regarding the significance of respiratory regulation by cell-to-cell communication molecules, a number of studies have demonstrated that respiratory chains are not only coupled to aerobic denitrification but may have significance for bacterial physiology. The biological roles of denitrification regulation by cell-to-cell communication signaling molecules, such as AHL and PQS, remain elusive, and it is reasonable to assume that there are biological functions yet to be uncovered.

ACKNOWLEDGMENTS

We thank Michael A. Kertesz for providing us with the pME4510 plasmid and Herbert P. Schweizer for providing us with the pUCP24 plasmid.

This study was partially supported by a grant to N.N. from the Industrial Technology Research Grant Program 2003–2005 of the New Energy and Industrial Technology Development Organization (NEDO) of Japan.

REFERENCES

1. Ariai, H., Y. Igarashi, and T. Kodama. 1995. The structural genes for nitric oxide reductase from Pseudomonas aeruginosa. Biochim. Biophys. Acta 1261: 279–286.
2. Ariai, H., T. Kodama, and Y. Igarashi. 1999. Effect of nitrogen oxides on expression of the nir and nor genes for denitrification in Pseudomonas aeruginosa. FEMS Microbiol. Lett. 170:19–24.
3. Ariai, H., M. Mizutani, and Y. Igarashi. 2003. Transcriptional regulation of the nos genes for nitrous oxide reductase in Pseudomonas aeruginosa. Microbiology 149:26–36.
4. Barraud, N., D. J. Hassett, S. H. Hwang, S. A. Rice, S. Kjelleberg, and J. S. Webb. 2006. Involvement of nitric oxide in biofilm dispersal of Pseudomonas aeruginosa. J. Bacteriol. 188:7344–7353.
5. Borriello, G., E. Werner, F. Roe, A. M. Kim, G. D. Ehrlich, and P. S. Stewart. 2004. Oxygen limitation contributes to antibiotic tolerance of Pseudomonas aeruginosa in biofilms. Antimicrob. Agents Chemother. 48:2659–2664.
6. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254.
7. Bredenbruch, F., R. Geffers, M. Nimtz, J. Buer, and S. Haussler. 2006. The Pseudomonas aeruginosa quinoline signal (PQS) has an iron-chedling activity. Environ. Microbiol. 8:1318–1329.
8. Bryan, L. E., T. Nicas, B. W. Holloway, and C. Crowther. 1980. Aminoglycoside-resistant mutation of Pseudomonas aeruginosa defective in cytochrome c₅₅₃ and nitrate reductase. Antimicrob. Agents Chemother. 17:71–79.
9. Bryan, L. E., and H. M. Van Den Elzen. 1977. Effects of membrane-energy mutations and cations on streptomycin and gentamicin accumulation by bacteria: a model for entry of streptomycin and gentamicin in susceptible and resistant bacteria. Antimicrob. Agents Chemother. 12:163–177.
10. Callee, M. W., J. G. Shelton, J. A. McCubrey, and E. C. Pesci. 2005. Solubility and bioactivity of the Pseudomonas quinolone signal are increased by a Pseudomonas aeruginosa-produced surfactant. Infect. Immun. 73:878–882.
11. Cao, H., G. Krishnan, B. Goumnerov, J. Tsongalis, R. Tompkins, and L. G. Rahme. 2001. A quorum sensing-associated virulence gene of Pseudomonas aeruginosa encodes a LyxR-like transcription regulator with a unique self-regulatory mechanism. Proc. Natl. Acad. Sci. USA 98:14613–14618.
12. Chen, F., Q. Xia, and L. K. Ju. 2003. Aerobic denitrification of Pseudomonas aeruginosa monitored by online NAD(P)H fluorescence. Appl. Environ. Microbiol. 69:7675–7672.
13. Comollì, J. C., and T. J. Donohue. 2002. Pseudomonas aeruginosa RoxR, a response regulator related to Rhodobactor sphaeroides PrA, activates expression of the cyanide-insensitive terminal oxidase. Mol. Microbiol. 45:755–766.
14. Daniels, R. D., E. De Vos, J. Desair, G. Raedschelders, E. Layten, V. Rose-meyer, C. Verreth, E. Schoeters, J. Vanderleyden, and J. Michiels. 2002. The cin quorum sensing locus of Rhizobium etli CNPAS512 affects growth and productivity nitrogen fixation. J. Bacteriol. 184:462–466.
15. Déziel, E., S. Gopalan, A. P. Tampakaki, F. Lépine, K. E. Padfield, M. Sauzier, G. Xiao, and L. G. Rahme. 2005. The contribution of MvfR to Pseudomonas aeruginosa pathogenesis: multiple quorum sensing-regulated genes are modulated without affecting las/ilm or the production of N-acetyl-homoserine lactones. Mol. Microbiol. 55:998–1014.
16. Déziel, E., F. Lépine, S. Milot, J. He, M. N. Mindrinos, R. G. Tompkins, and L. G. Rahme. 2004. Analysis of Pseudomonas aeruginosa 4-hydroxy-2-alkylquinolines (HAQs) reveals a role for 4-hydroxy-2-heptylquinoline in cell-to-cell communication. Proc. Natl. Acad. Sci. USA 101:1339–1344.
17. Diggle, S. P., P. Lumiätkase, F. Dipilato, K. Winzer, M. Kunakorn, D. A. Barrett, S. R. Chhabra, M. Câmara, and P. Williams. 2006. Functional genetic analysis reveals a 2-alkyl-4-quinolone signaling system in the human pathogen Burkholderia pseudomallei and related bacteria. Chem. Biol. 13:701–710.
18. Diggle, S. P., S. Matthias, V. J. Wright, M. P. Fletcher, S. R. Chhabra, I. L. Lamont, X. Kong, R. C. Hitler, P. Cornelis, M. Câmara, and P. Williams. 2007. The Pseudomonas aeruginosa 4-quinoline signal molecules HHQ and PQS play multifunctional roles in quorum sensing and iron entrapment. Chem. Biol. 14:87–96.
19. Diggle, S. P., K. Winzer, S. R. Chhabra, K. E. Worrall, M. Câmara, and P. Williams. 2003. The Pseudomonas aeruginosa quinoline signal molecule overcomes the cell density-dependency of the quorum sensing hierarchy, regulates rhf-dependent genes at the onset of stationary phase and can be produced in the absence of LasR. Mol. Microbiol. 50:429–433.
20. Farinha, M. A., and A. M. Kropinski. 1990. High efficiency electroporation of Pseudomonas aeruginosa using frozen cell suspensions. FEMS Microbiol. Lett. 70:221–226.
Holloway, B. W., V. Krishnapillai, and A. F. Morgan. 1979. Chromosomal genetics of Pseudomonas. Microbiol. Rev. 43:73–102.

Ka, J. O., J. Urbanche, R. W. Ye, T. Y. Ahn, and J. M. Tiedje. 2002. Anaerobic metabolism and quorum sensing by Pseudomonas aeruginosa biofilms in chronically infected cystic fibrosis airways: rethinking antibiotic treatment strategies and drug targets. Adv. Drug Deliv. Rev. 54:1425–1443.

Häussler, S., and T. Becker. 2004. The FNR family of transcriptional regulators. Antonie van Leeuwenhoek 85:109–1090.

He, X., W. Chang, D. L. Pierce, L. O. Seib, J. Wagner, and C. Fuqua. 2003. Quorum sensing in Rhizobium sp. strain NGR224 regulates conjugal transfer of the F plasmid. J. Bacteriol. 185:8651–8656.

Hoffman, L. R., E. Délé, D. A. D’Argenio, F. Lépine, J. Emerson, S. McNamara, R. L. Gibson, B. W. Ramsey, and S. I. Miller. 2006. Selection for Slhphytophthora aurea cell-clone variants due to growth in the presence of Pseudomonas aeruginosa. FEMS Microbiol. Lett. 56:55–60.

Koerner, H., and W. G. Zumft. 1989. Expression of denitrification enzymes in Pseudomonas stutzeri in response to the dissolved oxygen level and respiratory substrate in continuous culture of Pseudomonas stutzeri. J. Biol. Chem. 264:2702–2708.

Kawasaki, S., H. Arai, T. Kodama, and Y. Igarashi. 2000. Functions required for extracellular quinolone signaling by Pseudomonas aeruginosa. J. Bacteriol. 182:4672–4680.

Kawasaki, S., H. Arai, T. Kodama, and Y. Igarashi. 2000. Functions required for extracellular quinolone signaling by Pseudomonas aeruginosa. J. Bacteriol. 182:4672–4680.

Koerner, H., and W. G. Zumft. 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of Pseudomonas stutzeri. J. Biol. Chem. 264:2702–2708.

Koerner, H., and W. G. Zumft. 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of Pseudomonas stutzeri. J. Biol. Chem. 264:2702–2708.

Koerner, H., and W. G. Zumft. 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of Pseudomonas stutzeri. J. Biol. Chem. 264:2702–2708.

Koerner, H., and W. G. Zumft. 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of Pseudomonas stutzeri. J. Biol. Chem. 264:2702–2708.

Koerner, H., and W. G. Zumft. 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of Pseudomonas stutzeri. J. Biol. Chem. 264:2702–2708.

Koerner, H., and W. G. Zumft. 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of Pseudomonas stutzeri. J. Biol. Chem. 264:2702–2708.

Koerner, H., and W. G. Zumft. 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of Pseudomonas stutzeri. J. Biol. Chem. 264:2702–2708.

Koerner, H., and W. G. Zumft. 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of Pseudomonas stutzeri. J. Biol. Chem. 264:2702–2708.

Koerner, H., and W. G. Zumft. 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of Pseudomonas stutzeri. J. Biol. Chem. 264:2702–2708.

Koerner, H., and W. G. Zumft. 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of Pseudomonas stutzeri. J. Biol. Chem. 264:2702–2708.

Koerner, H., and W. G. Zumft. 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of Pseudomonas stutzeri. J. Biol. Chem. 264:2702–2708.

Koerner, H., and W. G. Zumft. 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of Pseudomonas stutzeri. J. Biol. Chem. 264:2702–2708.

Koerner, H., and W. G. Zumft. 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of Pseudomonas stutzeri. J. Biol. Chem. 264:2702–2708.

Koerner, H., and W. G. Zumft. 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of Pseudomonas stutzeri. J. Biol. Chem. 264:2702–2708.

Koerner, H., and W. G. Zumft. 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of Pseudomonas stutzeri. J. Biol. Chem. 264:2702–2708.

Koerner, H., and W. G. Zumft. 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of Pseudomonas stutzeri. J. Biol. Chem. 264:2702–2708.

Koerner, H., and W. G. Zumft. 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of Pseudomonas stutzeri. J. Biol. Chem. 264:2702–2708.

Koerner, H., and W. G. Zumft. 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of Pseudomonas stutzeri. J. Biol. Chem. 264:2702–2708.

Koerner, H., and W. G. Zumft. 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of Pseudomonas stutzeri. J. Biol. Chem. 264:2702–2708.

Koerner, H., and W. G. Zumft. 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of Pseudomonas stutzeri. J. Biol. Chem. 264:2702–2708.

Koerner, H., and W. G. Zumft. 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of Pseudomonas stutzeri. J. Biol. Chem. 264:2702–2708.

Koerner, H., and W. G. Zumft. 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of Pseudomonas stutzeri. J. Biol. Chem. 264:2702–2708.

Koerner, H., and W. G. Zumft. 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of Pseudomonas stutzeri. J. Biol. Chem. 264:2702–2708.

Koerner, H., and W. G. Zumft. 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of Pseudomonas stutzeri. J. Biol. Chem. 264:2702–2708.