

## Quorum Sensing Regulates Denitrification in *Pseudomonas aeruginosa* PAO1<sup>∇</sup>

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**Anaerobic growth of *Pseudomonas aeruginosa* PAO1 was affected by quorum sensing. Deletion of genes that produce *N*-acyl-L-homoserine lactone signals resulted in an increase in denitrification activity, which was repressed by exogenous signal molecules. The effect of the *las* quorum-sensing system was dependent on the *rhl* quorum-sensing system in regulating denitrification.**

Bacteria regulate their metabolism to adapt to various conditions by sensing environmental signals. Under anoxic conditions, many bacteria are able to use N-oxides as terminal electron acceptors. *Pseudomonas aeruginosa* is a denitrifying bacterium capable of anaerobic growth by utilizing N-oxides such as nitrate (NO<sub>3</sub><sup>-</sup>) and nitrite (NO<sub>2</sub><sup>-</sup>). Denitrification is induced under low-oxygen conditions when N-oxides are also present (1, 9, 11).

Recent studies on bacteria have revealed new types of environmental signals known as cell-to-cell communication signals (25). *P. aeruginosa* is reported to control gene expression globally in response to cell density by utilizing *N*-acyl-L-homoserine lactone (AHL) signals. This cell-density-dependent regulation is termed quorum sensing (5). *P. aeruginosa* possesses at least two quorum-sensing systems: the LasR-LasI (*las*) and RhlR-RhlI (*rhl*) systems (20). LasI directs the synthesis of the AHL signal *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C<sub>12</sub>-HSL) (17, 18), and RhlI directs the synthesis of another AHL signal, *N*-butyryl-L-homoserine lactone (C<sub>4</sub>-HSL) (19). The transcription regulatory proteins, LasR (6) and RhlR (16), are specifically activated by 3-oxo-C<sub>12</sub>-HSL and C<sub>4</sub>-HSL, respectively. Previous studies have indicated that production of virulence factors, such as protease, exotoxin A, rhamnolipids, and siderophores, is regulated by quorum sensing in *P. aeruginosa* (7, 12, 26), suggesting that quorum sensing is important in the pathogenesis of infection with this bacterium.

Recent transcriptomic and proteomic studies indicate that quorum sensing is a global regulation system in *P. aeruginosa* (3, 23, 28). From these findings, it can be presumed that quorum-sensing systems have ecologically important roles in addition to the control of pathogenesis for the bacterium. For example, recent work suggests that quorum sensing regulates the activities of denitrification enzymes. In a recent study by

Yoon et al. (31), the authors reported that levels of denitrifying enzyme activities of anaerobically grown *P. aeruginosa* cells are higher for an *rhlR* mutant than for its parent strain in an in vitro system. We were interested in further characterizing the potential anaerobic regulation of denitrification by the quorum-sensing system. Here we present a comprehensive analysis of the impact of quorum sensing on the denitrification pathway under anaerobic conditions by using in vivo and in vitro analyses.

**Effect of quorum sensing on denitrification activity.** *P. aeruginosa* PAO1 was cultured anaerobically in 17-ml Hungate tubes containing 5 ml Luria-Bertani (LB) medium supplemented with 100 mM KNO<sub>3</sub> with shaking at 200 rpm at 37°C. The tubes were sealed with rubber stoppers, and the air was replaced with argon. The growth of  $\Delta$ *rhlI* and  $\Delta$ *lasI* mutants, which are deficient in producing C<sub>4</sub>-HSL and 3-oxo-C<sub>12</sub>-HSL, respectively, was almost identical to that of the parental strain, PAO1. However, when C<sub>4</sub>-HSL was added to the  $\Delta$ *rhlI* mutant, the turbidity at 600 nm was decreased approximately 50% when cells entered the stationary phase (Fig. 1). No effect on growth was observed with addition of 3-oxo-C<sub>12</sub>-HSL to the  $\Delta$ *lasI* mutant. To further investigate the effect of quorum sensing on denitrification, the amounts of NO<sub>3</sub><sup>-</sup> reduced and N<sub>2</sub> produced during a 12-h incubation were measured. NO<sub>3</sub><sup>-</sup> was measured by the brucine method (15), and N<sub>2</sub> was measured by a gas chromatograph (GC-8AIT; Shimadzu) equipped with a Molecular Sieve 5A column. The quorum-sensing mutants reduced more NO<sub>3</sub><sup>-</sup> and produced more N<sub>2</sub> than PAO1, a pattern that was reversed by addition of 3-oxo-C<sub>12</sub>-HSL and C<sub>4</sub>-HSL (Fig. 2A). To investigate whether the observed effect of quorum sensing on NO<sub>3</sub><sup>-</sup> reduction is linked with the NO<sub>3</sub><sup>-</sup> respiratory chain, the cell membrane was collected and NO<sub>3</sub><sup>-</sup>-respiring activity was measured by using NADH as an electron donor. Cells were washed twice with 0.01 M potassium phosphate buffer, pH 7.0, containing 10% glycerol and were sonicated in the same buffer. The sonicated cells were centrifuged for 10 min at 2,000 × *g* and 4°C to remove unbroken cells and were then centrifuged at 104,000 × *g* and 4°C for 60 min. Pellets were resuspended in the same buffer and collected as membrane fractions. A 500- $\mu$ l volume of reaction mixture for the NADH-dependent NO<sub>3</sub><sup>-</sup> reductase (NAR) activity assay contained 1 mM NADH, 10 mM KNO<sub>3</sub>, and the membrane fraction of anaerobically cultured *P. aeruginosa* in 0.01 M potassium phosphate buffer (pH 7.0)–10% glycerol. The air of the

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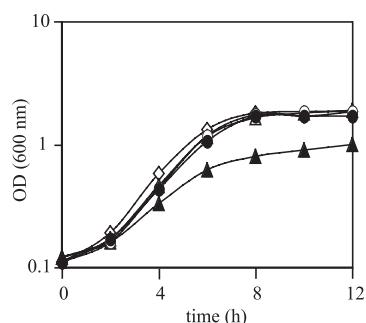


FIG. 1. Effects of AHL signals on anaerobic growth. Cells were grown anaerobically in LB medium supplemented with 100 mM  $\text{NO}_3^-$ . Symbols: open diamonds, PAO1; open triangles,  $\Delta rhlI$  mutant; solid triangles,  $\Delta rhlI$  mutant with 10  $\mu\text{M}$   $\text{C}_4$ -HSL; open circles,  $\Delta lasI$  mutant; solid circles,  $\Delta lasI$  mutant with 1  $\mu\text{M}$  3-oxo- $\text{C}_{12}$ -HSL. Three independent experiments were conducted, and representative data are shown.

reaction mixture was replaced with  $\text{N}_2$ , and incubation was carried out at 30°C for 30 min. The reaction was started by addition of NADH and was stopped by boiling at 100°C for 15 min.  $\text{NO}_2^-$  production was measured according to the method of Nicholas and Nason (15). To ensure that  $\text{NO}_3^-$ -respiring activity was measured in this assay, rotenone, which is an inhibitor of complex I in the respiratory chain, was added.  $\text{NO}_3^-$  reduction was repressed to approximately 70%, as has been observed for other *Pseudomonas* species (21). The  $\text{NO}_3^-$ -respiring activity in the  $\Delta rhlI$  mutant was 1.4-fold higher than that in PAO1 and was repressed 50% when the  $\Delta rhlI$  mutant was cultured with  $\text{C}_4$ -HSL (Fig. 2B). The  $\Delta lasI$  mutant showed

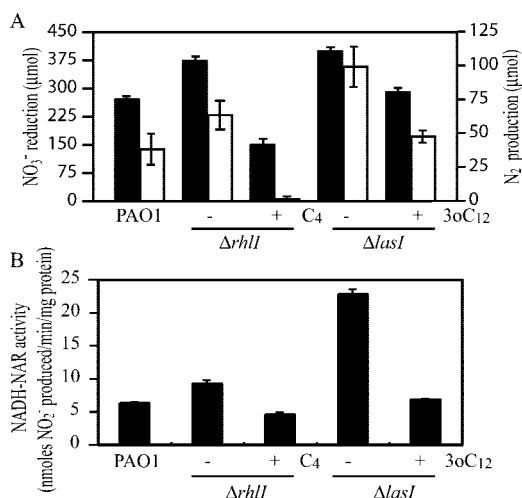


FIG. 2. Effects of AHL signals on denitrification. (A) Effects of AHL signals on  $\text{NO}_3^-$  reduction and  $\text{N}_2$  production. The amounts of  $\text{NO}_3^-$  reduced and  $\text{N}_2$  produced over 12 h were measured in PAO1 and in  $\Delta rhlI$  and  $\Delta lasI$  mutants. Solid bars,  $\text{NO}_3^-$  reduced; open bars,  $\text{N}_2$  produced.  $\text{C}_4$ -HSL and 3-oxo- $\text{C}_{12}$ -HSL were added to the cultures at final concentrations of 10  $\mu\text{M}$  and 1  $\mu\text{M}$ , respectively. Data are means  $\pm$  standard deviations from three independent experiments. (B) Effects of AHL signals on  $\text{NO}_3^-$ -respiring activity. NADH-dependent NAR activity was measured in cells grown anaerobically for 12 h.  $\text{C}_4$ -HSL at 10  $\mu\text{M}$  and 3-oxo- $\text{C}_{12}$ -HSL at 1  $\mu\text{M}$  were added to the cultures of  $\Delta rhlI$  and  $\Delta lasI$  mutants, respectively. The experiment was performed in triplicate.

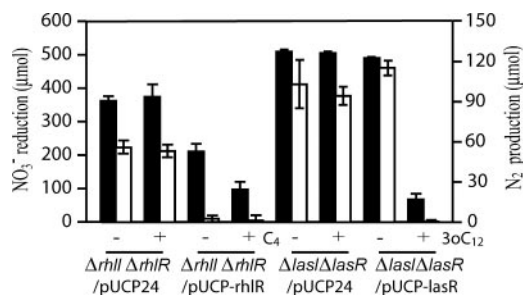


FIG. 3. Effects of RhlR and LasR transcriptional regulators on denitrification regulation. The amounts of  $\text{NO}_3^-$  reduced and  $\text{N}_2$  produced over 12 h were measured. Solid bars,  $\text{NO}_3^-$  reduced; open bars,  $\text{N}_2$  produced.  $\text{C}_4$ -HSL and 3-oxo- $\text{C}_{12}$ -HSL were added to the cultures at final concentrations of 10  $\mu\text{M}$  and 1  $\mu\text{M}$ , respectively. *rhlR* or *lasR* was inserted into a pUCP24 plasmid (pUCP-rhlR or pUCP-lasR) and transformed into a  $\Delta rhlI \Delta rhlR$  or  $\Delta lasI \Delta lasR$  double mutant for complementation. pUCP24 (29) was transformed into a  $\Delta rhlI \Delta rhlR$  or  $\Delta lasI \Delta lasR$  mutant as a control. Data are means  $\pm$  standard deviations from three independent experiments.

the highest activity (3.5-fold higher than that of PAO1), which could be repressed 70% by the addition of 3-oxo- $\text{C}_{12}$ -HSL in the culture. Taken together, these results indicate that the *rhl* and *las* quorum-sensing systems repress denitrification activity. Even though the  $\Delta rhlI$  and  $\Delta lasI$  mutants had higher denitrification activity than PAO1, no significant difference in growth could be observed between the mutants and PAO1. This may be due to the accumulation of toxic levels of nitric oxide (NO) in the quorum-sensing mutants (31), which may repress growth.

To investigate the involvement of the transcription regulatory proteins RhlR and LasR in the regulation of denitrification,  $\text{C}_4$ -HSL or 3-oxo- $\text{C}_{12}$ -HSL was added to a  $\Delta rhlI \Delta rhlR$  or  $\Delta lasI \Delta lasR$  double mutant, respectively. As a result, denitrification was not decreased by either of the AHLs when their binding proteins were deficient ( $\Delta rhlI \Delta rhlR$ /pUCP24,  $\Delta lasI \Delta lasR$ /pUCP24), and the regulation was restored by complementing each mutant with its appropriate wild-type copy ( $\Delta rhlI \Delta rhlR$ /pUCP-rhlR,  $\Delta lasI \Delta lasR$ /pUCP-lasR) (Fig. 3), indicating that denitrification regulation by  $\text{C}_4$ -HSL and 3-oxo- $\text{C}_{12}$ -HSL is dependent on their cognate transcription regulatory proteins. Interestingly, denitrification activity was decreased even in the absence of  $\text{C}_4$ -HSL when *rhlR* was complemented and was more repressed when  $\text{C}_4$ -HSL was added, as could be observed in the  $\text{NO}_3^-$  reduction data (Fig. 3). RhlR has been reported to regulate *rhlAB* oppositely in the presence and absence of  $\text{C}_4$ -HSL (14); however, in this study, denitrification was repressed by RhlR both in the presence and in the absence of  $\text{C}_4$ -HSL. Therefore, the  $\text{C}_4$ -HSL-RhlR complex and RhlR alone may regulate denitrification through different pathways, although it is still a question whether RhlR represses denitrification under physiological conditions, since RhlR was over-expressed in this experiment.

**Regulation of denitrification by *rhl* and *las* quorum-sensing systems.** The *rhl* and *las* quorum-sensing systems are reported as a hierarchy, in which the *las* quorum-sensing system regulates the *rhl* quorum-sensing system (20). To study whether the *rhl* and *las* quorum-sensing systems regulate denitrification concertedly or not,  $\text{C}_4$ -HSL and 3-oxo- $\text{C}_{12}$ -HSL were added to

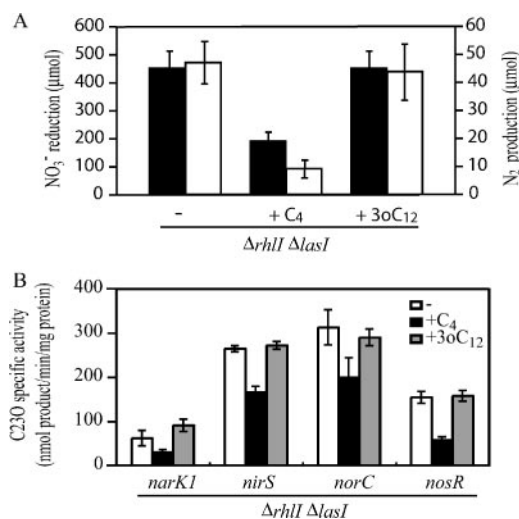


FIG. 4. Denitrification regulation by *rhl* and *las* quorum-sensing systems. (A) Effect of 10  $\mu\text{M}$  C<sub>4</sub>-HSL and 1  $\mu\text{M}$  3-oxo-C<sub>12</sub>-HSL on a  $\Delta rhlII \Delta lasI$  double mutant. The amounts of NO<sub>3</sub><sup>-</sup> reduced and N<sub>2</sub> produced over 12 h were measured. Solid bars, NO<sub>3</sub><sup>-</sup> reduced; open bars, N<sub>2</sub> produced. Data are means  $\pm$  standard deviations from three independent experiments. (B) Effects of 10  $\mu\text{M}$  C<sub>4</sub>-HSL and 1  $\mu\text{M}$  3-oxo-C<sub>12</sub>-HSL on the transcription of denitrifying genes in a  $\Delta rhlII$  mutant. C23O activity was measured in cells cultured for 6 h (late-exponential phase). Data are means  $\pm$  standard deviations from three independent experiments.

the culture of a  $\Delta rhlII \Delta lasI$  double mutant. The amounts of NO<sub>3</sub><sup>-</sup> reduced and N<sub>2</sub> produced during a 12-h incubation were measured. Addition of C<sub>4</sub>-HSL decreased the denitrification activity of the  $\Delta rhlII \Delta lasI$  mutant; however, 3-oxo-C<sub>12</sub>-HSL did not (Fig. 4A). To study whether denitrification is regulated at the transcriptional level, transcriptional fusion plasmids were constructed. During NO<sub>3</sub><sup>-</sup> reduction to N<sub>2</sub>, NAR, NO<sub>2</sub><sup>-</sup> reductase (NIR), NO reductase (NOR), and N<sub>2</sub>O reductase (NOS) are involved, and the genes encoding each of those enzymatic activities are organized into distinct operons (1, 2, 10, 24). The promoter regions of *narK1*, *nirS*, *norC*, and *nosR* were fused to a *xylE* gene on a pMEX9 plasmid, which was constructed by replacing a *lacZ $\alpha$*  gene of pME4510 (22) with *xylE*. Promoter regions of denitrifying genes were amplified by PCR using appropriate oligonucleotide primers. The amplified DNA fragments were digested with appropriate restriction enzymes and then inserted into a multicloning site of pMEX9. Each plasmid constructed, as well as a promoterless pMEX9 plasmid used as a reference, was transformed into a  $\Delta rhlII \Delta lasI$  mutant, and catechol 2,3-dioxygenase (C23O) activity was measured during the late-exponential phase (culture for 6 h). C23O activity was measured as described previously (13), and the promoter-dependent C23O activity was divided by the basal C23O activity, which was measured by using a  $\Delta rhlII \Delta lasI$  mutant transformed with pMEX9. Transcription of the denitrifying genes in the  $\Delta rhlII \Delta lasI$  mutant was repressed by C<sub>4</sub>-HSL; however, 3-oxo-C<sub>12</sub>-HSL had no effect (Fig. 4B). Thus, these results indicate that the denitrification regulation by the *las* quorum-sensing system is dependent on the *rhl* quorum-sensing system and that each denitrifying enzyme is regulated at the transcriptional level. The repression of the *narK1*, *nirS*, and *norC* promoters by quorum sensing is in accordance with

the previous result, which showed that NAR, NIR, and NOR activities are higher in an *rhlR* mutant than in its parental strain (31).

In conclusion, the denitrification activity in *P. aeruginosa* is regulated by C<sub>4</sub>-HSL and 3-oxo-C<sub>12</sub>-HSL. Regulation by the *las* quorum-sensing system was dependent on the *rhl* quorum-sensing system, suggesting a hierarchical regulation from the *las* to the *rhl* quorum-sensing system in denitrification regulation. However, regulation by the *las* quorum-sensing system may not be as simple, since NO<sub>3</sub><sup>-</sup>-respiring activity was higher in the  $\Delta lasI$  mutant than in the  $\Delta rhlII$  mutant, indicating that other, *rhl*-independent factors are involved. Although the mechanism of denitrification regulation by quorum sensing remains to be investigated, this is the first study, to our knowledge, to directly demonstrate the role of quorum sensing in controlling bacterial respiration.

Quorum sensing has been reported to affect butanediol fermentation in *Serratia* species (27) and the growth rate in *Rhizobium* species, although the mechanisms remain obscure (8, 30). A study by Wagner et al. using DNA microarray analysis (28) reported that exogenous C<sub>4</sub>-HSL and 3-oxo-C<sub>12</sub>-HSL affect the transcription of denitrifying genes in *P. aeruginosa* PAO1 under aerobic conditions. In addition, the results of Yoon et al. (31) indicate that quorum sensing affects biofilm formation under anaerobic conditions. Furthermore, it was reported that a denitrification gene, *nirS*, is expressed in aerobically grown *P. aeruginosa* biofilms (4), suggesting that regulation of denitrification by quorum sensing may be important in both aerobically and anaerobically grown biofilms. These studies, along with our data concerning the regulation of respiration by quorum sensing, may provide new insight into how bacteria regulate their energy metabolism during biofilm development.

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