Effects of exogenous short-chain N-acyl homoserine lactone on denitrifying process of *Paracoccus denitrificans*

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ABSTRACT

N-acyl-homoserine lactones (AHLs) serve as quorum-sensing signals, which control a number of bacterial processes in many proteobacteria. Here we report the effects of exogenous short-chain AHL on the denitrifying process of *Paracoccus denitrificans*, which are capable of aerobic and anaerobic growth by utilizing nitrate. The denitrification activity of these cells was monitored by measuring denitrification products (including nitrate, nitrite, and nitrous oxide), and the individual messenger ribonucleic acid (mRNA) levels of nitrate, nitrite, nitric oxide and nitrous oxide reductases. The results indicated that 2 μmol/L C6-homoserine lactone (HSL) has little effect on cell density under either anaerobic or aerobic culture conditions, and the nitrate reduction activity appeared slightly affected by N-hexanoyl-DL-homoserine lactone (C6-HSL). However, exogenous C6-HSL significantly affected the transcription of nitrite reductase and nitric oxide reductase genes in *P. denitrificans* regardless of the presence of oxygen, and N_2O accumulation activity in *P. denitrificans* was suppressed by C6-HSL under aerobic condition. In contrast, exogenous C6-HSL stimulated the production of N_2O under anaerobic condition, suggesting that the regulation of denitrification by quorum sensing may be important in N_2O release.

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Introduction

Denitrification is the biological removal of nitrogen, from nitrate to nitrogen gas. A large number of denitrifying bacteria have been isolated from soil and activated sludge (Chèneby et al., 2000; Heylen et al., 2006). Normally, the denitrification process involves the enzymes nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR), and nitrous oxide reductase (NOS) (Zumft, 1997). It has been widely accepted that denitrification requires completely anaerobic conditions. However, recent studies have shown that denitrification can also occur in fully aerobic conditions (Chen et al., 2003, 2012; Zhu et al., 2012). The aerobic denitrification mechanism shows that co-respiration is an important mechanism, and oxygen and nitrate are simultaneously utilized as terminal electron acceptors in aerobic denitrification (Takaya et al., 2003; Zheng et al., 2011). Aerobic denitrifying species isolated from ponds, soils, and activated sludge mainly include *Pseudomonas* (Li et al., 2012), *Alcaligenes* (Joo et al., 2006), *Paracoccus* (Shi et al., 2013) and *Bacillus* (Zhang et al., 2012a), and are likely either aerobic or facultative aerobic, and heterotrophic. Most denitrifiers produce nitrous oxide (N_2O) instead of dinitrogen (N_2) under aerobic conditions (Takaya et al., 2003).

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Paracoccus denitrificans is a non-motile coccoid, Gram-negative bacterium and was first isolated from soil in 1910 by Martinus Beijerinck. Because of the similarity to eukaryotic mitochondria and nutritional versatility, it has been an important model organism for studies of electron transfer and energy conservation (Janzon et al., 2008; Stouthamer, 1991). *P. denitrificans* can grow in either aerobic or anaerobic conditions due to its ability to utilize both oxygen and nitrogenous oxides.

It has been widely accepted that bacteria can produce some special signal molecules spontaneously and regulate behaviors and metabolisms, such as luminescence, virulence factors and biofilm formation, a phenomenon termed quorum sensing (QS) by responding to the signal concentrations (Miller and Bassler, 2001; Waters and Bassler, 2005). In many Gram-negative bacteria, the QS system is driven by *N*-acyl-homoserine lactone (AHL) molecules. The most classic QS system is LuxI/LuxR-type regulatory system. AHL biosynthesis depends primarily on a synthase protein (I), and target genes are then activated via the interaction between the signal molecules and a response regulator protein (R) (Williams and Cámara, 2009). However, little is known about the signals and mechanisms in *P. denitrificans*, although recently some QS regulatory genes have been found and the QS signals have been characterized in this bacterium. It has been reported that *P. denitrificans* can synthesize long-chain AHL (C16-HSL) signals (Llamas et al., 2004; Schaefer et al., 2002).

Recent studies revealed that the denitrification activity in *Pseudomonas aeruginosa* is regulated by *N*-butyryl homoserine lactone (C4-HSL) and *N*-(3-oxododecanoyl) homoserine lactone (3-oxo-C12-HSL) produced by the *rhl* and *las* QS systems, respectively (Pesci et al., 1997). Regulation by the *las* QS system was dependent on the *rhl* QS system, indicating a hierarchical regulation from the *las* to the *rhl* QS systems in the regulation of denitrification. Wagner et al. (2003) also discovered that the transcription of denitrifying genes in *P. aeruginosa* PAO1 was changed by exogenous C4-HSL and 3-oxo-C12-HSL under aerobic and anaerobic conditions using DNA microarray analysis.

Thus far, however, the effects on denitrification of a non-original signal have not been reported in denitrifiers. In this study, we studied *P. denitrificans* responses to exogenous *N*-hexanoyl-DL-homoserine lactone (C6-HSL) in aerobic and anaerobic conditions in terms of growth and denitrification efficiency. The results demonstrated that exogenous C6-HSL significantly affected the transcription of NIR and NOS gene in *P. denitrificans* and N_2O accumulation activity in *P. denitrificans* was affected by C6-HSL.

1. Materials and methods

1.1. Bacterial strains and culture conditions

P. denitrificans used in this study were purchased from German Collection of Microorganisms and Cell Cultures (DSMZ). *P. denitrificans* were cultured in 100 mL of beef extract peptone medium (10 g/L peptone, 3 g/L beef extract, 5 g/L NaCl) supplemented with 1 g/L KNO_3 under aerobic conditions or 10 g/L KNO_3 under anaerobic condition with the pH adjusted to 7.0. C6-HSL was added to the culture at a final concentration

of 2 μmol/L. An equivalent volume of ethanol was added as a control. Experiments were performed in triplicate. Autoinducer *N*-hexanoyl-DL-homoserine lactone (C6-HSL) was obtained from the Sigma-Aldrich Corporation (Shanghai, China) and dissolved in ethanol.

1.2. Growth curves

P. denitrificans was inoculated with 1% cell suspension and grown aerobically or anaerobically at 30°C, with shaking continually at 150 r/min. The optical densities of the bacterial suspensions were measured spectrophotometrically at 600 nm (Beckman Coulter DU730 spectrophotometer A23616, Life Sciences Corporation, America). The *p* value of logarithmic growth phase data using first-order kinetics was calculated. The experiment was performed in triplicate and one-way analysis of variance (ANOVA) significance analysis was performed.

1.3. Measurement of NO_3^-, NO_2^- and N_2O concentration

Nitrate (NO_3^-) and nitrite (NO_2^-) concentrations were measured by anion chromatography method using ICS-1500 ion chromatography system (ICS-1500ICS, Dionex Corporation, America) (Zhang et al., 2012b). To obtain standard curves, NO_3^-- and NO_2^--mixed standard stock solutions were diluted with ddH_2O to reach concentrations of 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, and 10.0 mg/L and 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, and 1.0 mg/L, respectively. Samples were obtained from *P. denitrificans* culture supernatants and were filtered through a 0.45-μm membrane filter. A 9.0 mmol/L Na_2CO_3 solution was used for the mobile phase of the ion chromatography at a rate of 1.0 mL/min.

N_2O concentrations were detected by gas chromatography–mass spectrometry (GC–MS) (GC-2010 Plus Shimadu Corporation, Japan). Ten microliters of *P. denitrificans* gas samples harvested at exponential time (*P. denitrificans* cultured aerobically: 10, 12 and 16 hr; cultured anaerobically: 18, 24 and 36 hr) was injected and quantified in 2 min.

1.4. Ribonucleic acid (RNA) extraction and quantitative polymerase chain reaction (qPCR)

Total RNA was isolated from *P. denitrificans* in the logarithmic growth phase by using TRIzol Reagent (Life Technologies Inc., America). Bacterial cells were harvested by centrifugation of 1 mL at 10,000 r/min for 5 min. RNA extraction was performed according to the manufacturer's protocol. The amounts of RNA were determined using a Nanodrop spectrophotometer (Nanodrop2000, Thermo Fisher Scientific Inc., America). Complementary deoxyribonucleic acid (cDNA) was synthesized from 1-μg RNA samples by a reverse-transcription reaction proceeding for 15 min by using Prime Script RT reagent Kit with gDNA Eraser (TaKaRa Biotechnology, China).

Real-time PCR was performed on a CFX96™ Real-Time PCR system (CFX96 PCR System, Bio-rad Laboratories, USA) with SYBR Premix Ex Taq™ (TaKaRa Biotechnology, China). Each reaction was performed with a total reaction mixture of 25 μL containing 12.5 μL of SYBR Premix Ex Taq™, 10 μmol/L of each primer and 1 ng of cDNA. The relevant primers are listed in Table 1. The RT-PCR procedures were as follows: initial denaturation at 95°C for 3 min, followed by 39 cycles of 95°C

Table 1 – Primers used in the quantitative PCR.

Name	Gene	Sequence (5′ to 3′)
16SrRNA-F	16S	ACTCCTACGGGAGGCAGCAGT
16SrRNA-R	16S	TATTACCGCGGCTGCTGGC
Pden-I-5	Pden-I	ACTTTCCTTCGCCAACCTTCA
Pden-I-3	Pden-I	TGGGCATCGCGGATCATAT
Pden-R-5	Pden-R	GCGGCATGACTACCTCACCAAG
Pden-R-3	Pden-R	AACGGTATGCTCTGAAATCTCCA
Pden-NarG-5	narG	CTGGAACCTGGAGCAGAAGG
Pden-NarG-3	narG	GGCTGTAATTGGCGGCAGAA
Pden-NarI-5	narI	ACGCCGGTCGCCGTATT
Pden-NarI-3	narI	TTGCGCCCAGGACATCA
Pden-NirS-5	nirS	GACCTGATGGCGAACTACCTG
Pden-NirS-3	nirS	GCGGACATGCGGCTGATA
Pden-NorC-5	norC	GCAAGCATATCTGGGAACGC
Pden-NorC-3	norC	ATCCCGGTGGGCATGGATT
Pden-NosZ-5	nosZ	CGGAAAGAGGCAAGCAGC
Pden-NosZ-3	nosZ	TGTCGAGGAACAGCGAGGTAT

PCR: polymerase chain reaction.

for 3 min, 58°C for 30 s and 72°C for 1 min. Negative controls treated without cDNA templates were used to ensure that there was no contamination. Experiments were performed in triplicate.

1.5. Data analysis

All data were analyzed and graphed by Origin 9.0 (OriginLab Corporation). Significance analysis was completed in SPSS (Statistical Product and Service Solutions) using one-way ANOVA.

2. Results

2.1. Effect of C6-HSL on cell growth and denitrification activity

The growth curves of *P. denitrificans* under aerobic/anaerobic conditions appeared to follow a first-order kinetic reaction at short-term exponential time, which used to compare cells growth in C6-HSL addition group and control group (Tables S1 and S2). The turbidity at 600 nm of *P. denitrificans* anaerobic culture was approximately 50% lower than the aerobic culture when cells entered the stationary phase (Fig. 1). However, it indicated that there is no significant difference between C6-HSL addition group and control group in cell growth (Tables S1 and S2).

The amounts of NO_3^- and NO_2^- and N_2O produced during the logarithmic growth phase were measured. The NO_3^- and NO_2^- concentrations were measured by ion chromatography. The retention times of the NO_3^- and NO_2^- standard samples were 8.36 and 6.16 min, respectively. Standard curves derived from nitrate nitrogen and nitrite nitrogen characteristic peak areas were obtained, and their correlation coefficients were 99.27% and 98.64%, respectively.

It appeared that 1 g/L nitrate was completely converted to nitrite after 24 hr cultivation of *P. denitrificans* grown aerobically. In anaerobic conditions, the concentration of nitrate also decreased gradually as time went by, and almost 90% of the nitrate was consumed by *P. denitrificans* after 24 hr, despite a higher nitrate initial concentration. There was clearly little difference between the nitrate removal efficiency changes in the aerobic C6-HSL-treated group and the control group (Fig. 2a). No nitrite accumulation was detected in control samples at 9 hr; however, nitrite still existed at low concentrations in the C6-HSL-treated group at the same time, which suggests that the short acyl-chain AHL slowed nitrite reduction progress during this growth period. In anaerobic groups, approximately 90% of nitrate consumption occurred after 12 hr incubation, and the rate of nitrate consumption was suppressed in C6-HSL-treated group (Fig. 2b). However, nitrite accumulation was not affected when C6-HSL was added to the culture.

NO and N_2O are both intermediate products in the denitrification process. Most denitrifiers produce nitrous oxide (N_2O) instead of dinitrogen (N_2) under aerobic conditions (Wrage et al., 2001). Therefore, to further investigate the effect of QS on denitrification, the efficiency of N_2O production during the exponential growth phase was measured. The accumulation of N_2O was estimated relative to the standard curve formed by different concentration gradients of standard N_2O. The corresponding R^2 value of the standard curve was 0.999. *P. denitrificans* emissions of N_2O were regulated at two levels: N_2O production and reduction.
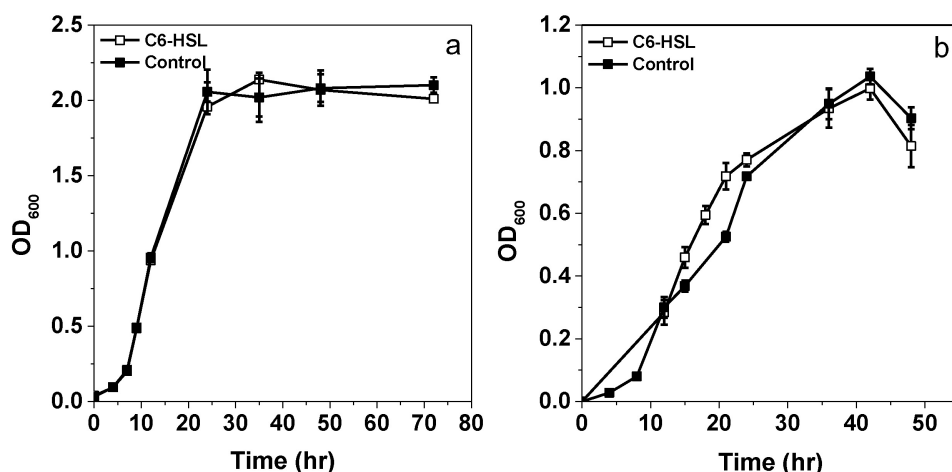

Fig. 1 – Growth curves of *P. denitrificans* under aerobic (a) and anaerobic (b) conditions. Black square, samples treated with 2 μmol/L C6-HSL; red circle, control group without AHLs. The experiment was performed in triplicate.
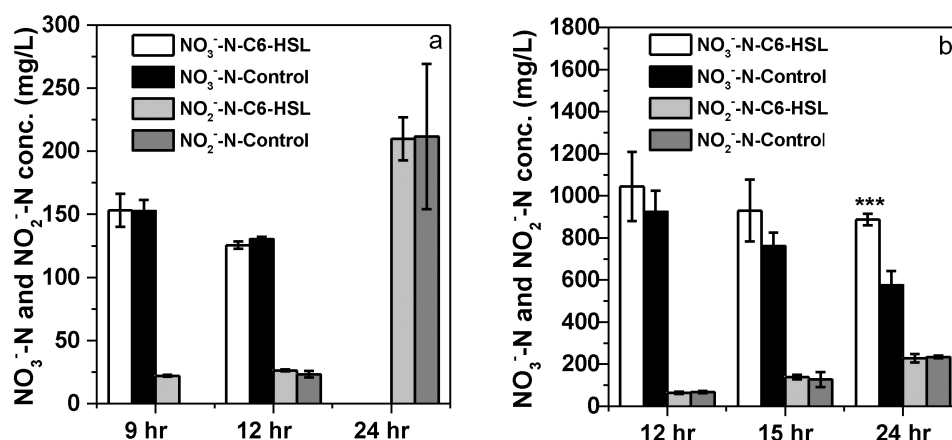
Fig. 2 – The effects of C6-HSL on nitrate/nitrite consumption of *P. denitrificans*. (a) Cells grew aerobically supplemented with 1 g/L KNO_3, NO_3^- and NO_2^- concentrations were measured at 9, 12, and 24 hr by ion chromatography; (b) Cells grew anaerobically supplemented with 10 g/L KNO_3, NO_3^- and NO_2^- concentrations were measured at 12, 15, and 24 hr. The experiment was performed in triplicate. Asterisks indicate a significant increase compared to the control in the same treatment period ($^{***}p < 0.01$).

In aerobic conditions, the maximum amounts of N_2O produced by the C6-HSL-treated group and the *P. denitrificans* control group were at 12 hr, with average values of 5092.4 and 5456.5 ppm, respectively, whereas maximum N_2O production was 1065.5 and 1196.2 ppm at 16 hr, respectively. Thus, it appeared that N_2O accumulated during the initial 12 hr, and then the consumption of N_2O continued in *P. denitrificans* culture. The relative rate of N_2O accumulation was severely reduced after treatment with 2 μmol/L C6-HSL from 10 to 12 hr (Fig. 3a). However, the nitrous oxide reduction rate was also significantly repressed by the exogenous AHL from 12 to 16 hr. The relative rate of N_2O production and reduction in the C6-HSL-treated group was 4.9- and 1.4-fold lower than that in control group, respectively.

Batch tests were performed for *P. denitrificans* under anaerobic conditions to observe cell growth, and anaerobic effects resulted in decreased growth rate compared to the aerobic environment (Fig. 1). The maximum amounts of N_2O produced by the C6-HSL-treated group and the control group were, at 36 hr, an average of 12,009.0 and 6972.7 ppm, respectively, whereas nitrous oxide was almost entirely consumed after 48 hr of culture. Therefore, N_2O accumulation during the logarithmic growth phase was measured during 18–36 hr in anaerobic conditions. In contrast to aerobic culture, the addition of 2 μmol/L C6-HSL significantly enhanced the relative rate of N_2O accumulation during 18–36 hr (Fig. 3b). The relative rate of N_2O production in the C6-HSL-treated group was 2.4- and 1.6-fold higher than that in the control group at mid-logarithmic phase, respectively.
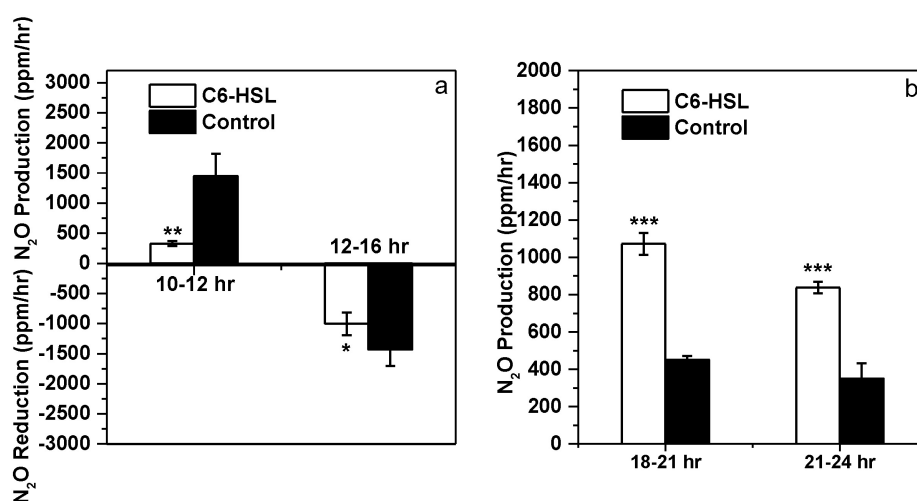

Fig. 3 – The effects of C6-HSL on N_2O production and reduction of *P. denitrificans* under aerobic (a) and anaerobic (b) conditions. (a) The relative rate of N_2O production in 2 hr from 10 to 12 hr, was measured. Concurrently, the N_2O reduction rate from 12 to 16 hr was calculated; (b) The relative rate of N_2O production during 18 to 36 hr was measured. C6-HSL was added to the medium at a final concentration of 2 μmol/L. The data are the mean values and standard deviations from three parallel experiments. The standard deviations are from three parallel experiments. Asterisks indicate a significant increase compared to the control in the same treatment period ($^*p < 0.1$, $^{**}p < 0.05$, $^{***}p < 0.01$).

2.2. Transcriptional regulation of denitrification by C6-HSL

To study whether denitrification is regulated at the transcriptional level, an expression assay of genes encoding NAR, NIR, NOR and NOS was constructed. Moreover, the expression of potential I/R genes was also examined in C6-HSL-treated samples. The $2^{-\Delta\Delta Ct}$ method was used to calculate cycle threshold (CT) value data from PCR (Livak and Schmittgen, 2001). The standard deviation was determined from three independent experiments. Transcription of the denitrifying genes in the C6-HSL-treated sample was repressed in aerobic conditions; however, C6-HSL had little effect on QS I/R genes transcription (Fig. 4a). It is noteworthy that the relative levels of *nirS*, *norC* and *nosZ* mRNA in the C6-HSL group were repressed to almost 50% compared with the control group (Fig. 4a). The repression of the *nirS*, *norC* and *nosZ* genes by short acyl-chain AHL is in accordance with a previous result, which showed that N_2O production and reduction processes were repressed by exogenous C6-HSL. Interestingly, in a striking contrast, approximately 2.74- and 2.65-fold more *nirS* and *norC* were expressed in C6-HSL-treated groups than in control groups under anaerobic conditions, respectively (Fig. 4b). The addition of synthetic AHLs significantly influenced the N_2O production rate under anaerobic conditions. C6-HSL also improved the expression *narI* gene, although an inconsistent regulatory pattern was observed with nitrate consumption under anaerobic growth (Fig. 2b).

3. Discussion

In this study, we added exogenous *N*-hexanoyl-DL-homoserine lactone (C6-HSL) into *P. denitrificans* to investigate the growth and metabolism response under aerobic and anaerobic condition. We have discovered that 2 μmol/L C6-HSL had little effect on cell density, and there were few differences between the C6-HSL-treated group and the control group in nitrate removal under aerobic conditions. However, in anaerobic groups, the rate of nitrate consumption was suppressed due to the addition of AHL (Fig. 2b). In contrast, the *narI* mRNA relative amount in C6-HSL group was increased compared with control group. Nar initiates a complete denitrification pathway, and four genes of the *nar* operon (*narGHJI*) encode the three classic subunits of the heterotrimeric NAR (NarG, NarH, and NarI) and the corresponding chaperone required for its maturation (NarJ) (Doi et al., 2009; Zumft, 1997). The transcription of *narI* was upregulated by the presence of the AHL signal, whereas the differential expression of *narG* was not found to be statistically significant in *P. denitrificans* anaerobic cultures. In contrast to the rate of nitrate reduction, the opposite result was obtained (Fig. 4b) from two genes involved in coding NARs, suggesting that the AHL regulation mechanism was likely related to other nitrogen and energy metabolism pathways.

It should be noted that nitrous oxide production and reduction activities in *P. denitrificans* were regulated by C6-HSL regardless of the presence of oxygen. The amounts of nitrous oxide produced and reduced during a 36-hr incubation were measured. The relative rate of N_2O accumulation and reduction was repressed by the exogenous AHL in aerobic culture, but the addition of C6-HSL continuously enhanced the relative rate of N_2O accumulation in the mid-logarithmic phase. This was consistent with fluctuation in the transcription of genes responsible for converting nitrite (NO_2^-) to nitrous oxide (N_2O) and N_2O to dinitrogen gas (N_2), compared with the control group under these conditions. The addition of C6-HSL decreased the denitrification activity of *P. denitrificans* under aerobic conditions, in contrast to anaerobic conditions. Our results indicate that the short acyl-chain AHL signal alone was sufficient to induce the expression of many enzymes involved in denitrification regardless of the presence or absence of oxygen. NIR, NOR and NOS were regulated markedly at the transcriptional level by the AHL signal, suggesting that the regulation of denitrification by QS was important in the sequential induction of NIR, NOR and NOS activity, causing the accumulation and consumption of N_2O.
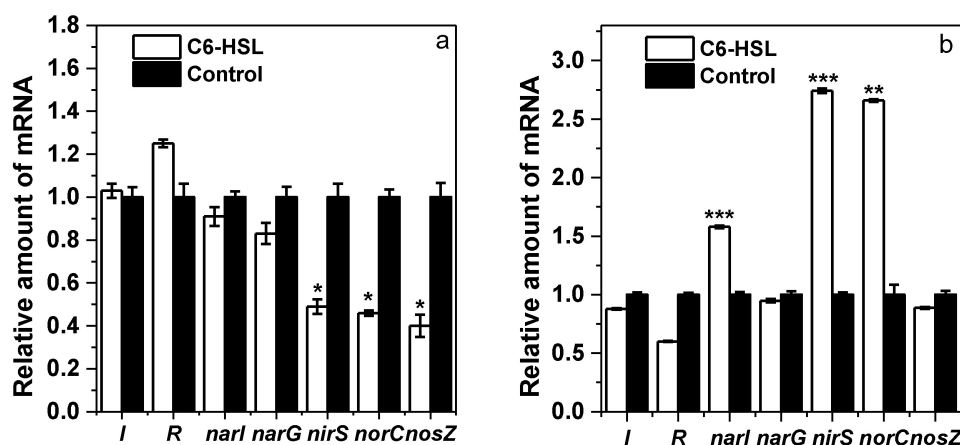

Fig. 4 – The effects of C6-HSL on the transcription of *Pden-I(I)*, *Pden-R(R)*, *narI*, *narG*, *nirS*, *norC* and *nosZ* genes in *P. denitrificans* under aerobic (a) and anaerobic (b) conditions. C6-HSL was added to the medium at final concentration of 2 μmol/L. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method. Three replicate samples were analyzed, and error bars represent the standard errors. Asterisks indicate a significant increase compared to the control in the same treatment period ($^*p < 0.1$, $^{**}p < 0.05$, $^{***}p < 0.01$).

From these findings, it can be presumed that QS systems have ecologically important roles in addition to the control of denitrification process for the denitrifying bacterium (Salles et al., 2009; Toyofuku et al., 2007). Wagner et al. (2003) found that exogenous C4-HSL and 3-oxo-C12-HSL affect the transcription of denitrifying genes in *P. aeruginosa* PAO1 under aerobic conditions using DNA microarray analysis. Furthermore, it was found by Toyofuku et al. (2007) that NO_3^- reduction and N_2 production were decreased after the addition of C4-HSL to QS mutants in *P. aeruginosa* PAO1, which indicated that denitrification was controlled by *rhl* QS in *P. aeruginosa*. Recently, a third intercellular signal molecule, the *Pseudomonas* quinolone signal (PQS) was found to regulate the denitrification process by iron chelation and to be related to oxygen respiration (Toyofuku et al., 2008). Our results have arrived at the similar conclusion that denitrification was repressed by short acyl-chain AHL in the presence of oxygen. However, unlike *P. aeruginosa* PAO1, it has been reported that *P. denitrificans* can synthesize the long-chain AHL (C16-HSL) signal instead of short acyl-chain AHL. Thus, we cannot rule out the possibility that the denitrification activity of *P. denitrificans* might be regulated by a short-chain autoinducer produced by other microorganisms in the natural environment. It has been reported that *P. aeruginosa* PQS repressed the growth of other bacterial species, including both Gram-negative and Gram-positive bacteria, which demonstrated that it may influence the development of bacterial communities by regulating bacterial growth (Toyofuku et al., 2010). These studies, along with our data, suggest that AHLs may regulate coordinated behaviors in different bacteria to balance nitrogen cycling under natural conditions. Whether QS effects confer a broad regulation to *P. denitrificans* remains to be investigated.

4. Conclusions

In this study, 2 μmol/L exogenous C6-HSL repressed the denitrifying process of *P. denitrificans* under aerobic culture conditions; however, in contrast, C6-HSL enhanced its anaerobic denitrifying process. The effects of C6-HSL on N_2O accumulation activity were especially prominent. We also found that the QS system gene *Pden-R* was affected simultaneously by exogenous signaling molecules, which suggested QS regulation may be important in the process of denitrification. By regulation of the key enzyme gene expression, such as *nirS*, *norB*, *norC* and *nosZ*, QS influences the enzymes activities of NAR, NIR, NOR, and NOS and eventually affects the transformation of nitrite to nitric oxide and its reversion to nitrous oxide gas. One of major findings of the present study was suggesting that the exit of cell-to-cell communication between bacteria in nitrogen cycles was driven by AHL molecules. Our results also provided a method to control the process of denitrification by *P. denitrificans,* and this may play an important role in the application of wastewater treatment system. However, the molecular mechanisms underlying C6-HSL regulation of the denitrifying process remain unclear. In future studies, we will try to focus on the molecular mechanisms of regulation of denitrification by C6-HSL and other types of AHLs in *P. denitrificans*.

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