

## ORIGINAL ARTICLE

# Denitrification versus respiratory ammonification: environmental controls of two competing dissimilatory $\text{NO}_3^-/\text{NO}_2^-$ reduction pathways in *Shewanella loihica* strain PV-4

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**Denitrification and respiratory ammonification are two competing, energy-conserving  $\text{NO}_3^-/\text{NO}_2^-$  reduction pathways that have major biogeochemical consequences for N retention, plant growth and climate. Batch and continuous culture experiments using *Shewanella loihica* strain PV-4, a bacterium possessing both the denitrification and respiratory ammonification pathways, revealed factors that determine  $\text{NO}_3^-/\text{NO}_2^-$  fate. Denitrification dominated at low carbon-to-nitrogen (C/N) ratios (that is, electron donor-limiting growth conditions), whereas ammonium was the predominant product at high C/N ratios (that is, electron acceptor-limiting growth conditions). pH and temperature also affected  $\text{NO}_3^-/\text{NO}_2^-$  fate, and incubation above pH 7.0 and temperatures of 30 °C favored ammonium formation. Reverse-transcriptase real-time quantitative PCR analyses correlated the phenotypic observations with *nirK* and *nosZ* transcript abundances that decreased up to 1600-fold and 27-fold, respectively, under conditions favoring respiratory ammonification. Of the two *nrfA* genes encoded on the strain PV-4 genome, *nrfA*<sub>0844</sub> transcription decreased only when the chemostat reactor received medium with the lowest C/N ratio of 1.5, whereas *nrfA*<sub>0505</sub> transcription occurred at low levels ( $\leq 3.4 \times 10^{-2}$  transcripts per cell) under all growth conditions. At intermediate C/N ratios, denitrification and respiratory ammonification occurred concomitantly, and both *nrfA*<sub>0844</sub> (5.5 transcripts per cell) and *nirK* (0.88 transcripts per cell) were transcribed. Recent findings suggest that organisms with both the denitrification and respiratory ammonification pathways are not uncommon in soil and sediment ecosystems, and strain PV-4 offers a tractable experimental system to explore regulation of dissimilatory  $\text{NO}_3^-/\text{NO}_2^-$  reduction pathways.**

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## Introduction

Phylogenetically diverse groups of microorganisms use the nitrogen (N) oxyanions nitrate ( $\text{NO}_3^-$ ) and nitrite ( $\text{NO}_2^-$ ) as terminal electron acceptors in anoxic environments (Sørensen, 1978; Tiedje *et al.*, 1982; Zumft, 1997; Burgin and Hamilton, 2007). During denitrification,  $\text{NO}_3^-$  is reduced to the

gaseous products, nitrous oxide ( $\text{N}_2\text{O}$ ) and dinitrogen gas ( $\text{N}_2$ ), in a step-wise manner via  $\text{NO}_2^-$  and nitric oxide (NO) as intermediates (Zumft, 1997; Burgin and Hamilton, 2007).  $\text{N}_2\text{O}$  and  $\text{N}_2$  release to the atmosphere causes N loss from terrestrial and aquatic environments, and  $\text{N}_2\text{O}$  is an ozone-depleting greenhouse gas (Lashof and Ahuja, 1990; Ravishankara *et al.*, 2009). Alternatively, many microbes reduce  $\text{NO}_3^-$  via respiratory ammonification (also referred to as dissimilatory nitrate reduction to ammonium), a pathway that shares the  $\text{NO}_3^-$  to  $\text{NO}_2^-$  reaction step with denitrification but reduces  $\text{NO}_2^-$  to  $\text{NH}_4^+$  (Tiedje *et al.*, 1982; Silver *et al.*, 2001, 2005; Templer *et al.*, 2008; Simon and Klotz, 2013). In contrast to  $\text{NO}_3^-$  and  $\text{NO}_2^-$ ,

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positively charged ammonium ( $\text{NH}_4^+$ ) is retained in soils and sediments and has a higher tendency for incorporation into microbial or plant biomass (Laima *et al.*, 1999; Silver *et al.*, 2001; Fitzhugh *et al.*, 2003). Hence, the relative contributions of denitrification versus respiratory ammonification activities have important consequences for N retention, plant growth and climate.

Because of the biogeochemical consequences of denitrification versus respiratory ammonification, many studies aimed at elucidating the environmental controls of the two competing  $\text{NO}_3^-/\text{NO}_2^-$  reduction pathways (Tiedje *et al.*, 1982; Nägele and Conrad, 1990; Ogilvie *et al.*, 1997; Fazzolari *et al.*, 1998; Stevens *et al.*, 1998; Silver *et al.*, 2001; Nizzoli *et al.*, 2010; Dong *et al.*, 2011). Based on empirical observations, Tiedje *et al.* (1982) suggested that the carbon-to-nitrogen (C/N) ratio regulates  $\text{NO}_3^-$  fate. Theoretically, denitrification yields more free energy per electron but respiratory ammonification yields more free energy per molecule of  $\text{NO}_3^-$  reduced than complete denitrification to  $\text{N}_2$  (Strohm *et al.*, 2007). Pure culture growth experiments demonstrated that heterotrophic denitrifier biomass yields were lower than those obtained with organisms performing respiratory ammonification, suggesting more efficient energy conservation in the latter process (Strohm *et al.*, 2007). Therefore, it is sensible that denitrification occurs under electron donor-limiting conditions (that is, low C/N ratios), whereas respiratory ammonification is preferred under N oxyanion-limiting conditions (that is, high C/N ratios). In support of this hypothesis, Fazzolari *et al.* (1998) demonstrated that high glucose-to- $\text{NO}_3^-$  ratios increased  $\text{NH}_4^+$  and lowered  $\text{N}_2\text{O}$  production. Nijburg *et al.* (1997) observed that increased  $\text{NO}_3^-$  loading favored denitrifying bacteria, and Schmidt *et al.* (2011) observed a strong correlation between C/N ratio and respiratory ammonification activity in arable soils; however, other studies failed to establish a relationship between C/N ratios and  $\text{NO}_3^-$  fate (Kelso *et al.*, 1997; Stevens *et al.*, 1998). Furthermore, the effects of varying C/N ratios on the expression of key genes implicated in denitrification and respiratory ammonification are unclear.

The C/N ratio is not the only environmental parameter hypothesized to affect the environmental fate of  $\text{NO}_3^-/\text{NO}_2^-$ . Increased temperature has been linked with elevated respiratory ammonification activity (Ogilvie *et al.*, 1997; Silver *et al.*, 2001; Tomaszek and Rokosz, 2007; Nizzoli *et al.*, 2010; Dong *et al.*, 2011). pH also influenced the relative contributions of denitrification versus respiratory ammonification, although with no consistent patterns. Stevens *et al.* (1998) observed significantly higher respiratory ammonification activity in a surface water gley soil at pH 8.0 than at pH 6.5 and 6.0, whereas Nägele and Conrad (1990) reported elevated  $\text{NH}_4^+$  production under acidic conditions. Many studies have explored how microbial community composition and geochemical parameters

affect the fate of  $\text{NO}_3^-/\text{NO}_2^-$  via the two competing dissimilatory pathways but these efforts yielded inconsistent results and have led to conflicting conclusions.

Until recently, the general understanding had been that denitrification and respiratory ammonification pathways do not coexist within a single organism. This apparent pathway incompatibility limited experimental designs exploring the environmental factors controlling dissimilatory  $\text{NO}_3^-/\text{NO}_2^-$  reduction pathways to mixed cultures, or, at best, co-cultures of microorganisms performing either pathway (Rehr and Klemme, 1989). Because of organism-specific characteristics in terms of growth kinetics and growth yields under different cultivation conditions, experiments aimed at delineating conditions that favor denitrification over respiratory ammonification, or vice versa, yielded inconclusive results. *Opitutus terrae*, *Marivirga tractuosa* and *Shewanella loihica* possess the complete sets of genes encoding both pathways and 16S rRNA gene surveys, as well as metagenomic analyses, suggested that bacteria harboring the pathways for denitrification and respiratory ammonification might not be rare in the environment (Hengstmann *et al.*, 1999; Sanford *et al.*, 2012; Mania *et al.*, 2014). *S. loihica* strain PV-4 possesses two copies of *nrfA*, as well as the complete suite of genes encoding denitrification enzymes (*nirK*, *norB* and *nosZ*) (Sanford *et al.*, 2012; Yoon *et al.*, 2013). Inconsistent with the genome information, *S. loihica* was initially characterized as a non- $\text{NO}_3^-/\text{NO}_2^-$ -reducing bacterium (Gao *et al.*, 2006) but a recent study demonstrated growth via denitrification (Yoon *et al.*, 2013). Here, we demonstrate that strain PV-4 also performs respiratory ammonification and provide evidence that a single organism can reduce  $\text{NO}_3^-/\text{NO}_2^-$  to  $\text{NH}_4^+$  and/or  $\text{N}_2\text{O}/\text{N}_2$ . Using batch and continuous (chemostat) cultures of *S. loihica* strain PV-4, the effects of C/N ratio, pH and temperature on the selection of a dissimilatory  $\text{NO}_3^-/\text{NO}_2^-$  reduction pathway were explored.

## Materials and methods

### Media and culture conditions

For batch experiments with *S. loihica* strain PV-4, completely synthetic, anoxic, phosphate-buffered basal salt medium was prepared as previously described (Yoon *et al.*, 2013). The medium (100 ml) was distributed to 160 ml serum bottles using the Hungate technique, and the bottles were sealed with black butyl-rubber stoppers (Geo-Microbial Technologies, Inc., Ochelata, OK, USA). After autoclaving, vitamins (Wolin *et al.*, 1964) were added from a degassed and filter-sterilized 200-fold concentrated stock solution. Electron donor (varying amounts of lactate), electron acceptors (varying amounts of  $\text{NO}_3^-$ ) and  $\text{NH}_4^+$  (0.1 mM) were added from sterilized and degassed stock solutions. Sodium nitrate

( $\geq 99\%$ , Fisher Scientific, Pittsburg, PA, USA) and ammonium chloride ( $\geq 99\%$ , Fisher Scientific) stock solutions were prepared in distilled water at concentrations of 0.1 M. Sodium lactate stock solutions (0.5 M) were prepared from a 60% lactate syrup (Sigma-Aldrich, St Louis, MO, USA). The initial substrate concentrations and the medium pH varied depending on the objectives of each experiment (see below). Early stationary phase *S. loihica* strain PV-4 cultures grown with 2.0 mM lactate and 1.0 mM  $\text{NO}_3^-$  served as inocula (0.5%, vol/vol) after  $\text{NO}_3^-$  and  $\text{NO}_2^-$  were depleted. The sum of the amounts of lactate and acetate transferred with the inocula to the fresh medium did not exceed 0.01  $\mu\text{mol}$ . To inhibit nitrous oxide reductase (NosZ) activity and measure  $\text{N}_2\text{O}$  as a proxy for denitrification activity, 6 ml  $\text{N}_2$  headspace was replaced with acetylene gas (99.6%, Airgas, Knoxville, TN, USA) (Yoshinari *et al.*, 1977). Unless mentioned otherwise, all batch experiments were performed at room temperature (21 °C). The medium for the chemostat experiment was slightly modified and used higher phosphate (25 mM) and ammonium chloride (0.5 mM) concentrations to increase the buffering capacity and provide sufficient N for assimilation and cell growth. To prevent precipitate formation, trace metals were added to the chemostat from a degassed and autoclaved 200-fold concentrated stock solution.

#### Analytical procedures

The Dionex ICS-2100 system (Sunnyvale, CA, USA) was used to measure  $\text{NO}_3^-$  and  $\text{NO}_2^-$  and the Dionex ICS-1100 system was used to measure  $\text{NH}_4^+$  concentrations (Yoon *et al.*, 2013). Lactate and acetate were quantified using an Agilent 1200 Series high-performance liquid chromatography system (Palo Alto, CA, USA). For  $\text{N}_2\text{O}$  measurements, 1 ml of headspace gas was collected for analysis with an Agilent 3000A MicroGC. Aqueous concentrations of  $\text{N}_2\text{O}$  were calculated using a dimensionless Henry's constant for a temperature of 21 °C that was corrected for the medium's ionic strength to 1.751 (Schumpe *et al.*, 1982; Schumpe, 1993; Sander, 1999).  $\text{N}_2\text{O}$  measurements for the culture bottles incubated at 30 °C and 37 °C were made after the bottles had been equilibrated to room temperature (21 °C). Biomass estimates assumed a mass of  $2.77 \times 10^{-13}$  g for a single strain PV-4 cell (Yoon *et al.*, 2013). Cell numbers were calculated from quantitative real-time PCR (qPCR) enumeration of 16S rRNA genes corrected for the presence of nine 16S rRNA gene operons on the *S. loihica* strain PV-4 genome (NCBI Reference Sequence: NC\_009092). The C and N content calculations used the empirical formula  $\text{C}_5\text{H}_7\text{O}_2\text{N}$  for biomass.

#### Batch experiments

The effects of the C/N ratio (the ratio of C atoms in the electron donor to N atoms in electron acceptor), pH and temperature were explored in batch systems.

To examine the effect of C/N ratios, the 160 ml culture vessels were amended with 0.2 mM sodium nitrate and varying concentrations of sodium lactate (0.1, 0.2, 0.5, 1.0, 2.0 and 10.0 mM). The vessels were incubated at room temperature (21 °C) without shaking. Liquid (1.5 ml) and headspace samples (1 ml) were withdrawn immediately after inoculation and after 5 days when  $\text{NO}_3^-$  reduction was complete.

For the pH experiments, 5.0 mM potassium phosphate or 20 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) buffers were used. The ratios of monobasic potassium phosphate ( $\text{KH}_2\text{PO}_4$ ) (Fisher Scientific, St Louis, MO, USA) and dibasic potassium phosphate ( $\text{K}_2\text{HPO}_4$ ) (JT Baker, Phillipsburg, NJ, USA) were varied to achieve pH values of 6.0, 6.5, 7.0, 7.5 and 8.0. If necessary, 10.0 M HCl or 5.0 M NaOH solutions were used to adjust the medium pH to the desirable values. The Tris buffer was adjusted to pH values of 8.0, 8.5 and 9.0 with 10.0 M HCl. The degassed medium was dispensed under a stream of nitrogen gas and autoclaved. The pH measurements at the conclusions of the experiments verified that the pH values remained unchanged during the incubation period. Temperature effects were examined by incubating culture vessels amended with 5.0 mM sodium lactate and 1.0 mM sodium nitrate at 21 °C, 30 °C and 37 °C. All batch experiments were performed in triplicate and repeated in at least one independent experiment to verify reproducibility.

#### Chemostat experiments

Continuous culture experiments were performed in an anoxic chemostat reactor (DS0200TBSC, DASCIP, Jülich, Germany). The total capacity of the reactor was 475 ml and the volume of the aqueous phase was maintained at 200 ml. A syringe pump (PHD 2000, Harvard Apparatus, Holliston, MA, USA) was employed to simultaneously deliver fresh medium and remove bioreactor waste at a constant rate of 20 ml  $\text{h}^{-1}$  that equals a dilution rate of 0.1  $\text{h}^{-1}$ . The headspace of the bioreactor and the influent medium was constantly flushed with  $\text{N}_2$  to maintain anoxic conditions. The medium was prepared as described above in 2 l glass bottles that were subsequently connected to the chemostat influent (Supplementary Figure S1). C/N ratios of 1.5, 3.0, 4.5, 6.0 and 7.5 were established by amending the medium with 1, 2, 3, 4 or 5 mM lactate and 2 mM  $\text{NO}_3^-$  as the electron acceptor. To initiate each chemostat experiment, 2 mM lactate, 1 mM  $\text{NO}_3^-$ , the vitamin solution and trace metal solution were added to the autoclaved reactor containing 200 ml of medium. The reactor vessel was flushed with  $\text{N}_2$  for 1 h before inoculation with *S. loihica* strain PV-4. Following a 2-day incubation period, the syringe pump was turned on, and the bioreactor was continuously stirred to evenly distribute substrates and cells. Constant concentrations of  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ , lactate and acetate over a 6-h time interval indicated

steady-state conditions that were reached in 72 h for all experimental conditions tested. Under steady-state reactor operating conditions, the cell density and the concentrations of substrates and products remained constant over time. Lactate, acetate,  $\text{NO}_3^-$ ,  $\text{NO}_2^-$  and  $\text{NH}_4^+$  concentrations were measured in 1.5 ml samples collected from the bioreactor. After each sampling event, the valve controlling the outflow was closed for 4.5 min to adjust the aqueous volume to 200 ml. To quantify denitrification activity, the valves controlling the flow of  $\text{N}_2$  gas were closed and 10% of the headspace gas was replaced with acetylene. Headspace  $\text{N}_2\text{O}$  concentrations were measured every 30–40 min to determine  $\text{N}_2\text{O}$  production rates. The loss of dissolved  $\text{N}_2\text{O}$  from the reactor vessel via the effluent was calculated by integrating the  $\text{N}_2\text{O}$  effluent rate (aqueous  $\text{N}_2\text{O}$  concentration multiplied by the flow rate) over time. The  $\text{N}_2\text{O}$  production rate was corrected by this  $\text{N}_2\text{O}$  loss rate to calculate the actual  $\text{N}_2\text{O}$  production rate. After sampling, the reactor operation continued overnight before a 15-ml sample was collected for gene expression analyses. After sampling, the effluent valve was closed for 45 min to adjust the reactor volume to 200 ml. The measurements of steady-state lactate, acetate,  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ , and  $\text{NH}_4^+$  concentrations, as well as  $\text{N}_2\text{O}$  production rates, were repeated 24 h following the initial sampling event.

**RNA extraction, purification and reverse transcription**  
Biomass for RNA extraction was collected from steady-state chemostat reactors operated under different feeding regimes (that is, C/N ratios of 1.5, 3.0, 4.5, 6.0 and 7.5; fumarate as electron acceptor). Sample aliquots (0.5 ml) were immediately mixed with 1.0 ml of RNA Protect Bacteria Reagent (Qiagen, Germantown, MD, USA), centrifuged for 10 min at  $5000 \times g$  and stored at  $-80^\circ\text{C}$  after the supernatant had been removed. Total RNA was extracted from the frozen samples within 1 week of sampling using an established protocol (Amos *et al.*, 2008) with the following modifications. Luciferase control mRNA (Promega, Madison, WI, USA) was diluted to  $10^{10}$  copies per ml and 1  $\mu\text{l}$  of the diluted control mRNA was added as an internal standard to account for RNA loss during the extraction and purification process (Amos *et al.*, 2008; Ritalahti *et al.*, 2010). Cell pellets were then suspended in 350  $\mu\text{l}$  of buffer RLT provided with RNeasy Mini Kit (Qiagen). The cell suspensions were transferred to 2 ml safe-lock tubes containing 50 mg of 200  $\mu\text{m}$  zirconium beads (OPS Diagnostics, Lebanon, NJ, USA) and disrupted with the Omni Bead Ruptor 24 Homogenizer (Omni, Kennesaw, GA, USA) at  $5.65 \text{ m s}^{-1}$  for 5 min. After a brief 10-s centrifugation step ( $16\,000 \times g$ ), the supernatants of each tube were transferred to new 1.5 ml tubes. The RNeasy Mini Kit (Qiagen) was then used following the manufacturer's recommendations to obtain the

RNA in a final volume of 60  $\mu\text{l}$  RNase-free water. The RNase-free DNase Set Kit (Qiagen) was used to remove any residual DNA. To 60  $\mu\text{l}$  of the RNA solution, 10  $\mu\text{l}$  of buffer RDD, 26.5  $\mu\text{l}$  of RNase-free water and 3.5  $\mu\text{l}$  of DNase I stock solution (all supplied with the Set Kit) were added. The reaction was incubated at room temperature for 15 min. After digestion, RNA was purified using the RNA MinElute Kit (Qiagen) according to the Qiagen protocol. The final step used 20  $\mu\text{l}$  of nuclease-free water to elute the RNA from the column. Reverse transcription was performed with Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). One microliter of 10 mM dNTP mix (Invitrogen) and 1  $\mu\text{l}$  of random hexamers (Invitrogen) diluted to  $50 \text{ ng } \mu\text{l}^{-1}$  were added to 11  $\mu\text{l}$  of the purified RNA solution. After incubation at  $65^\circ\text{C}$  for 5 min, the reaction vials were immediately placed on ice for 1 min. Then, 4  $\mu\text{l}$  of fivefold concentrated first-strand buffer, 1  $\mu\text{l}$  of 0.1 M dithiothreitol and 1  $\mu\text{l}$  of RNaseOUT ( $40 \text{ U } \mu\text{l}^{-1}$ ; Invitrogen) were added. After incubation at room temperature for 2 min, 1  $\mu\text{l}$  of Superscript III Reverse Transcriptase ( $200 \text{ U } \mu\text{l}^{-1}$ ) was added. The mixture was incubated at  $25^\circ\text{C}$  for 10 min, at  $42^\circ\text{C}$  for 3 h and at  $72^\circ\text{C}$  for 15 min (Ritalahti *et al.*, 2010). Then, 1  $\mu\text{l}$  of RNase H ( $2 \text{ U } \mu\text{l}^{-1}$ ; Invitrogen) was added to remove remaining RNA during a 20-min incubation period at  $37^\circ\text{C}$ .

#### Genomic DNA extraction

For DNA extraction, the biomass from 1.5 ml aliquots was collected by centrifugation at  $16\,000 \times g$  for 5 min at room temperature, and the cell pellets were immediately stored at  $-80^\circ\text{C}$ . Genomic DNA was extracted from *S. loihica* strain PV-4 cell pellets using the DNeasy Blood and Tissue Kit (Qiagen) as previously described (Yoon *et al.*, 2013) and quantified spectrophotometrically with a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

#### Quantitative real-time PCR

Primer sets targeting the *S. loihica* strain PV-4 16S rRNA, *nirK*, *nosZ* and *nrFA* genes were designed using Primer3 software (Rozen and Skaletsky, 2000) (Table 1). *In silico* PCR (Bikandi *et al.*, 2004) and Primer-BLAST (Ye *et al.*, 2012) were used to assess primer specificity. *S. loihica* strain PV-4 harbors two nonidentical *nrFA* gene copies, *nrFA*<sub>0505</sub> (Shew\_0505) and *nrFA*<sub>0844</sub> (Shew\_0844). The translated proteins share 44% amino acid identity, and both genes/transcripts were targeted in this study. These targets were amplified using complementary DNA (cDNA) and genomic DNA as templates and quantified using Power SYBR Green detection chemistry (Life Technologies, Carlsbad, CA, USA) and the ABI ViiA7 real-time PCR system (Life Technologies) using default parameters (Yoon *et al.*, 2013). PCR amplicons obtained with these

**Table 1** Primers used for RT-qPCR analyses and qPCR calibration curve parameters

Primer set	Target gene (locus tag)	Amplicon length (bp)	Slope	y-Intercept	Amplification efficiency	R <sup>2</sup>	Reference
SlonirK853f: 5'-AAGGTGGGTGAGTCTGTGCT-3'	<i>nirK</i> (Shew_3335)	188	-3.526	35.565	92.1	0.999	This study
SlonirK1040r: 5'-GGCTGGCGGAAGGTGTAT-3'							
SlonrfA1083f: 5'-GGATATCCGTCACGCTCAAT-3'	<i>nrfA</i> (Shew_0844)	226	-3.384	34.555	97.5	0.998	Yoon <i>et al.</i> (2013)
SlonrfA1308r: 5'-GTCATACCCAATGCAGCTT-3'							
SlonrfA818f: 5'-AGGGCAAGGCCTATACCAAC-3'	<i>nrfA</i> (Shew_0505)	149	-3.332	35.975	99.6	0.996	This study
SlonrfA950r: 5'-TTCTCGGCTATCTGCGACTT-3'							
SlonosZ599f: 5'-ATGGTAAGGAGACGCTGGAA-3'	<i>nosZ</i> (Shew_3400)	160	-3.515	34.451	92.3	0.996	This study
SlonosZ758r: 5'-TTGTAGCAGGTAGAGGGCAAG-3'							
Slo16Sf: 5'-CACACTGGGACTGAGACACG-3'	16S rRNA genes <sup>a</sup>	191	-3.380	34.240	97.6	0.991	This study
Slo16Sr: 5'-TGCTTCTTCTGCGAGTAACG-3'							
lucrefA: 5'-TACAACACCCCAACATCTTCGA-3'	<i>luciferase</i> control mRNA	67	-3.380	36.817	97.6	0.999	Johnson <i>et al.</i> (2005)
lucrefB: 5'-GGAAGTTCACCGCGTCAT-3'							

Abbreviation: RT-qPCR, reverse-transcriptase real-time quantitative PCR.

<sup>a</sup>*Shewanella loihica* strain PV-4 possesses nine 16S rRNA gene copies that all amplified with the Slo16Sf and Slo16Sr primer sets, as suggested by *in silico* PCR (Bikandi *et al.*, 2004).

primers were inserted into the PCR2.1 vector using the TOPO TA Cloning Kit (Invitrogen). The plasmids were extracted with the QIAprep Spin Miniprep Kit (Qiagen) and used to construct qPCR calibration curves (Table 1). Triplicate serial 10-fold dilutions yielded 10<sup>8</sup> to 10<sup>2</sup> target genes per µl and were used as template DNAs in qPCR assays. Using the default instrument parameters and automated C<sub>q</sub> determination, standards resulted in linear calibration curves and amplification efficiencies ranging from 92.1% to 99.6% (Hatt and Löffler, 2012). The standard deviation of C<sub>q</sub> values for 10<sup>2</sup> copies (C<sub>q</sub> ~ 29.0) were lower than 0.16 for all calibration qPCR runs, indicating the limit of detection was below 10<sup>2</sup> target sequences. All qPCR assays were performed in triplicate for each cDNA and genomic DNA sample. Luciferase cDNA recovery was used to correct for mRNA losses during extraction, purification and the reverse transcription step (Johnson *et al.*, 2005). Consistent melting temperatures suggested qPCR assay specificity and no amplification occurred in no-template controls. The calibration curve for luciferase DNA was constructed by reverse transcription of the luciferase control mRNA and insertion of the PCR product (amplified with primers lucrefF and lucrefR) into the PCR2.1 vector (Amos *et al.*, 2008). Based on the amount of luciferase mRNA recovered, the RNA recovery ranged from 11.3% to 20.7%. The qPCR assay results were analyzed with ViiA7 Software (Life Technologies) provided with the system. The two-tailed Student's *t*-tests were performed using the SPSS 21.0.0 software package (IBM Corp, Armonk, NY, USA) to verify statistical significance of the qPCR results.

## Results

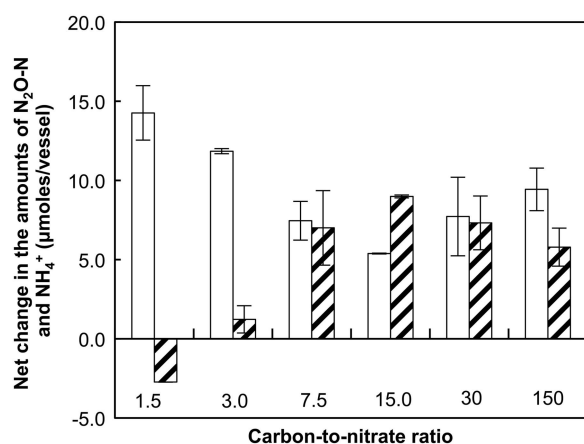
*Effects of C/N ratios on NO<sub>3</sub><sup>-</sup>/NO<sub>2</sub><sup>-</sup> reduction pathways*  
*S. loihica* strain PV-4 batch culture experiments with 0.1–10 mM lactate and 0.2 mM NO<sub>3</sub><sup>-</sup> suggested

that denitrification predominated at low C/N ratios (Figure 1). At an initial C/N ratio of 1.5, the product of dissimilatory NO<sub>3</sub><sup>-</sup> reduction was exclusively N<sub>2</sub>O, with 14.3 ± 1.7 µmol N<sub>2</sub>O-N recovered from 19.5 ± 0.1 µmol of NO<sub>3</sub><sup>-</sup>. The amount of NH<sub>4</sub><sup>+</sup> in the medium decreased during cultivation, presumably because of NH<sub>4</sub><sup>+</sup> incorporation into biomass. In batch incubations with initial C/N ratios higher than 1.5, both N<sub>2</sub>O and NH<sub>4</sub><sup>+</sup> were produced concomitantly. Significantly more N<sub>2</sub>O-N and less NH<sub>4</sub><sup>+</sup> were produced at an initial C/N ratio of 3.0 than at a C/N ratio of 7.5 (*P* < 0.01); however, increasing the C/N ratio beyond 7.5 did not affect the denitrification/respiratory ammonification ratio significantly (one-way analysis of variance, *P* > 0.05). When the NO<sub>3</sub><sup>-</sup> concentration was increased to 1 mM, N<sub>2</sub>O was the predominant product regardless of the C/N ratio. These observations suggested that the batch systems cannot resolve C/N ratio effects on pathway selection presumably because of changing C/N ratios during growth.

To explore NO<sub>3</sub><sup>-</sup> reduction pathways under controlled conditions with consistent limitation of either electron donor or electron acceptor (that is, a constant C/N ratio in the feed medium), *S. loihica* strain PV-4 was grown in a chemostat vessel. Supplying feed solutions with C/N ratios ≥ 6.0 resulted in steady-state conditions with no measurable NO<sub>3</sub><sup>-</sup>/NO<sub>2</sub><sup>-</sup> in the reactor, indicating the electron acceptor is limiting (Table 2). Chemostat vessel operation at C/N ratios between 3.0 and 4.5 resulted in steady-state conditions with no measurable lactate or NO<sub>3</sub><sup>-</sup>/NO<sub>2</sub><sup>-</sup> concentrations in the reactor. At a C/N ratio of 1.5, the steady-state nitrate concentration was 0.18 ± 0.14 mM, indicating electron donor-limiting conditions. Acetate concentrations ranged from 1.47 ± 0.06 to 3.98 ± 0.09 mM in the reactor at C/N ratios of ≥ 3.0 but dropped to below the detection limit of ~ 10 µM at a C/N ratio of 1.5.

The net NH<sub>4</sub><sup>+</sup> and N<sub>2</sub>O production rates served as measures of respiratory ammonification and denitrification activities, respectively. At C/N ratios of

$\leq 3.0$  in the chemostat feed, production of  $\text{N}_2\text{O}$ , but not of  $\text{NH}_4^+$ , was observed (Figure 2). At a C/N ratio of 1.5, denitrification was the predominant pathway with  $76 \pm 10\%$  of the consumed  $\text{NO}_3^-$  recovered as  $\text{N}_2\text{O}$ . Assimilation of  $\text{NH}_4^+$  exceeded production, resulting in a net  $\text{NH}_4^+$  consumption (Figure 2). Previous growth experiments with *S. loihica* strain PV-4 suggested that approximately half of the biomass N originated from  $\text{NH}_4^+$  and the other half from  $\text{NO}_3^-$  when grown under denitrifying conditions with acetate as electron donor (Yoon *et al.*, 2013). Assuming an even split of assimilated N compounds with lactate as electron donor,  $\text{NH}_4^+$  production from respiratory ammonification was negligible at a C/N ratio of 1.5. Similar observations were made at a C/N ratio of 3.0, with 82% of  $\text{NO}_3^-$  reduced to  $\text{N}_2\text{O}$  and a net  $\text{NH}_4^+$  consumption indicating the absence of significant respiratory ammonification activity. Higher C/N ratios in the chemostat feed led to increased  $\text{NH}_4^+$  formation and correspondingly lower  $\text{N}_2\text{O}$  production rates. Simultaneous production of  $\text{NH}_4^+$  and  $\text{N}_2\text{O}$  was observed at a C/N ratio of 4.5. At C/N ratios of 6.0 and 7.5, only  $\text{NH}_4^+$  and no  $\text{N}_2\text{O}$  was produced (Figure 2). Similar results were obtained in an independent replicate chemostat experiment (Supplementary



**Figure 1** Net production of  $\text{N}_2\text{O}$  (white bars) and  $\text{NH}_4^+$  (hatched bars) over a 5-day incubation period at 21 °C of *S. loihica* strain PV-4 batch cultures with 0.2 mM  $\text{NO}_3^-$  and 0.1, 0.2, 0.5, 1.0, 2.0 and 10.0 mM lactate. Each bar represents the average of triplicate samples, with error bars representing the s.d.

**Table 2** Concentrations of carbon (C) and nitrogen (N) compounds in the influent medium and the steady-state reactor in experiments examining the effect of C/N ratios on respiratory ammonification and denitrification pathways in *Shewanella loihica* strain PV-4

C/N ratio	Measured influent concentrations (mM)			Measured steady-state concentrations (mM)				
	Lactate	$\text{NO}_3^-$	$\text{NH}_4^+$	$\text{NO}_3^-$	$\text{NO}_2^-$	$\text{NH}_4^+$	Lactate	Acetate
7.5	5.04 (0.03)	1.96 (0.10)	0.46 (0.03)	0	0	1.79 (0.01)	0.51 (0.01)	3.98 (0.09)
6.0	3.91 (0.18)	2.03 (0.01)	0.47 (0.01)	0	0	1.48 (0.01)	0.05 (0.01)	2.93 (0.05)
4.5	2.82 (0.07)	2.03 (0.02)	0.44 (0.02)	0	0	0.88 (0.01)	0	2.33 (0.10)
3.0	2.15 (0.00)	1.89 (0.00)	0.50 (0.02)	0	0	0.29 (0.05)	0	1.47 (0.06)
1.5	1.04 (0.03)	1.90 (0.03)	0.48 (0.03)	0.18 (0.15)	0.14 (0.13)	0.20 (0.02)	0	0

The averages of duplicate measurements are presented with the s.d. indicated in parentheses.

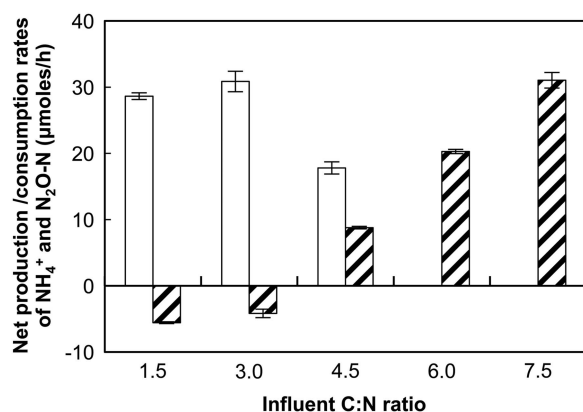
Figure S2). Under the experimental conditions promoting denitrification activity (that is, C/N ratio  $\leq 3.0$ ), biomass yields were lower than under conditions favoring respiratory ammonification (that is, C/N ratio  $\geq 6.0$ ; Table 3 and Supplementary Figure S3), indicating that more energy was conserved during  $\text{NO}_3^-$  to  $\text{NH}_4^+$  reduction than during  $\text{NO}_3^-$  to  $\text{N}_2\text{O}$  reduction. N mass balances for the different C/N ratios ranged from 98.5% to 106.8% in terms of  $\text{NO}_3^-$ -N added to the bioreactor and recovered as  $\text{NO}_2^-$ ,  $\text{NH}_4^+$ ,  $\text{N}_2\text{O}$  and biomass N (Table 3). The DNA yields mirrored the biomass production rate estimates (Table 3).

At an influent C/N ratio of 7.5, a steady-state acetate concentration of  $3.98 \pm 0.09$  mM was observed that was significantly higher ( $P < 0.05$ ) than the steady-state lactate concentration of  $0.51 \pm 0.01$  mM (Table 3). *S. loihica* strain PV-4 can use acetate for denitrification (Yoon *et al.*, 2013); however, the presence of acetate did not lead to  $\text{N}_2\text{O}$  production, indicating that acetate did not affect dissimilatory pathway selection (that is, denitrification vs respiratory ammonification) under the chemostat cultivation conditions. When the feed contained both 1.5 mM  $\text{NO}_2^-$  and 0.5 mM  $\text{NO}_3^-$ , only  $\text{NH}_4^+$  or  $\text{N}_2\text{O}$  were formed at C/N ratios above or below 4.5, respectively, and both products were observed at a C/N ratio of 4.5 (data not shown). These findings were consistent with the observations made in the  $\text{NO}_3^-$ -fed reactor, indicating that the electron acceptor (that is,  $\text{NO}_3^-$  vs  $\text{NO}_2^-$ ) did not alter reduced product(s) formation.

#### Effects of C/N ratios on the expression of genes encoding denitrification and respiratory ammonification pathways

Reverse-transcriptase qPCR (RT-qPCR) was used to enumerate *nirK*, *nosZ* and two *nrfA* transcripts in cDNA derived from RNA of steady-state chemostat cultures grown under different C/N ratios. The mRNA abundances in cells incubated with fumarate as the electron acceptor benchmarked the expression of genes involved in denitrification and/or respiratory ammonification (Supplementary Table S1). No more than 0.01 *nrfA*<sub>0505</sub>, *nirK* (Shew\_3335) and *nosZ* (Shew\_3400) transcripts per strain PV-4

cell were measured in fumarate-grown control cultures, whereas the abundance of *nrfA*<sub>0844</sub> transcripts was significantly higher (4.44 transcripts per cell) (Supplementary Table S1). In lactate/NO<sub>3</sub><sup>-</sup>-fed chemostat cultures with a C/N ratio of 1.5, the abundance of *nrfA*<sub>0844</sub> mRNA was two orders of magnitude lower than in fumarate-grown cells, suggesting an effect of the C/N ratio on *nrfA*<sub>0844</sub> gene expression (Figure 3). At a C/N ratio of 3.0, no NH<sub>4</sub><sup>+</sup> was produced but *nrfA*<sub>0844</sub> was expressed and 2.5 ± 0.2 transcripts per cell were measured. *nrfA*<sub>0844</sub> transcripts at higher C/N ratios were in the same order of magnitude and the maximum abundance of *nrfA*<sub>0844</sub> transcripts was observed at a C/N ratio of 6.0 with 8.0 ± 0.35 transcripts per cell. Interestingly, the abundance of *nrfA*<sub>0844</sub> at a C/N ratio of 7.5 (3.8 ± 0.04 transcripts per cell) was only 1.5-fold higher



**Figure 2** Net production of N<sub>2</sub>O (white bars) and NH<sub>4</sub><sup>+</sup> (shaded bars) in *S. loihica* strain PV-4 chemostat cultures with C/N ratios varying from 1.5 to 7.5 in the feed solution. The feed solution contained 2 mM NO<sub>3</sub><sup>-</sup> and was delivered at a rate of 20 ml h<sup>-1</sup>. Rates of respiratory ammonification were calculated from the input and steady-state concentrations of NH<sub>4</sub><sup>+</sup>, whereas denitrification rates were calculated by measuring N<sub>2</sub>O production rates following the addition of 10% (vol/vol) acetylene gas to the reactor vessel headspace. At C/N ratios of 1.5 and 3.0, net consumption of NH<sub>4</sub><sup>+</sup> occurred. The bars represent the averages of two separate measurements taken on different days after steady-state conditions had been reached. The error bars show the s.d. of two measurements taken with a 24-h interval.

**Table 3** Substrate consumption and metabolite production rates in the steady-state reactor receiving feed with different carbon-to-nitrogen (C/N) ratios

C/N ratio	Rates of production/consumption <sup>a</sup> in the steady-state reactor (µmol h <sup>-1</sup> )							DNA (ng µl <sup>-1</sup> ) <sup>b</sup>		
	Lactate	Acetate	NO <sub>3</sub> <sup>-</sup>	NO <sub>2</sub> <sup>-</sup>	NH <sub>4</sub> <sup>+</sup>	N <sub>2</sub> O-N	Biomass N	Biomass C		
7.5	-90.6 (0.4)	79.6 (1.9)	-39.2 (2.0)	0 (0)	26.5 (0.2)	0	14.5 (1.2)	72.5 (6.0)	10.2 (1.2)	
6.0	-77.2 (2.3)	58.56 (1.1)	-40.6 (0.1)	0 (0)	20.2 (0.3)	0	19.8 (0.8)	98.8 (4.1)	14.5 (1.1)	
4.5	-56.4 (1.5)	46.6 (2.0)	-40.6 (0.1)	0 (0)	8.8 (0.2)	17.8 (0.9)	14.1 (0.6)	70.5 (3.0)	9.8 (0.5)	
3.0	-43.0 (0.1)	29.4 (1.1)	-37.8 (0.1)	0 (0)	-4.2 (0.6)	30.9 (1.6)	11.1 (0.5)	55.7 (2.4)	9.0 (0.2)	
1.5	-20.8 (0.6)	0 (0)	-34.4 (2.4)	2.8 (2.7)	-5.6 (0.1)	28.6 (0.5)	11.3 (0.1)	56.7 (0.6)	8.4 (0.4)	

The production rates of dissolved compounds and biomass N and C were calculated from the concentrations in the influent medium and steady-state aqueous phase concentrations. The reported N<sub>2</sub>O production rates account for N<sub>2</sub>O loss because of the flux of the dissolved N<sub>2</sub>O with the effluent (see Materials and methods section for details). Except for biomass C and N, the values are the average of duplicate measurements and the s.d. is indicated in parentheses. Biomass C and N data were calculated from the cell quantification data presented in Supplementary Figure S2.

<sup>a</sup>A minus sign denotes consumption.

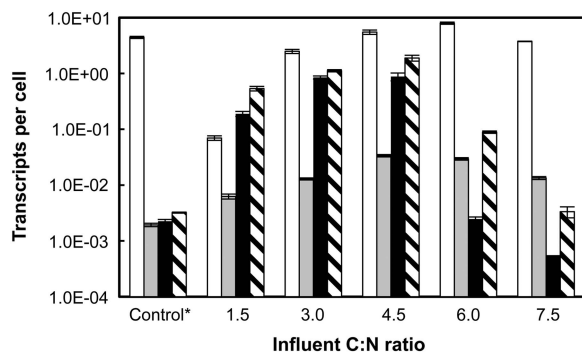
<sup>b</sup>Data shown are average and s.d. of triplicate DNA extracts; 260/280 ratios were between 1.78 and 1.87.

( $P < 0.05$ ) than at the C/N ratio of 3.0 despite the significant difference observed in N<sub>2</sub>O versus NH<sub>4</sub><sup>+</sup> production (Figure 2). *nrfA*<sub>0505</sub> was expressed at orders of magnitude lower levels than *nrfA*<sub>0844</sub> under all growth conditions tested.

In contrast to *nrfA*<sub>0505</sub> and *nrfA*<sub>0844</sub> expression, pronounced *nirK* transcriptional changes were observed in response to changing the C/N ratios (Figure 3). At C/N ratios of 6.0 and 7.5, the *nirK* mRNA abundances exceeded those measured at lower C/N ratios by up to three orders of magnitude (that is, ~1600-fold). The *nosZ* expression followed a profile similar to that of *nirK*, and at C/N ratios of 6.0 and 7.5, *nosZ* transcription was up to 27-fold lower compared with the transcript levels observed at lower C/N ratios. The observation of active *nirK* transcription (that is, ≥ 0.19 transcript per cell) coincided with N<sub>2</sub>O production, whereas cells with ≤ 2.5 × 10<sup>-3</sup> *nirK* transcripts per cell produced no N<sub>2</sub>O. An independent chemostat experiment demonstrated the reproducibility of the reverse-transcriptase qPCR analysis. The *nrfA*<sub>844</sub> mRNA abundances at C/N ratios of 3.0 and 6.0 were < 3% different from the *nrfA*<sub>844</sub> expression data of the first experiment and a 2.5-fold difference was observed at a C/N ratio of 1.5 (Supplementary Figure S4). The *nirK* transcript abundance data exhibited < 25% deviation from the *nirK* expression data presented in Figure 3 for all three C/N ratios tested.

#### Effects of pH and temperature on NO<sub>3</sub><sup>-</sup>/NO<sub>2</sub><sup>-</sup> reduction pathways

For assessing the effects of pH and temperature on dissimilatory NO<sub>3</sub><sup>-</sup>/NO<sub>2</sub><sup>-</sup> reduction pathways, the medium received 5 mM lactate and 1 mM NO<sub>3</sub><sup>-</sup> (C/N = 15) to avoid electron donor limitations. A pH increase from 6.5 to 8.0 shifted the reduced product distribution from entirely N<sub>2</sub>O to predominantly NH<sub>4</sub><sup>+</sup>. At pH 6.5, 92.1 ± 3.4 µmol of the 105.9 µmol of NO<sub>3</sub><sup>-</sup> were reduced to N<sub>2</sub>O (Figure 4a). In these cultures, NH<sub>4</sub><sup>+</sup> decreased by 10.0 ± 0.03 µmol, presumably because of assimilation into biomass.



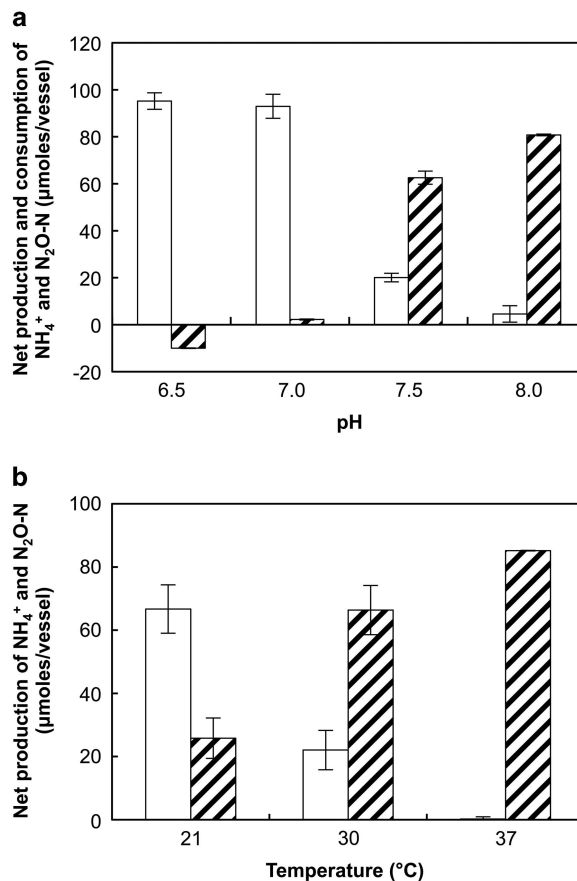
**Figure 3** Reverse-transcriptase (RT)-qPCR analyses of *nrfA<sub>0844</sub>* (white bars), *nrfA<sub>0505</sub>* (gray bars; respiratory ammonification), *nirK* (black bars) and *nosZ* (hatched bars; denitrification) transcripts in *S. loihica* strain PV-4 under varying C/N ratios. The error bars represent the s.d. of triplicate per-cell transcript copy numbers calculated from the s.d. of qPCR measurements for cDNA and genomic DNA using the error propagation method. The results of an independent replicate experiment are shown in Supplementary Figure S3. \*The control is the transcript copy number of each gene in the sample extracted from the steady-state reactor incubated with fumarate as the electron acceptor.

The proportion of  $\text{NH}_4^+$  increased as the pH was raised in 0.5 increments, and at pH 8.0,  $\text{NH}_4^+$  was the predominant product of  $\text{NO}_3^-$  reduction with  $80.8 \pm 0.36 \mu\text{mol}$  formed, whereas  $\text{N}_2\text{O}$  accounted for only  $4.4 \pm 3.4 \mu\text{mol}$  (Figure 4a). In Tris-buffered medium with  $\text{pH} \geq 8.0$ , exclusively  $\text{NH}_4^+$  was produced (data not shown). Consistent results obtained with different buffer systems in overlapping pH ranges indicated that the buffer type (that is, phosphate versus Tris buffer) did not affect  $\text{NO}_3^-/\text{NO}_2^-$  reduction pathways in *S. loihica* strain PV-4. These findings demonstrate that pH has a strong effect on the selection of dissimilatory  $\text{NO}_3^-$  reduction pathways in strain PV-4, and  $\text{N}_2\text{O}$  is the major product under acidic conditions whereas  $\text{NH}_4^+$  predominated at alkaline pH.

*S. loihica* strain PV-4 was reported to grow over a temperature range of  $0^\circ\text{C}$  to  $42^\circ\text{C}$  (Gao *et al.*, 2006), and the effects of temperature on the two  $\text{NO}_3^-$  reduction pathways were explored. Batch cultures incubated at  $21^\circ\text{C}$  transformed  $98.7 \pm 0.8 \mu\text{mol}$  of  $\text{NO}_3^-$  to  $33.2 \pm 3.1 \mu\text{mol}$   $\text{N}_2\text{O}$  and  $22.1 \pm 7.8 \mu\text{mol}$   $\text{NH}_4^+$  (Figure 4b). Higher incubation temperatures shifted the reduced product distribution toward  $\text{NH}_4^+$  and  $65.6 \pm 6.1 \mu\text{mol}$   $\text{NH}_4^+$  and  $10.9 \pm 3.9 \mu\text{mol}$   $\text{N}_2\text{O}$  were produced at  $30^\circ\text{C}$ . At  $37^\circ\text{C}$ , the highest temperature tested,  $\text{NH}_4^+$  ( $84.3 \pm 0.9 \mu\text{mol}$ ) was the predominant product and  $<0.3 \mu\text{mol}$  of  $\text{N}_2\text{O}$  was detected at the end of the incubation. Despite numerous attempts, cultivation of *S. loihica* strain PV-4 with  $\text{NO}_3^-$  as electron acceptor was not successful at temperatures below  $20^\circ\text{C}$ .

## Discussion

The batch culture experiments with *S. loihica* strain PV-4 had shortcomings in unraveling C/N ratio effects on the selection of denitrification versus



**Figure 4** Net change of  $\text{N}_2\text{O}$  (white bars) and  $\text{NH}_4^+$  (hatched bars) in *S. loihica* strain PV-4 batch cultures amended with 5.0 mM lactate and 1.0 mM  $\text{NO}_3^-$  (C/N = 15:1) under varying (a) pH and (b) temperature conditions. The bars represent the average of triplicate samples, with error bars representing the s.d.

respiratory ammonification pathways. At C/N ratios of  $\geq 7.5$ ,  $\text{N}_2\text{O}$  and  $\text{NH}_4^+$  were produced in similar amounts (one-way analysis of variance,  $P > 0.05$ ). Respiratory ammonification yields more biomass per molecule of  $\text{NO}_3^-/\text{NO}_2^-$  reduced than denitrification and thus should be a competitive process under  $\text{NO}_3^-/\text{NO}_2^-$ -limiting conditions (Tiedje *et al.*, 1982; Strohm *et al.*, 2007). Hence, the prediction is that organisms select the pathway that maximizes energy conservation, in particular under conditions of substrate limitation. In batch cultures with 0.2 mM  $\text{NO}_3^-$  and low initial cell numbers ( $<10^6$  cells per ml),  $\text{NO}_3^-/\text{NO}_2^-$  was, at least initially, not limiting and therefore should not have affected pathway selection. At C/N ratios of  $\leq 3.0$ , the formation and consumption of acetate complicated data interpretation. *Shewanella* spp. couple denitrification to acetate oxidation but acetate does not support  $\text{NO}_2^-$  reduction to  $\text{NH}_4^+$  (Yoon *et al.*, 2013). As lactate oxidation to acetate was insufficient to deplete the  $\text{NO}_3^-/\text{NO}_2^-$  pool at these C/N ratios, the ensuing utilization of acetate yielded exclusively  $\text{N}_2\text{O}$ . A similar bias because of selective acetate oxidation was previously observed in a mixed culture experiment where denitrification coupled to acetate oxidation obscured C/N ratio effects



(Rehr and Klemme, 1989). Therefore, the batch culture results could not resolve the effects of different C/N ratios on pathway selection. The chemostat experiments in conjunction with gene-targeted reverse-transcriptase qPCR expression analyses resolved these complications and demonstrated the C/N ratio effects on the selection of dissimilatory  $\text{NO}_3^-/\text{NO}_2^-$  reduction pathways in *S. loihica* strain PV-4. Chemostats can achieve steady-state substrate concentrations near zero while maintaining a constant substrate supply, and cellular responses to substrate limitations can be observed (Friedrich, 1982; Durner *et al.*, 2000). In the experiments with *S. loihica* strain PV-4, high C/N ratios in the influent resulted in  $\text{NO}_3^-$  limitations that reduced *nirK* expression levels and led to the predominance of respiratory ammonification. Thus, the chemostat cultures demonstrated the effects of high C/N ratios on pathway selection, and the findings are consistent with previous observations that respiratory ammonification predominates in carbon-rich environments.

Although *S. loihica* strain PV-4 can couple acetate oxidation to denitrification but not respiratory ammonification, the chemostat supplied with a C/N ratio of 7.5 demonstrated that the presence of acetate (up to 3.92 mM) did not result in increased *nirK/nosZ* expression levels or denitrification activity (Figures 2 and 3). Thus, acetate oxidation occurred only under conditions that favored denitrification, suggesting that acetate did not affect the outcome and interpretation of the chemostat experiments. Although the steady-state lactate concentrations in the reactor were below the method detection limit of  $\sim 10\ \mu\text{M}$  at C/N ratios of  $\leq 3.0$  in the feed solution, lactate was constantly introduced into the reactor at rates of  $20\text{--}40\ \mu\text{mol min}^{-1}$ , and lactate oxidation sustained denitrification activity but not respiratory ammonification. Consistent with these observations, the reverse-transcriptase qPCR results demonstrated elevated *nirK* and reduced *nrfA* expression levels under lactate-limiting conditions (that is, at low C/N ratios).

The transcription levels of *nrfA* and *nirK* did not decrease at intermediate C/N ratios of 3.0 and 4.5, at which lactate and nitrate concentrations in steady-state chemostats were both below the detection limits (Table 3). Nevertheless, the influent C/N ratio caused a subtle (for example, twofold and statistically distinguishable) shift in *nrfA* or *nirK* transcription levels (Figure 3 and Supplementary Table S2), resulting in significantly different outcomes at these two C/N ratios (that is, exclusively  $\text{N}_2\text{O}$  production at a C/N ratio of 3.0 versus production of both  $\text{N}_2\text{O}$  and  $\text{NH}_4^+$  at a C/N ratio of 4.5; Figure 2). Taken together, the phenotypic observations and the *nrfA*<sub>0844</sub>, *nirK* and *nosZ* transcription levels (Figure 3) indicated that both pathways were active at a C/N ratio of 4.5. This ambivalent regulation of the two pathways at intermediate C/N ratios is a likely cause for the inconsistent batch experiment

results, where neither electron donor nor electron acceptor was limiting until the end of the experiment.

*S. loihica* strain PV-4 possesses two nonidentical *nrfA* genes, *nrfA*<sub>0505</sub> and *nrfA*<sub>0844</sub>, and the expression levels of *nrfA*<sub>0844</sub> exceeded those of *nrfA*<sub>0505</sub> by at least one order of magnitude. NrfA<sub>0844</sub> shares 78% amino acid identity to the NrfA of *S. oneidensis* strain MR-1, whose function as an ammonia-forming nitrite reductase has been confirmed (Cruz-Garcia *et al.*, 2007). On the other hand, NrfA<sub>0505</sub> has not been functionally characterized but shares up to 50% similarity with functionally verified NrfA proteins from *S. oneidensis* strain MR-1 and *Escherichia coli*. The low expression level of *nrfA*<sub>0505</sub> suggests that NrfA<sub>0844</sub> is responsible for the observed respiratory ammonification activity; however, a detailed biochemical characterization of NrfA<sub>0505</sub>-type proteins to elucidate their functional roles, if any, in respiratory ammonification has yet to be accomplished. A microarray-based transcriptome analysis of *S. oneidensis* strain MR-1 (Beliaev *et al.*, 2005) found the *nrfA*<sub>0844</sub>-like gene highly expressed when grown with  $\text{NO}_3^-$ ; however, our study found that *nrfA*<sub>0844</sub> was also expressed in cultures grown with fumarate in the absence of  $\text{NO}_3^-$ . The chemostat experiments demonstrated downregulation of *nrfA*<sub>0844</sub> under electron donor-limiting conditions (that is, at a C/N ratio of 1.5), suggesting that carbon availability may be a controlling factor in *nrfA*<sub>0844</sub> gene transcription. At a C/N ratio of 4.5 with simultaneous production of  $\text{NH}_4^+$  and  $\text{N}_2\text{O}$ , *nrfA*<sub>0844</sub> transcription was higher than at a C/N ratio of 7.5 ( $P < 0.05$ ), where the product was predominantly  $\text{NH}_4^+$ . These observations indicate that the measurement of *nrfA* transcripts as a proxy for respiratory ammonification activity in an environmental sample must be interpreted cautiously, and should be at least accompanied by additional measurements such as *nirK* and *nirS* mRNA abundance.

Although pure cultures studies have limitations to predict environmental processes, the observations made with *S. loihica* strain PV-4 can explain at least some previous field observations. Soils and sediments receiving high  $\text{NO}_3^-$  loadings showed increased denitrification rates, whereas elevated respiratory ammonification rates were observed in soils and sediments receiving high carbon loadings (Koop-Jakobsen and Giblin, 2010; Nizzoli *et al.*, 2010; Schmidt *et al.*, 2011; Fernandes *et al.*, 2012; Dunn *et al.*, 2013). In carbon-rich environments, the N oxyanion availability in anoxic zones depends on  $\text{NO}_3^-$  or  $\text{NO}_2^-$  fluxes from the oxic zone (that is, through organic N compound degradation and nitrification). Hence, carbon-rich environments are generally  $\text{NO}_3^-$  and  $\text{NO}_2^-$  limited, conditions that were mimicked in chemostats operated under high C/N ratios, under which respiratory ammonification activity predominated. In events of heavy N input such as fertilizer application, bioavailable electron

donor and carbon sources in anoxic zones may become limiting (DeSimone and Howes, 1996; Inwood *et al.*, 2007; Koop-Jakobsen and Giblin, 2010). Such conditions were simulated in chemostat experiments receiving feed with low C/N ratios that increased  $\text{NH}_4^+$  production rates but decreased  $\text{N}_2\text{O}$  production rates and *nirK* transcription levels. A recent study using a chemostat inoculated with a tidal flat sediment community also reported that the C/N ratio was a major factor determining the end product of bacterial  $\text{NO}_3^-$  reduction (Kraft *et al.*, 2014). Nevertheless, a number of studies found no consistent correlation between C/N ratios and dissimilatory  $\text{NO}_3^-$  reduction pathways (Nizzoli *et al.*, 2006; Schmidt *et al.*, 2011). This inconsistency may be attributed to balanced input of C and N, as observed in the chemostats receiving intermediate C/N ratios, the degradability of the bioavailable C substrate or the heterogeneity of the soil and sediment environment studied. Another possibility is that respiratory ammonification activity is only upregulated when labile C sources, such as root exudates, are available (Schmidt *et al.*, 2011). To further explore the effects of C or N limitation on  $\text{NO}_3^-/\text{NO}_2^-$  fate via dissimilatory pathways, controlled mesocosm-scale experiments should be performed to carefully determine whether the strain PV-4 pure culture results have bearing for predicting  $\text{NO}_3^-/\text{NO}_2^-$  fate in complex environmental systems. Other  $\text{NO}_3^-/\text{NO}_2^-$  reduction pathways may also play a role, including the recently discovered reverse-HURM (hydroxylamine:ubiquinone reductase module) pathway in *Nautilia* and *Campylobacter* spp. (Hanson *et al.*, 2013). This  $\text{NO}_3^-/\text{NO}_2^-$  ammonification pathway provides the organism with ammonium for biomass synthesis; however, its significance for dissimilatory  $\text{NO}_3^-/\text{NO}_2^-$  reduction remains to be determined. Nevertheless, the possibility that dissimilatory processes in addition to denitrification and respiratory ammonification contribute to  $\text{NO}_3^-/\text{NO}_2^-$  fate should be considered.

In addition to C/N ratios, other environmental factors, including pH and temperature, can influence pathway selection. Previous reports about pH effects on  $\text{NO}_3^-$  fate were largely contradictory (Nägele and Conrad, 1990; Stevens *et al.*, 1998). The pure culture experiments with *S. loihica* strain PV-4 revealed that respiratory ammonification was favored at elevated pH and denitrification was favored under lower pH conditions. The catalytic subunit (*NirK*) of the copper-dependent nitrite reductase (CuNIR) and the ammonia-forming nitrite reductase, NrfA, are both located in the periplasm. The periplasmic pH of *E. coli*, a neutrophilic Gammaproteobacterium like *S. loihica*, is influenced by the extracellular pH (Wilks and Slonczewski, 2007), suggesting that the *in vivo* activities of these enzymes could be affected by the pH of the surrounding matrix. Previous enzyme characterization studies demonstrated that CuNIR and NrfA proteins have distinct pH optima and

ranges for activity. For example, the pH optima of characterized CuNIR enzyme systems are below 7 (Iwasaki and Matsubara, 1972; Abraham *et al.*, 1997; Jacobson *et al.*, 2007). The CuNIR isolated from the Betaproteobacterium *Alcaligenes xylosoxidans* showed optimal catalytic performance at pH 5.2 that diminished to negligible levels above pH 7.5 (Abraham *et al.*, 1997). Interestingly, the other group of NO-forming nitrite reductases, the periplasmic cytochrome *cd<sub>1</sub>* nitrite reductases (catalytic subunit encoded by *nirS*) (Yamazaki *et al.*, 1995), also showed maximum activity under acidic pH conditions (Lam and Nicholas, 1969; Singh, 1974; Richter *et al.*, 2002), suggesting that  $\text{NO}_2^-$  reduction to NO is favored at  $\text{pH} < 7$ . In contrast, the *E. coli* strain K-12 NrfA protein exhibited optimal activity around pH 7.5, with negligible activity below pH 7.0, and the *Desulfovibrio desulfuricans* NrfA protein demonstrated maximum activity between pH 8.0 and 9.5 (Liu and Peck, 1981; Kajie and Anraku, 1986). The *S. loihica* strain PV-4 NirK shares 76% amino acid sequence similarity with the NirK of *Alcaligenes xylosoxidans*, whereas NrfA<sub>0844</sub> is 76% similar to the *E. coli* NrfA. Thus, it is possible that the physicochemical properties of NirK and NrfA contributed to the observed pH effects on denitrification and respiratory ammonification activity in strain PV-4. Although temperature effects on dissimilatory  $\text{NO}_3^-/\text{NO}_2^-$  reduction pathways were observed with *S. loihica* strain PV-4, the mechanistic underpinning is unclear. A possible explanation for the observed temperature effects may be the direct responses of the NrfA and NirK enzyme systems. The *E. coli* NrfA showed maximum activity at 57 °C (Kajie and Anraku, 1986); however, no information regarding temperature effects on NirK activity is available. Although the mechanism for temperature regulation is unknown, the strain PV-4 experimental findings are in agreement with previous field observations, as respiratory ammonification activity had a stronger influence on  $\text{NO}_3^-$  fate in tropical climates and during the warmer summer season (Ogilvie *et al.*, 1997; Nizzoli *et al.*, 2006; Dong *et al.*, 2011; Dunn *et al.*, 2013).

In summary, the experiments with *S. loihica* strain PV-4 capable of  $\text{NO}_3^-/\text{NO}_2^-$  reduction to  $\text{N}_2$  or  $\text{NH}_4^+$  revealed the specific conditions that favor either denitrification or respiratory ammonification.  $\text{NO}_3^-/\text{NO}_2^-$  limitations at high C/N ratios decreased transcription levels of denitrification genes (*nirK* and *nosZ*) and led to the predominance of the respiratory ammonification pathway, whereas electron acceptor limitations at low C/N ratios increased *nirK* and *nosZ* transcription, leading to the predominance of denitrification. At elevated temperatures and alkaline pH conditions, the respiratory ammonification pathway predominated over the denitrification pathway. The *S. loihica* strain PV-4 experiments suggest that pure culture studies can contribute to a better understanding of the environmental controls governing the fate of  $\text{NO}_3^-/\text{NO}_2^-$  in soils and sediments.

## Conflict of Interest

The authors declare no conflict of interest.

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