JOURNAL OF BACTERIOLOGY, July 2007, p. 4969–4972 Vol. 189, No. 13
0021-9193/07/$08.00+0 doi:10.1128/JB.00289-07
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Quorum Sensing Regulates Denitrification in *Pseudomonas aeruginosa* PAO1[▽]

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Received 24 February 2007/Accepted 16 April 2007

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_3^-) and nitrite (NO_2^-). 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_{12}-HSL) (17, 18), and RhlI directs the synthesis of another AHL signal, *N*-butyryl-L-homoserine lactone (C_4-HSL) (19). The transcription regulatory proteins, LasR (6) and RhlR (16), are specifically activated by 3-oxo-C_{12}-HSL and C_4-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_3 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 Δ*rhlI* and Δ*lasI* mutants, which are deficient in producing C_4-HSL and 3-oxo-C_{12}-HSL, respectively, was almost identical to that of the parental strain, PAO1. However, when C_4-HSL was added to the Δ*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_{12}-HSL to the Δ*lasI* mutant. To further investigate the effect of quorum sensing on denitrification, the amounts of NO_3^- reduced and N_2 produced during a 12-h incubation were measured. NO_3^- was measured by the brucine method (15), and N_2 was measured by a gas chromatograph (GC-8AIT; Shimadzu) equipped with a Molecular Sieve 5A column. The quorum-sensing mutants reduced more NO_3^- and produced more N_2 than PAO1, a pattern that was reversed by addition of 3-oxo-C_{12}-HSL and C_4-HSL (Fig. 2A). To investigate whether the observed effect of quorum sensing on NO_3^- reduction is linked with the NO_3^- respiratory chain, the cell membrane was collected and NO_3^--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-μl volume of reaction mixture for the NADH-dependent NO_3^- reductase (NAR) activity assay contained 1 mM NADH, 10 mM KNO_3, 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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▽ Published ahead of print on 20 April 2007.

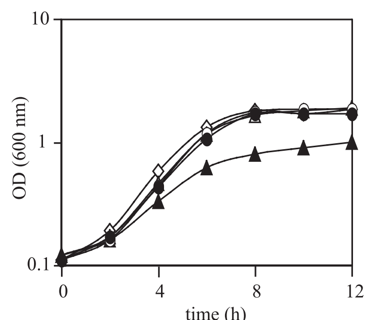

FIG. 1. Effects of AHL signals on anaerobic growth. Cells were grown anaerobically in LB medium supplemented with 100 mM NO_3^-. Symbols: open diamonds, PAO1; open triangles, Δ*rhlI* mutant; solid triangles, Δ*rhlI* mutant with 10 μM C_4-HSL; open circles, Δ*lasI* mutant; solid circles, Δ*lasI* mutant with 1 μM 3-oxo-C_{12}-HSL. Three independent experiments were conducted, and representative data are shown.

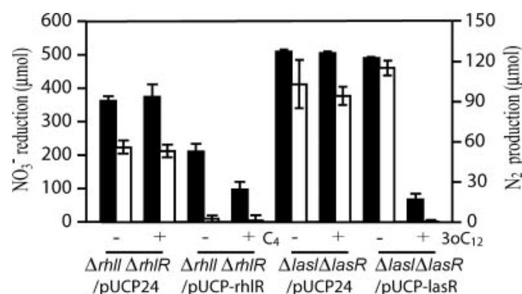

FIG. 3. Effects of RhlR and LasR transcriptional regulators on denitrification regulation. The amounts of NO_3^- reduced and N_2 produced over 12 h were measured. Solid bars, NO_3^- reduced; open bars, N_2 produced. C_4-HSL and 3-oxo-C_{12}-HSL were added to the cultures at final concentrations of 10 μM and 1 μM, respectively. *rhlR* or *lasR* was inserted into a pUCP24 plasmid (pUCP-rhlR or pUCP-lasR) and transformed into a Δ*rhlI* Δ*rhlR* or Δ*lasI* Δ*lasR* double mutant for complementation. pUCP24 (29) was transformed into a Δ*rhlI* Δ*rhlR* or Δ*lasI* Δ*lasR* mutant as a control. Data are means ± standard deviations from three independent experiments.

reaction mixture was replaced with 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. NO_2^- production was measured according to the method of Nicholas and Nason (15). To ensure that NO_3^--respiring activity was measured in this assay, rotenone, which is an inhibitor of complex I in the respiratory chain, was added. NO_3^- reduction was repressed to approximately 70%, as has been observed for other *Pseudomonas* species (21). The NO_3^--respiring activity in the Δ*rhlI* mutant was 1.4-fold higher than that in PAO1 and was repressed 50% when the Δ*rhlI* mutant was cultured with C_4-HSL (Fig. 2B). The Δ*lasI* mutant showed the highest activity (3.5-fold higher than that of PAO1), which could be repressed 70% by the addition of 3-oxo-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 Δ*rhlI* and Δ*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.

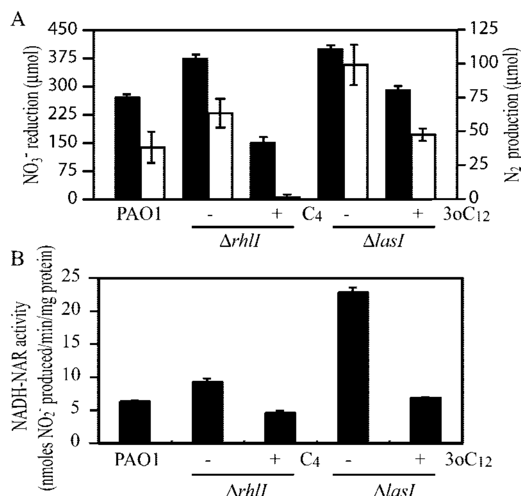

FIG. 2. Effects of AHL signals on denitrification. (A) Effects of AHL signals on NO_3^- reduction and N_2 production. The amounts of NO_3^- reduced and N_2 produced over 12 h were measured in PAO1 and in Δ*rhlI* and Δ*lasI* mutants. Solid bars, NO_3^- reduced; open bars, N_2 produced. C_4-HSL and 3-oxo-C_{12}-HSL were added to the cultures at final concentrations of 10 μM and 1 μM, respectively. Data are means ± standard deviations from three independent experiments. (B) Effects of AHL signals on NO_3^--respiring activity. NADH-dependent NAR activity was measured in cells grown anaerobically for 12 h. C_4-HSL at 10 μM and 3-oxo-C_{12}-HSL at 1 μM were added to the cultures of Δ*rhlI* and Δ*lasI* mutants, respectively. The experiment was performed in triplicate.

To investigate the involvement of the transcription regulatory proteins RhlR and LasR in the regulation of denitrification, C_4-HSL or 3-oxo-C_{12}-HSL was added to an Δ*rhlI* Δ*rhlR* or Δ*lasI* Δ*lasR* double mutant, respectively. As a result, denitrification was not decreased by either of the AHLs when their binding proteins were deficient (Δ*rhlI* Δ*rhlR*/pUCP24, Δ*lasI* Δ*lasR*/pUCP24), and the regulation was restored by complementing each mutant with its appropriate wild-type copy (Δ*rhlI* Δ*rhlR*/pUCP-rhlR, Δ*lasI* Δ*lasR*/pUCP-lasR) (Fig. 3), indicating that denitrification regulation by C_4-HSL and 3-oxo-C_{12}-HSL is dependent on their cognate transcription regulatory proteins. Interestingly, denitrification activity was decreased even in the absence of C_4-HSL when *rhlR* was complemented and was more repressed when C_4-HSL was added, as could be observed in the NO_3^- reduction data (Fig. 3). RhlR has been reported to regulate *rhlAB* oppositely in the presence and absence of C_4-HSL (14); however, in this study, denitrification was repressed by RhlR both in the presence and in the absence of C_4-HSL. Therefore, the 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 overexpressed 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, C_4-HSL and 3-oxo-C_{12}-HSL were added to

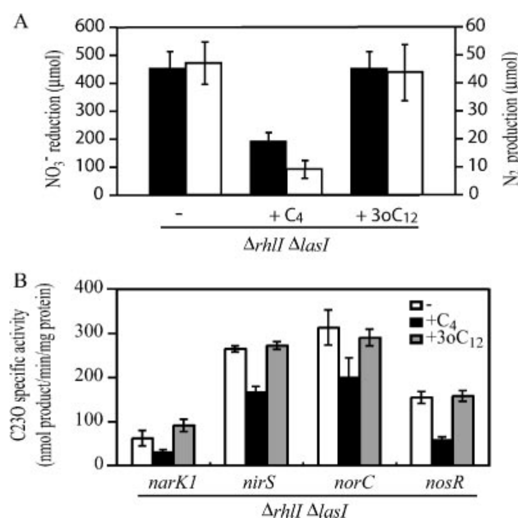

FIG. 4. Denitrification regulation by *rhl* and *las* quorum-sensing systems. (A) Effect of 10 μM C_4-HSL and 1 μM 3-oxo-C_{12}-HSL on a Δ*rhlI* Δ*lasI* double mutant. The amounts of NO_3^- reduced and N_2 produced over 12 h were measured. Solid bars, NO_3^- reduced; open bars, N_2 produced. Data are means ± standard deviations from three independent experiments. (B) Effects of 10 μM C_4-HSL and 1 μM 3-oxo-C_{12}-HSL on the transcription of denitrifying genes in a Δ*rhlI* Δ*lasI* mutant. C23O activity was measured in cells cultured for 6 h (late-exponential phase). Data are means ± standard deviations from three independent experiments.

the culture of a Δ*rhlI* Δ*lasI* double mutant. The amounts of NO_3^- reduced and N_2 produced during a 12-h incubation were measured. Addition of C_4-HSL decreased the denitrification activity of the Δ*rhlI* Δ*lasI* mutant; however, 3-oxo-C_{12}-HSL did not (Fig. 4A). To study whether denitrification is regulated at the transcriptional level, transcriptional fusion plasmids were constructed. During NO_3^- reduction to N_2, NAR, NO_2^- reductase (NIR), NO reductase (NOR), and N_2O 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*α 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 Δ*rhlI* Δ*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 Δ*rhlI* Δ*lasI* mutant transformed with pMEX9. Transcription of the denitrifying genes in the Δ*rhlI* Δ*lasI* mutant was repressed by C_4-HSL; however, 3-oxo-C_{12}-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_4-HSL and 3-oxo-C_{12}-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_3^--respiring activity was higher in the Δ*lasI* mutant than in the Δ*rhlI* 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_4-HSL and 3-oxo-C_{12}-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.

We thank Kiwamu Minamisawa, Yuichi Suwa, Sherry L. Kuchma, Daniel. P. MacEachran, and E. Peter Greenberg for helpful comments on the manuscript and Emiko Matsuzaka and Norihisa Okada for technical advice in determining denitrification activities. We also thank Michael A. Kertesz for providing the pME4510 plasmid and Herbert P. Schweizer for providing 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.

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