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Nitrous Oxide Reduction Kinetics Distinguish Bacteria Harboring Clade I NosZ from Those Harboring Clade II NosZ

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ABSTRACT

Bacteria capable of reduction of nitrous oxide (N_2O) to N_2 separate into clade I and clade II organisms on the basis of *nos* operon structures and *nosZ* sequence features. To explore the possible ecological consequences of distinct *nos* clusters, the growth of bacterial isolates with either clade I (*Pseudomonas stutzeri* strain DCP-Ps1, *Shewanella loihica* strain PV-4) or clade II (*Dechloromonas aromatica* strain RCB, *Anaeromyxobacter dehalogenans* strain 2CP-C) *nosZ* with N_2O was examined. Growth curves did not reveal trends distinguishing the clade I and clade II organisms tested; however, the growth yields of clade II organisms exceeded those of clade I organisms by 1.5- to 1.8-fold. Further, whole-cell half-saturation constants (K_ss) for N_2O distinguished clade I from clade II organisms. The apparent K_s values of 0.324 ± 0.078 μM for *D. aromatica* and 1.34 ± 0.35 μM for *A. dehalogenans* were significantly lower than the values measured for *P. stutzeri* (35.5 ± 9.3 μM) and *S. loihica* (7.07 ± 1.13 μM). Genome sequencing demonstrated that *Dechloromonas denitrificans* possessed a clade II *nosZ* gene, and a measured K_s of 1.01 ± 0.18 μM for N_2O was consistent with the values determined for the other clade II organisms tested. These observations provide a plausible mechanistic basis for why the relative activity of bacteria with clade I *nos* operons compared to that of bacteria with clade II *nos* operons may control N_2O emissions and determine a soil's N_2O sink capacity.

IMPORTANCE

Anthropogenic activities, in particular fertilizer application for agricultural production, increase N_2O emissions to the atmosphere. N_2O is a strong greenhouse gas with ozone destruction potential, and there is concern that nitrogen may become the major driver of climate change. Microbial N_2O reductase (NosZ) catalyzes N_2O reduction to environmentally benign dinitrogen gas and represents the major N_2O sink process. The observation that bacterial groups with clade I *nosZ* versus those with clade II *nosZ* exhibit distinct affinities to N_2O has implications for N_2O flux models, and these distinct characteristics may provide opportunities to curb N_2O emissions from relevant soil ecosystems.

Nitrous oxide (N_2O) is a potent greenhouse gas with a strong potential to drive climate change (1, 2) and will continue to be the largest contributor to ozone depletion in the stratosphere (2, 3). Anthropogenic activities, predominantly, fertilizer application in agricultural production, have contributed to a steady increase in atmospheric N_2O concentrations, and a continued upward trend is expected (4–6). Particularly troublesome are the findings of a recent study that concluded that without solutions for the N_2O problem, carbon dioxide (CO_2) emission reductions even greater than those already proposed will be required to avoid climate change (7). Due to its environmental impact, the pathways leading to the generation and consumption of N_2O have received heightened interest.

In the environment, N_2O is predominantly formed as an intermediate of denitrification and a nitrification by-product (8). Denitrification is the stepwise reduction of NO_3^-/NO_2^- to gaseous products (i.e., N_2O, N_2), with each step being mediated by distinct enzyme systems (9). A kinetic imbalance in the rates of reactions producing and consuming N_2O during denitrification leads to the release of N_2O to the atmosphere (8, 10). In nitrification, N_2O is generated by nitrifier denitrification and as a by-product of ammonia oxidation (8, 11, 12). A recent report indicated that nitrifiers, rather than denitrifiers, may be the primary source of N_2O in agricultural soils (12). Other processes contributing to N_2O formation include respiratory ammonification (also known as dissimilatory nitrate/nitrite reduction to ammonium [DNRA]) and chemodenitrification (i.e., the abiotic reaction of NO_2^- with ferrous iron) (13, 14). In contrast to the diverse pathways of N_2O generation, the only known major biological pathway for the removal of N_2O is by reduction to N_2, catalyzed by the enzyme nitrous oxide reductase (NosZ) (8, 15–17). Recent efforts have identified a new cluster of atypical *nosZ* genes, now designated clade II *nosZ* genes (15, 16). Interestingly, many clade II *nosZ* genes were found on genomes lacking the *nirS* and/or *nirK* gene, suggesting that nondenitrifiers contribute to N_2O reduction (18). Growth experiments with *Anaeromyxobacter dehalogenans*,

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TABLE 1 Bacteria harboring *nosZ* used to obtain kinetic data during growth with N_2O provided as the electron acceptor and acetate as the electron donor

Organism	Type culture collection accession no.	*nosZ* clade (GenBank accession no.)	GenBank accession no. of genome sequence
Dechloromonas aromatica strain RCB	ATCC BAA-1848	II (YP_284794.1)	CP000089.1
Dechloromonas denitrificans strain ED-1	ATCC BAA-841, DSM 15892	II (KT592356.1)[a]	GCA_001551835.1[b]
Anaeromyxobacter dehalogenans strain 2CP-C	ATCC BAA-259	II (YP_002491966.1)	CP000251.1
Pseudomonas stutzeri strain DCP-Ps1	Not deposited	I (JQ513867)	Not sequenced
Shewanella loihica strain PV-4	ATCC BAA-1088, DSM 17748	I (YP_001095525.1)	CP000606.1

[a] *Dechloromonas denitrificans* strain ED-1 was initially characterized as a clade I bacterium harboring the typical *nosZ* (GenBank accession number AJ704212.1). Genome sequencing performed in this study revealed that strain ED-1 harbors a clade II *nosZ*.
[b] GenBank assembly accession number (draft genome).

an organism carrying the clade II *nosZ*, demonstrated that clade II NosZ functions as a respiratory oxidoreductase (15). PCR and metagenome analyses demonstrated the clade II *nosZ* gene abundance in different soils and suggested a correlation between the abundance and diversity of microorganisms carrying the clade II *nosZ* and a soil's N_2O sink capacity (16, 18, 19).

The clade II NosZ enzymes have <50% amino acid sequence similarity with the clade I NosZ enzymes, but both NosZ enzymes catalyze the same reaction. While clade I NosZ enzymes have been studied in detail (17, 20, 21), only the clade II NosZ enzyme of *Wolinella succinogenes* has been characterized to some extent biochemically (22, 23). The detailed kinetic parameters of the clade I and II NosZ enzymes have not been determined *in vivo*. Thus, the kinetic properties of clade I NosZ and those of clade II NosZ may differ, and these different kinetic properties may reflect distinguishing phenotypic characteristics of the N_2O-consuming hosts. To explore whether organisms carrying clade I *nosZ* and organisms carrying clade II *nosZ* exhibit distinct responses to N_2O, the growth characteristics of N_2O-respiring pure cultures representing both clades were examined. The measurement of growth yields and whole-cell N_2O consumption kinetics demonstrated that bacteria with clade I *nosZ* and bacteria with clade II *nosZ* exhibit distinct properties, suggesting niche differentiation and a relevant role for clade II organisms for controlling N_2O emissions to the atmosphere.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Five different bacterial isolates harboring *nosZ* were selected for a comparison of growth characteristics with N_2O as the electron acceptor. Included in the analysis were *Anaeromyxobacter dehalogenans*, a nondenitrifying deltaproteobacterium with clade II *nosZ*; the denitrifying betaproteobacterium *Dechloromonas aromatica* with clade II *nosZ*; and the denitrifying gammaproteobacteria *Pseudomonas stutzeri* and *Shewanella loihica*, both of which carry a clade I *nosZ*. Also included in the initial analysis was *Dechloromonas denitrificans*, an organism that shares physiological features with *Dechloromonas aromatica* but that apparently possesses a clade I *nosZ* (Table 1). *D. aromatica* strain RCB was kindly provided by John D. Coates, and *D. denitrificans* strain ED-1 was acquired from the American Type Culture Collection (ATCC BAA-841T). *S. loihica* strain PV-4 was grown in saline (2.0% [wt/vol] NaCl) phosphate-buffered basal salts medium as described previously (24). The same medium with a lower NaCl concentration of 0.05% was used to grow *D. aromatica* strain RCB, *D. denitrificans* strain ED-1, and *P. stutzeri* strain DCP-Ps1. *A. dehalogenans* strain 2CP-C was grown in 30 mM bicarbonate-buffered basal salts medium with an N_2-CO_2 headspace (80:20, vol/vol) (25). The final pH was 7.0 in all media used. Culture bottles were prepared using the Hungate technique and sealed with black butyl rubber stoppers (Geo-Microbial Technologies, Inc., Ochelata, OK, USA) to maintain anoxic conditions. Cultures were initiated from freezer stocks and maintained in 100 ml of the respective medium in 160-ml glass serum bottles amended with 5 mM acetate and 1 mM NO_3^-. NH_4Cl was used as a nitrogen source and was added to a final concentration of 0.5 mM. All culture vessels were incubated horizontally at 30°C with shaking at 110 rpm.

Bacterial growth and N_2O consumption were monitored following inoculation of 0.5 ml (0.5%, vol/vol) of a cell suspension from cultures that had consumed all NO_3^- and NO_2^- to fresh medium amended with 2 ml (~83 μmol) of N_2O gas (>99%; Sigma-Aldrich, St. Louis, MO). Acetate (500 μmol) was added in 24-fold excess in terms of electron equivalents (i.e., $\text{acetate}^- + 2H_2O \rightarrow 2CO_2 + 8e^- + 7H^+$; $N_2O + 2H^+ + 2e^- \rightarrow N_2 + H_2O$) to ensure that N_2O reduction activity was not electron donor limited. The optical density at 600 nm (OD_{600}) values of the cultures and N_2O concentrations were monitored until all N_2O was consumed and growth ceased. N_2O-negative controls exhibited no measurable growth.

Analytical procedures. N_2O consumption in growing cultures was monitored by measuring culture headspace samples with an Agilent 3000A MicroGC gas chromatograph (Palo Alto, CA) equipped with a Plot Q column and a thermal conductivity (TCD) detector (26). This instrument has a detection limit of ~50 ppmv N_2O. For measurement of the kinetic parameters half-saturation constant (K_s) and V_{max}, N_2O was quantified using an Agilent (HP) 7890A gas chromatograph equipped with an HP-Plot Q column (30 m by 0.320 mm in diameter; film thickness, 20 μm) and a micro-electron capture detector (μECD). This analytical system, which had a detection limit of ~0.3 ppmv N_2O, offered more sensitive quantification than the 3000A MicroGC instrument. The injector, oven, and detector temperatures were set to 200°C, 100°C, and 250°C, respectively. Helium (>99.999% purity) and N_2 (>99.999% purity) (Airgas, Knoxville, TN) served as the carrier and makeup gases, respectively. The split ratio of the injector was set to 20 when the initial N_2O concentration was below 200 ppmv and was increased to 100 when the initial N_2O concentrations exceeded 200 ppmv. For each measurement, 0.1 ml headspace gas was withdrawn from the culture vessel and manually injected into the gas chromatograph. A calibration curve with the appropriate split ratio for the experimental samples was generated immediately before each experiment. The aqueous concentration (in micromolar) of N_2O was calculated from the headspace concentrations using equation 1 as follows and a dimensionless Henry's constant of 1.94 for N_2O at 30°C (27):

$$C_{aq} = \frac{C_g}{H} \times \frac{1}{24.86} \tag{1}$$

where C_{aq}, C_g, and H are the aqueous concentration (in moles liter^{-1}), the headspace concentration (in moles liter^{-1}), and Henry's constant (dimensionless), respectively. A Henry's constant of 2.21 was used to correct for the ionic strength effects in the saline medium used for culturing *S. loihica* (28–30). OD_{600} measurements were performed with a Spectronic 20D+ spectrophotometer (Thermo Scientific, Waltham, MA) using disposable plastic cuvettes with a 1-cm path length.

Growth yield determination. The growth yields were measured for the bacteria listed in Table 1. Triplicate 160-ml serum bottles with 100 ml fresh medium received 1.0 ml of inoculum from the respective precultures grown with 5 mM acetate and 2 ml N_2O (>99%). After inoculation, each vessel was amended with 4 ml N_2O (165.8 μmol), and the initial amount of N_2O was quantified following equilibration at 30°C. Immediately after N_2O depletion, 40-ml cell suspensions were withdrawn from each bottle and the biomass was collected on 0.22-μm-pore-size membrane filters (Millipore, Billerica, MA) by vacuum filtration. The filters were dried at 100°C for 24 h and placed in a desiccator to cool to room temperature, and the weight was recorded after weight consistency was achieved. The biomass dry weight was calculated by subtraction of the premeasured weight of the dry membrane filter from the weight of the dried filter holding the biomass. The measurements were adjusted for the weight of the salts in the culture medium that stayed on the membrane filter after filtration. This adjustment was necessary because media with different ionic strengths were used for the cultivation of the microorganisms (30).

Determination of Michaelis-Menten parameters. The rates of N_2O consumption by *D. aromatica* strain RCB, *D. denitrificans* strain ED-1, *A. dehalogenans* strain 2CP-C, *P. stutzeri* strain DCP-Ps1, and *S. loihica* strain PV-4 cultures were measured. The bacterial cultures tested exhibited different growth characteristics and N_2O reduction rates. In order to determine Michaelis-Menten parameters, extensive preliminary experiments were performed to elucidate conditions that maintained pseudo-steady-state substrate and biomass concentrations. Initially, cells were grown with 4 ml of N_2O and 5 mM acetate in 160-ml serum bottles containing 100 ml of medium. Immediately following the consumption of 4 ml of N_2O, 0.4 ml of 5% (vol/vol) N_2O in N_2 was added to the vessels and its consumption was monitored. All culture bottles were incubated at 30°C on a shaker platform set to 110 rpm. The concentrations of dissolved N_2O were plotted against the rate of N_2O consumption to determine whether saturation of N_2O consumption activity (i.e., V_{max}) was observed within the examined concentration range. When saturation in the Michaelis-Menten curve was not observed, the experiments were repeated with increased amounts of N_2O so that consumption rates could be determined at higher N_2O concentrations. Since the added amount of N_2O was small (<1%) relative to the amount already consumed by the cultures, additional growth was considered negligible and confirmed by constant OD_{600} values. Thus, the amount of catalyst (i.e., the amount of NosZ per cell) was assumed to be constant. This approach was applied to all cultures to determine the optimal N_2O concentration ranges for the kinetic measurements. In addition, the preliminary experiments determined the appropriate cell densities to avoid N_2O concentration decreases exceeding 5% between consecutive measurements.

For each experiment, 0.5 ml of culture grown with 1 mM NO_3^- was inoculated into vessels containing fresh medium. Subsequently, N_2O (2 ml for *D. aromatica* strain RCB and *D. denitrificans* strain ED-1, 4 ml for *A. dehalogenans* strain 2CP-C and *S. loihica* strain PV-4) was added to the headspace and the culture bottles were incubated at 30°C on a shaker platform set to 110 rpm until the N_2O concentration reached the detection limit of the Agilent 7890 gas chromatograph. The *D. aromatica* strain RCB and *D. denitrificans* strain ED-1 cultures used for rate determination were prepared by transferring 5-ml aliquots from N_2O-depleted culture bottles with these organisms to autoclaved 160-ml serum bottles filled with N_2 gas at atmospheric pressure. Each vessel received 0.4 ml of 5% (vol/vol) N_2O (N_2O gas diluted with N_2). The remaining cell suspensions were used for the dry weight measurements described below. After *A. dehalogenans* strain 2CP-C and *S. loihica* strain PV-4 cultures had consumed the initial amount of N_2O, additional N_2O (0.4 ml of 5% N_2O for *A. dehalogenans* strain 2CP-C cultures, 0.3 ml of 99% N_2O for *S. loihica* strain PV-4 cultures) was provided for construction of progression curves for N_2O consumption. Following a 10-min equilibration period, gas chromatography measurements were taken at 2- to 5-min intervals. The total amounts of N_2O in the bottles at these time points were calculated from the headspace N_2O concentrations. Progression curves were prepared from three independent experiments, each of which was performed with a single culture bottle for each measurement. From each progression curve, 5 to 6 linear sections, each with at least three datum points, were selected. The slope of each linear section (i.e., a rate value) generated a datum point in the Michaelis-Menten plot. For each linear region, the averages of the aqueous-phase N_2O concentrations served as the representative N_2O concentrations, and the N_2O consumption rates were determined from the slope of the linear regression lines. Immediately following the completion of the kinetic measurements, the culture suspensions were used for cell dry weight determinations.

Due to the fast growth and rapid consumption of N_2O in *P. stutzeri* strain DCP-Ps1 cultures, the determination of the Michaelis-Menten parameters required a different approach. Following growth in 160-ml serum bottles containing 100 ml of medium with 5 mM acetate and 4 ml of N_2O (i.e., the N_2O was completely consumed), aliquots of 5-ml cell suspensions were transferred to serum bottles filled with N_2 gas and sealed with black butyl rubber stoppers. After adjustment to atmospheric pressure, 0.5 ml of 5% N_2O was injected, and after 10 min of equilibration at 30°C and agitation at 110 rpm, up to six gas chromatography measurements were taken at 4-min intervals. The gas chromatography measurements were repeated with various initial quantities of N_2O (1.0 ml of 5% N_2O; 0.1, 0.2, 0.5, and 1.0 ml of >99% N_2O) and various intervals between each measurement. The Michaelis-Menten plot was generated as described above. This entire procedure was independently repeated three times. The data from each individual experiment were combined after normalization by the total biomass (dry weight) of the culture and used in the nonlinear regression analysis.

The volumes of cell suspensions remaining after the rate measurements (i.e., 30 ml for the *D. aromatica* strain RCB, *D. denitrificans* strain ED-1, and *P. stutzeri* strain DCP-Ps1 cultures and 15 ml for the *A. dehalogenans* strain 2CP-C and *S. loihica* strain PV-4 cultures) were used to determine the dry weight of the biomass.

The Michaelis-Menten parameters K_s and V_{max} were determined from the nonlinear regression analysis of the plotted data using SPSS 22 for Mac (IBM Corp., Armonk, NY, USA).

Identification of *nosZ* in *D. denitrificans* strain ED-1. *D. denitrificans* strain ED-1 was grown in a 160-ml serum bottle amended with 5 mM acetate and 2 mM NO_3^- and incubated until the NO_3^- was consumed. From a 50-ml cell suspension, genomic DNA was isolated using the cetyltrimethylammonium bromide (CTAB) method (http://jgi.doe.gov/collaborate-with-jgi/pmo-overview/protocols-sample-preparation-information/). RNA was removed with RNase I (Thermo Scientific, Waltham, MA), and the quantity and purity of the extracted genomic DNA were confirmed with a Qubit fluorometer (Life Technologies, Carlsbad, CA) and a NanoDrop 1100 spectrophotometer (NanoDrop Technologies, Wilmington, DE), respectively. The genomic DNA was sequenced using the Illumina MiSeq platform, and 2,193,064 paired-end sequences (total length, 293 Mbp) were obtained. To confirm that the sequenced organism was *D. denitrificans* strain ED-1 and to identify the *nosZ* gene(s) in the genomic fragments, CLC Genomic Workbench software (CLC bio, Aarhus, Denmark) was used to map the sequence reads onto the 16S rRNA gene sequence of *D. denitrificans* strain ED-1 (GenBank accession number AJ318917.1) and *nosZ* sequences of *D. aromatica* strain RCB (Daro_1571 and Daro_1575 in CP000089.1) and *D. denitrificans* strain ED-1 (AJ704212.1) obtained from the NCBI database.

Nucleotide sequence accession numbers. The *nosZ* consensus sequence of *D. denitrificans* and the *D. denitrificans* draft genome sequence have been submitted to the NCBI GenBank database (accession numbers KT592356.1 and GCA_001551835.1, respectively).

RESULTS

Growth with N_2O as the electron acceptor. All tested cultures grew with N_2O provided as the electron acceptor, whereas no growth was observed in the same medium without N_2O (data not shown). The bacterial cultures exhibited distinct growth charac-

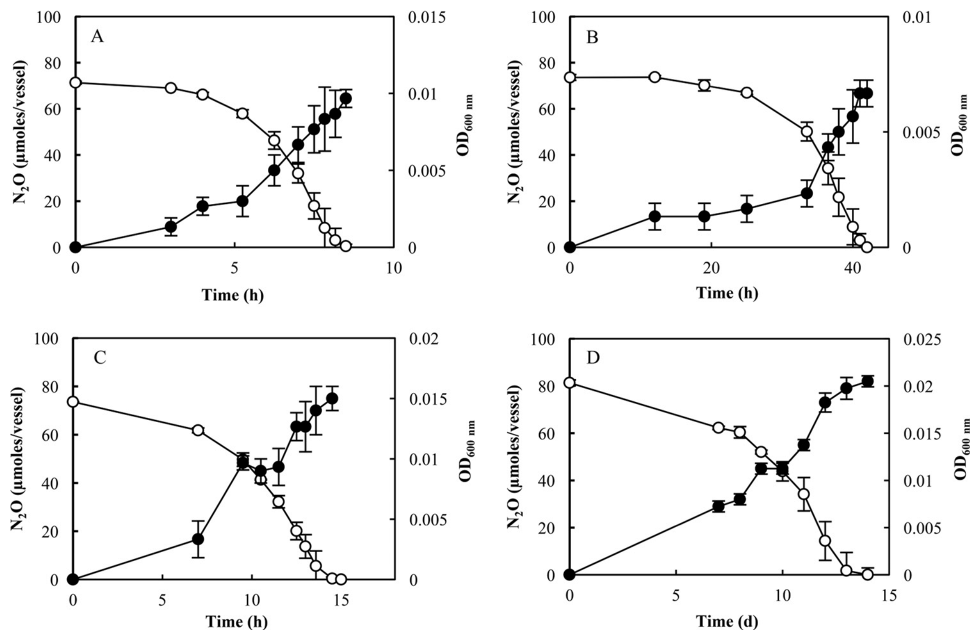

FIG 1 Growth (●) and N_2O consumption (○) in cultures of *P. stutzeri* strain DCP-Ps1 (clade I) (A), *S. loihica* strain PV-4 (clade I) (B), *D. aromatica* strain RCB (clade II) (C), and *A. dehalogenans* strain 2CP-C (clade II) (D). The error bars represent the standard deviations of measurements made with samples extracted from three separate culture vessels.

teristics with N_2O (Fig. 1; Table 2). *P. stutzeri* strain DCP-Ps1 cultures consumed 71.4 ± 0.6 μmol N_2O and grew to a final OD_{600} of 0.009 ± 0.001. *S. loihica* strain PV-4 grew to a final OD_{600} value of 0.007 ± 0.001 after 42 h of incubation with the concomitant consumption of 73.6 ± 1.2 μmol of N_2O. *D. aromatica* strain RCB and *A. dehalogenans* strain 2CP-C grew to significantly higher OD_{600} values but consumed similar amounts of N_2O. *D. aromatica* strain RCB consumed 73.6 ± 0.1 μmol N_2O in <15 h, growing to a final cell density of OD_{600} of 0.015 ± 0.001. *A. dehalogenans* strain 2CP-C required 14 days to consume 81.3 ± 2.4 μmol N_2O and reach OD_{600} readings of 0.021 ± 0.001. The highest rate of N_2O consumption was observed for *P. stutzeri* strain DCP-Ps1, with the maximum consumption rate being 282 nmol h^{-1} per ml of cell culture. The minimum rate of 8.3 nmol h^{-1} per ml of cell culture was observed for *A. dehalogenans* strain 2CP-C. No consistency in terms of the growth rates between bacteria with the same type of NosZ was observed; however, the final OD_{600} values suggested that the clade II N_2O reducers consistently yielded more biomass than clade I N_2O reducers (Table 2).

The biomass yield measurements were within the range of values previously reported for cultures grown with N_2O as the sole electron acceptor (15, 31); however, higher yields of clade II organisms suggest differences in energy conservation efficiencies during growth with N_2O (Table 2). The biomass yields of *A. dehalogenans* strain 2CP-C (11.2 ± 0.5 mg cell dry weight mmol N_2O^{-1}) and *D. aromatica* strain RCB (10.2 ± 0.7 mg cell dry weight mmol N_2O^{-1}) were significantly greater ($P < 0.05$) than the biomass yields of *P. stutzeri* strain DCP-Ps1 (7.24 ± 0.45 mg cell dry weight mmol N_2O^{-1}) and *S. loihica* strain PV-4 (6.31 ± 1.31 mg cell dry weight mmol N_2O^{-1}) (Table 2). The clade II N_2O reducers with the clade II *nosZ* had significantly higher biomass yields than the clade I N_2O reducers with the clade I *nosZ*, suggesting that the clade II NosZ allows more efficient energy conservation.

Michaelis-Menten parameters of N_2O consumption. Whole-cell Michaelis-Menten parameters K_s and V_{max} were determined for the bacterial strains listed in Table 1 (Fig. 2). *P. stutzeri* strain DCP-Ps1 exhibited the highest V_{max} of 4.16 ± 0.44 μmol min^{-1} mg $biomass^{-1}$, a value at least five times greater than the V_{max} values of any other organism tested. As implicated from its low

TABLE 2 Growth characteristics and K_s values of bacterial species with clade I or clade II *nosZ* in batch culture experiments[a]

Organism	*nosZ* clade	Exponential growth rate (h^{-1})	Final OD_{600}	Biomass yield (mg [dry wt]/mmol N_2O consumed)	K_s (μM) for N_2O	Maximum N_2O consumption rate (μmol/min/mg biomass)
Pseudomonas stutzeri strain DCP-1	I	0.326 (0.033)	0.009 (0.001)	7.24 (0.45)	35.5 (9.3)	4.16 (0.44)
Shewanella loihica strain PV-4	I	0.0892 (0.0131)	0.007 (0.001)	6.31 (1.31)	7.07 (1.13)	0.446 (0.024)
Dechloromonas aromatica strain RCB	II	0.182 (0.030)	0.015 (0.001)	10.2 (0.7)	0.324 (0.078)	0.461 (0.034)
Anaeromyxobacter dehalogenans strain 2CP-C	II	0.00762 (0.00072)	0.021 (0.001)	11.2 (0.5)	1.34 (0.35)	0.0171 (0.0024)

[a] For yield measurements, cell biomass was determined following the complete consumption of 0.16 mmol N_2O. The electron donor acetate was provided in excess (0.5 mmol). The numbers in parentheses represent the standard errors. The standard errors of the growth rates measured during exponential growth, half-saturation constants, and maximum rates of N_2O consumption were determined from the linear or nonlinear regression analysis using SPSS 22 for Mac with data obtained from triplicate experiments. The standard errors of the final OD_{600} and biomass yields were determined from the standard deviations of data for triplicate samples.

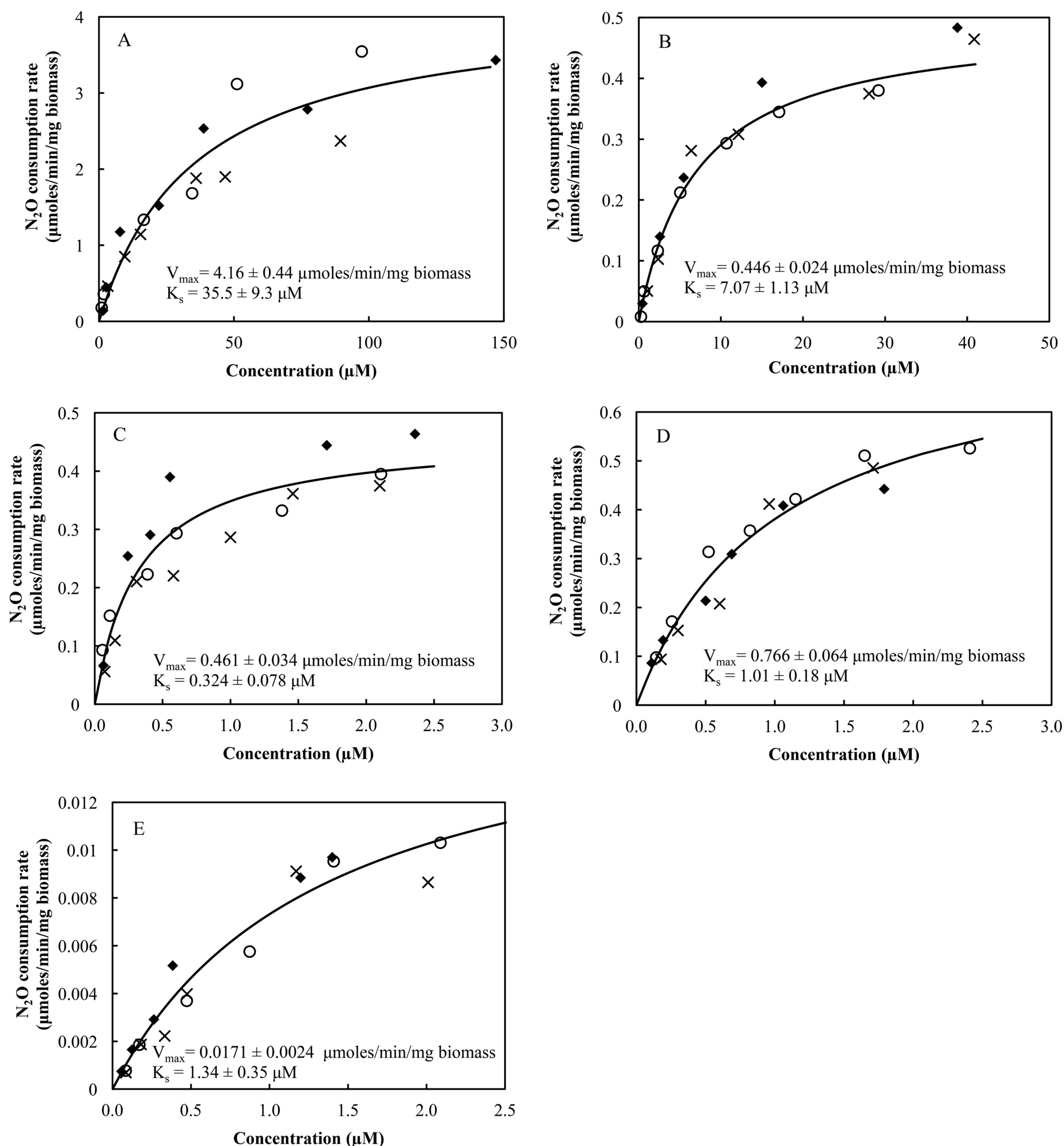

FIG 2 Whole-cell Michaelis-Menten kinetics curves of *P. stutzeri* strain DCP-Ps1 (clade I) (A), *S. loihica* strain PV-4 (clade I) (B), *D. aromatica* strain RCB (clade II) (C), *D. denitrificans* strain ED-1 (clade II) (D), and *A. dehalogenans* strain 2CP-C (clade II) (E). The different symbols (○, ×, and ◆) represent the results from independent experiments.

growth rate on N_2O, the lowest V_{max} value (0.0171 ± 0.0024 μmol min^{-1} mg $biomass^{-1}$) was observed for *A. dehalogenans* strain 2CP-C. The lowest K_s value of 0.324 ± 0.078 μM was observed for *D. aromatica* strain RCB; that value was ~100 times lower than the K_s value observed for *P. stutzeri* strain DCP-Ps1 (35.5 ± 9.3 μM). *D. denitrificans* strain ED-1 and *A. dehalogenans* strain 2CP-C demonstrated statistically significantly similar K_s values of 1.01 ± 0.18 μM and 1.34 ± 0.35 μM, respectively ($P > 0.05$). The K_s value of *S. loihica* strain PV-4 was 7.07 ± 1.13 μM, which fell between the K_s values of N_2O reducers harboring a clade II *nosZ* and the K_s value of *P. stutzeri* strain DCP-Ps1 harboring a clade I *nosZ* ($P < 0.05$).

Identification of *nosZ* in *D. denitrificans*. On the basis of *nosZ* amplicon sequence analysis, the genome of *D. denitrificans* strain ED-1 was originally reported to encode a clade I NosZ with 92% amino acid sequence identity to the NosZ of *Bradyrhizobium japonicum* strain USDA110 (32). Unexpectedly, we determined similar K_s and V_{max} values for *D. denitrificans* strain ED-1 (reported to possess a clade I *nosZ*) and *D. aromatica* strain RCB with a clade II *nosZ*. To verify the type of NosZ produced by strain ED-1, the genome of this organism was sequenced. Assuming that *D. denitrificans* strain ED-1 and *D. aromatica* strain RCB share similarly sized genomes of 4.5 Mbp, coverage of the *D. denitrificans* strain ED-1 genome of ~50 times was obtained. No reads

mapped to the *D. denitrificans nosZ* nucleotide sequence in the NCBI database (GenBank accession number AJ704212.1), suggesting that the *nosZ* sequence previously reported does not belong to strain ED-1. Instead, 1,044 sequence reads mapped onto the *D. aromatica* strain RCB *nosZ* gene, and the 2,292-bp consensus sequence (GenBank accession number KT592356.1) shared 89% nucleotide sequence identity with the *nosZ* sequence of strain RCB. Mapping of the reads onto the 16S rRNA gene sequence of *D. denitrificans* strain ED-1 (GenBank accession number AJ318917.1) resulted in a consensus sequence with only one mismatch over the 1,487-bp gene length, which confirmed that strain ED-1 was sequenced. The genome sequencing efforts indicated that *D. denitrificans* strain ED-1 does not harbor a clade I *nosZ* gene but instead possesses a clade II *nosZ* gene.

DISCUSSION

The whole-cell Michaelis-Menten N_2O reduction kinetics measurements performed with five proteobacterial isolates revealed differences between organisms harboring a clade I *nosZ* and organisms harboring a clade II *nosZ*. The organisms with a clade II *nosZ*, *D. aromatica* strain RCB and *A. dehalogenans* strain 2CP-C, had K_s values up to 2 orders of magnitude lower than those of the organisms harboring a clade I *nosZ*, *P. stutzeri* strain DCP-Ps1 and *S. loihica* strain PV-4. *D. denitrificans* strain ED-1, chosen as a *Dechloromonas* strain with a clade I *nosZ* (32), exhibited an unexpectedly low K_s that was similar to the values measured for the organisms with a clade II *nosZ*. Sequencing of strain ED-1 genomic DNA revealed that this organism actually possesses a clade II *nosZ* with 89% nucleotide sequence identity (94% amino acid sequence identity) to the *nosZ* of *D. aromatica* strain RCB. Taken together, these findings suggest that clade II organisms exhibit lower K_s values for N_2O than clade I organisms. The notion that clade II organisms have a higher affinity to N_2O than clade I organisms is also supported by previous observations. Betlach and Tiedje (33) observed a K_s value for N_2O reduction by a *Flavobacterium* sp. of ~0.4 μM. Although the *nosZ* gene of the *Flavobacterium* sp. used in that experiment has not been sequenced, all *Flavobacterium nosZ* sequences deposited in the NCBI database belong to clade II and share >89% translated amino acid sequence similarity with each other. On the other hand, the N_2O consumption curves of soybean nodules inoculated with *Bradyrhizobium japonicum* strain USDA110, possessing a clade I *nosZ*, indicated a K_s value for N_2O of greater than 15 μM (34). Thus, the substrate affinity constants reported in prior work support the hypothesis that clade II organisms exhibit a higher affinity to N_2O and play a crucial role in attenuating N_2O emissions. Of course, a broader diversity of organisms harboring *nosZ* should be investigated to conclusively demonstrate that differences in NosZ kinetic properties distinguish the ecophysiology of clade I and clade II organisms.

The distinguishing K_s values, growth yields with N_2O, and N_2O consumption rates may have implications for the competition between the clade I and clade II organismal groups when N_2O is used as the electron acceptor. N_2O concentrations rarely exceed 5 ppm (~0.10 μM in pore water at 30°C and atmospheric pressure) in soils, except for agricultural soils following intensive fertilizer or manure applications (35, 36). Thus, under most environmental conditions, clade II organisms should outcompete clade I N_2O reducers with higher K_s values. The analyses of soil metagenomes support this conclusion, and clade II *nosZ* dominated the *nosZ* gene pool in temperate grassland and tropical forest soils where no fertilizer was applied and elevated N_2O concentrations were not likely (19, 37). In a fertilized agricultural soil ecosystem, N_2O concentrations reaching 1,200 ppmv were observed following the application of cattle urine, and elevated N_2O concentrations exceeding 100 ppmv were sustained for at least 10 days (38). Nitrogen fertilizer application can also increase N_2O flux for prolonged time periods (39). Under such conditions, organisms with high V_{max} values, such as *P. stutzeri*, would have a competitive advantage, regardless of the NosZ substrate affinity, and are predicted to be the dominant N_2O consumers.

Along with the higher affinity to N_2O, the tested clade II organisms produced at least 1.5 times more biomass per mole of N_2O consumed than the tested clade I organisms. Growth yield is a measure of metabolic efficiency (40), and higher growth yields indicate tight coupling between catabolism and energy conservation. A mechanistic understanding about the differences in electron transfer and energy conservation between clade I NosZ and clade II NosZ is lacking, and detailed structural studies are warranted to explain the observed growth yield differences (15, 23, 41, 42). Organisms with more efficient energy conservation mechanisms can direct a greater fraction of electrons toward biomass synthesis (i.e., increased f_s), which provides a competitive advantage, particularly in oligotrophic environments.

Quantitative PCR data and metagenomic analyses revealed that *nosZ* genes affiliated with the *Anaeromyxobacter* genus are abundant in agricultural soils of the U.S. Midwest corn belt (15, 19). The results of *Anaeromyxobacter* genome analyses suggest that members of this genus do not possess NO-forming nitrite reductase genes (*nirK* or *nirS*) but share a clade II *nosZ* gene (15). Members of the *Anaeromyxobacter* genus are versaphiles but have apparently adapted to utilize the N_2O released in N_2O-producing biogeochemical reactions occurring in the soil environment, as suggested by the high growth yield of strain 2CP-C (~1.8-fold higher than that of *P. stutzeri*) and the low K_s of strain 2CP-C (~22-fold lower than that of *P. stutzeri*). Despite the lower K_s, the N_2O consumption rate calculated with experimentally acquired V_{max} and K_s values was orders of magnitudes lower for strain 2CP-C than for *P. stutzeri* strain DCP-Ps1 at any N_2O concentration. V_{max} is less relevant than K_s for survival and growth under famine (i.e., low N_2O concentration) conditions in the environment, as would be expected in natural, nonfertilized soils. Observations made with high-affinity methanotrophs support this conclusion, and the half-saturation constants and threshold concentrations for methane were more relevant for sustenance of methane oxidation than V_{max} or catalytic efficiency (i.e., V_{max}/K_s) (43). Fast-growing r-strategists, such as *Pseudomonas* spp., generally have high V_{max} values for substrate consumption when the substrate is plentiful and, thus, an advantage in the competition for shared resources; however, a trade-off for fast growth is the loss of efficiency and a high maintenance energy (44, 45). Indeed, *P. stutzeri* had a growth yield 1.8-fold lower than the value measured for *Anaeromyxobacter dehalogenans* strain 2CP-C. Fast-growing r-strategists are rarely abundant in nutrient-limited soil environments, and the slow-growing K strategists (e.g., *Anaeromyxobacter*, *Opitutus*, and *Gemmatimonas* populations) are generally more robust under such conditions (19, 46, 47). Competition experiments performed under controlled N_2O flux conditions will provide further insights and directly reveal the relevance of clade I versus clade II organisms for modulating soil N_2O emissions.

Several studies have reported on the N_2O sink capacities of natural soils as well as agricultural soils managed under various conditions (18, 48–50). The reason why soils act as net N_2O sources or sinks is a controversial topic (10, 18, 49, 51–55). To provide an explanation in an ecological context, the abundance of denitrifiers lacking *nosZ* genes was used to explain N_2O emission from soil (10, 54, 56). A recent study correlated the soil N_2O sink capacity with the abundance and phylogenetic diversity of clade II organisms (18); however, causality was not demonstrated. The findings reported here provide a plausible mechanistic explanation for the observed correlation between clade II *nosZ* abundance and a soil's N_2O sink capacity. The observation that NosZ kinetic properties distinguish groups of N_2O-consuming bacteria is an example of how a detailed understanding of the microbiology can provide relevant information to advance models of N_2O emission from diverse soil biomes. The specific stimulation of bacteria with clade II *nosZ* may provide opportunities to curb N_2O emissions from relevant soil ecosystems.

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