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Biokinetic Characterization and Activities of N_2O-Reducing Bacteria in Response to Various Oxygen Levels

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Nitrous oxide (N_2O)-reducing bacteria, which reduce N_2O to nitrogen in the absence of oxygen, are phylogenetically spread throughout various taxa and have a potential role as N_2O sinks in the environment. However, research on their physiological traits has been limited. In particular, their activities under microaerophilic and aerobic conditions, which severely inhibit N_2O reduction, remain poorly understood. We used an O_2 and N_2O micro-respirometric system to compare the N_2O reduction kinetics of four strains, i.e., two strains of an *Azospira* sp., harboring clade II type *nosZ*, and *Pseudomonas stutzeri* and *Paracoccus denitrificans*, harboring clade I type *nosZ*, in the presence and absence of oxygen. In the absence of oxygen, the highest N_2O-reducing activity, $V_{m,N2O}$, was 5.80 ± 1.78 × 10^{-3} pmol/h/cell of *Azospira* sp. I13, and the highest and lowest half-saturation constants were 34.8 ± 10.2 μM for *Pa. denitirificans* and 0.866 ± 0.29 μM for *Azospira* sp. I09. Only *Azospira* sp. I09 showed N_2O-reducing activity under microaerophilic conditions at oxygen concentrations below 110 μM, although the activity was low (10% of $V_{m,N2O}$). This trait is represented by the higher O_2 inhibition coefficient than those of the other strains. The activation rates of N_2O reductase, which describe the resilience of the N_2O reduction activity after O_2 exposure, differ for the two strains of *Azospira* sp. (0.319 ± 0.028 h^{-1} for strain I09 and 0.397 ± 0.064 h^{-1} for strain I13) and *Ps. stutzeri* (0.200 ± 0.013 h^{-1}), suggesting that *Azospira* sp. has a potential for rapid recovery of N_2O reduction and tolerance against O_2 inhibition. These physiological characteristics of *Azospira* sp. can be of promise for mitigation of N_2O emission in industrial applications.

Keywords: nitrous oxide reduction, O_2 inhibition, heterotrophic denitrification, biokinetic analysis, microsensor

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INTRODUCTION

Nitrous oxide (N_2O) is an ozone-depleting and greenhouse gas (Ravishankara et al., 2009), therefore it is important to decrease N_2O emissions from natural ecosystems, agriculture, and industrial systems. A large fraction of N_2O is emitted from agricultural croplands (IPCC, 2013; Harter et al., 2014). Emissions from industrial systems, mainly wastewater treatment plants, have become more significant as a result of upgrading of biological nitrogen removal processes, i.e., nitrification–denitrification or partial nitrification–anammox processes (Law et al., 2012). N_2O

is produced via multiple biological and abiotic pathways, e.g., in denitrification as an intermediate (Philippot et al., 2011; Wunderlin et al., 2011; Ishii et al., 2014), in the nitrifier denitrification of ammonia-oxidizing microorganisms (Zhu et al., 2013; Ali et al., 2016), and in chemical oxidation of hydroxylamine (Soler-Jofra et al., 2016; Terada et al., 2017). It is consumed mainly by denitrifying bacteria harboring a N_2O reductase system (Nos) (Henry et al., 2006; Zumft and Kroneck, 2006; Jones et al., 2008; Pauleta et al., 2013). In subsequent denitrification steps, N_2O reduction is severely affected by environmental factors, i.e., pH, availability of electron donors, and dissolved oxygen (DO) (Law et al., 2012; Pan et al., 2013). Although the physiological traits on N_2O reduction by canonical denitrifying species, i.e., the genera *Pseudomonas* and *Paracoccus* (Zumft, 1997; Vollack and Zumft, 2001; Philippot, 2002; Read-Daily et al., 2016), have been studied to date, knowledge is still limited. Comprehensive and thorough physiological research on N_2O-reducing bacteria under various environmental conditions is therefore warranted.

Recent metagenomic analyses have shown that N_2O-reducing bacteria that harbor Nos can be classified into two clade types: clade I and clade II (Sanford et al., 2012; Jones et al., 2013). It has been reported that the abundances of the two clades potentially depend on environmental conditions, e.g., pH, concentration of calcium ion, and C/N ratio, and niche differentiation probably occurs because of their physiological characteristics (Jones et al., 2014; Domeignoz-Horta et al., 2015; Juhanson et al., 2017). Some clade II type N_2O-reducing bacteria reportedly have lower half-saturation constants for N_2O than do those affiliated to clade I type bacteria, suggesting that a low N_2O concentration favors growth of clade II type N_2O-reducing bacteria (Yoon et al., 2016). Additionally, most non-denitrifying N_2O-reducing bacteria, which are unable to reduce nitrite and nitrate, are clade II type (Sanford et al., 2012; Hallin et al., 2018). Given these traits of clade II type bacteria, reports suggest that they potentially play an important role as N_2O sinks (Jones et al., 2014; Orellana et al., 2014).

Studies of the oxygen effects are of fundamental and engineering importance. The DO concentration determines N_2O emissions from soils and wastewater treatment plants via mainly nitrifier–denitrification and heterotrophic denitrification (Tallec et al., 2006; Morley et al., 2008; Riya et al., 2012). Studies on gene transcription and enzyme expression have suggested that Nos expression is regulated by the DO concentration (Körner and Zumft, 1989; Bergaust et al., 2012) and, more importantly, that the Nos enzyme is inactivated by oxygen (Pauleta et al., 2013). In addition, the susceptibility of N_2O-reducing activity to the oxygen concentration is distinct at species or strain levels, as reported for the genera *Thauera* and *Pseudomonas* (Miyahara et al., 2010; Liu et al., 2013; Zheng et al., 2014). However, the influence of O_2 on N_2O reduction by clade II type N_2O-reducing bacteria has not been comprehensively studied, except *Gemmatimonas aurantiaca* strain T-27 (Park et al., 2017). To enable their use in engineering applications as N_2O sinks, the effects of oxygen on the N_2O reduction activities of clade I and II type N_2O-reducing bacteria need to be compared based on biokinetic analysis.

In this study, we, for the first time, compared the N_2O reduction kinetics of clade I and clade II type N_2O-reducing bacteria in the presence and absence of oxygen. *Pseudomonas stutzeri* and *Paracoccus denitrificans,* harboring *nosZ* clade I type, and two strains of *Azospira* sp., isolated from a N_2O-fed enrichment device (unpublished data) inoculated with municipal wastewater treatment biomass, were subject to biokinetic comparison.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Two *Azospira* sp. strains and two strains of canonical denitrifiers, i.e., *Ps. stuzeri* strain JCM5965 (ATCC17588) and *Pa. denitrificans* strain NBRC102528 (ATCC17741), were used in this study. *Azospira* sp. strains I09 and I13, classified as Betaproteobacteria, were isolated from enrichment devices supplying N_2O as an electron acceptor (unpublished data). *Ps. stutzeri* and *Pa. denitrificans* were chosen because they are widely used as canonical denitrifiers (Körner et al., 1987; Baumann et al., 1996; Miyahara et al., 2010; Black et al., 2016). For comparison with other biokinetic studies, weight of a bacterial cell was determined as 0.388, 0.604, 0.743, and 0.787 pg-dry weight/cell for *Azospira sp.* strain I09, I13, *Ps. stutzeri* and *Pa. denitrificans*, respectively.

All the strains were aerobically pre-grown in an autoclaved nutrient medium containing (per liter of distilled water) 5.0 g of Bacto Peptone (BD-Difco, NJ), 3.0 g of Oxoid™ Lab-Lemco meat extract (Thermo Scientific, MA), and 5.0 g of NaCl.

Chemical Analyses

Dissolved organic carbon and dissolved total nitrogen were determined with a TOC analyzer, installing a total nitrogen measurement unit (5000A, Shimadzu, Kyoto, Japan). pH was measured using a pH meter (F-52, HORIBA, Kyoto, Japan). The gaseous N_2O concentration was determined using a gas chromatograph with an electron-capture detector (GC-14B, Shimadzu, Kyoto, Japan) instrument. The measurement conditions were described in a previous paper (Terada et al., 2013).

Activity Measurements

The pre-grown bacterial strains were harvested in centrifuge tubes at the early stationary phase. Two strains of *Azospira* spp. (I09 and I13) were washed twice with 0.05 × phosphate-buffered saline (PBS) by centrifugation at 5,000 rpm for 5 min, and re-suspended in the experimental medium. Our preliminary experiment showed that the N_2O-reducing activities of *Ps. stutzeri* JCM5965 and *Pa. denitrificans* NBRC102528 were significantly reduced by washing by centrifugation (data not shown). The pre-incubated cell suspensions of these two strains were therefore diluted with the experimental medium instead of washing. The experimental medium was placed in a conical flask, and the flask was sealed with a silicone cap (Shin-Etsu Polymer Co., Tokyo, Japan). The medium was mixed by shaking at 100 rpm at 30°C. The phosphate buffer medium, devoid of electron donors, contained (per liter of distilled water) 100 mg

of KH_2PO_4, 6.6 mg of NaCl, 8.20 mg of $MgSO_4 \cdot 7H_2O$, 13.4 mg of KCl, 115 mg of NH_4Cl, 188 mg of $NaHCO_3$, and 1 mL of a trace element solution consisting of (per liter of distilled water) 10 g of $FeSO_4$ $7H_2O$, 10 g of $FeCl_3$ $7H_2O$, 2 g of $ZnSO_4$ $7H_2O$, 4 g of $CuSO_4$ $7H_2O$, 0.5 g of $NaMoO_4$ $2H_2O$, 0.1 g of $MnCl_2$ $4H_2O$, 0.1 g of H_3BO_4, 0.3 g of Na_2SeO_3, and 10 g of citric acid (Miyahara et al., 2010). After sterilization, pH of the medium was adjusted to 7.5 with 1 M HCl.

The O_2 and N_2O consumption behaviors were investigated using an O_2 and N_2O micro-respiration system with amperometric microsensors (Unisense, Aarhus, Denmark). This system consisted of a Clark-type N_2O sensor (N2O-MR), O_2 sensor (OX-MR), double-port chamber (10 mL, MR-Ch double port), and stirrers installed in a sensor stand (**Figure 1**). The temperature during the experiments was controlled at 30 ± 0.2°C by a water bath. The experimental sequences of O_2 and N_2O consumption are shown in **Figure 1B**. The chamber was filled with the cell suspension, in which electrodes were inserted. Highly concentrated N_2O water (27.05–24.09 mM at 20–25°C and 0% salinity Weiss and Price, 1980) was prepared by pouring 25 mL of deionized water in a 50 mL vial, supplying pure N_2O gas to the water for 10 min at a room temperature and subsequently sealing the vial with a butyl rubber stopper. The highly concentrated N_2O water with a volume of 15–20 μL was injected into the chamber from the injection port (**Figure 1A**) using a Hamilton syringe, to replace the bacterial cell suspension and to give an initial dissolved N_2O concentration of approximately 50 μM. Then 250 mM sodium acetate solution (25 μL) was added to adjust the initial concentration to 625 μM. To achieve a homogeneous condition immediately, the glass-coated stir bar, was stirred at 600 rpm for 5 min after N_2O and carbon source injection and then at 300 rpm during the measurements. O_2 and N_2O concentrations were continuously monitored using SensorTrace Suite ver.2.8.0 (Unisense, Aarhus, Denmark). After the N_2O was completely consumed, highly concentrated N_2O water was injected again. The number of N_2O injections was changed depending on the distinct trends in the N_2O reduction rates observed among the tested strains. After the measurements, a sample of the cell suspension was immediately taken from the chamber and fixed with a 2% glutaraldehyde solution to determine the cell number, as previously described (Lunau et al., 2005). Briefly, the cell-counting procedure was as follows. Ethanol was added to the fixed cell suspension to achieve a final concentration of 10%. The mixture was homogenized for 10 s at 10 W (VP-050, Taitec, Tokyo, Japan), followed by dilution with 0.05 × PBS. The cells were trapped on a 0.2 μm membrane filter (Isopore, Merck Millipore, Germany), and washed twice with 1 mL of sterilized Tris-acetate EDTA buffer. The cells on

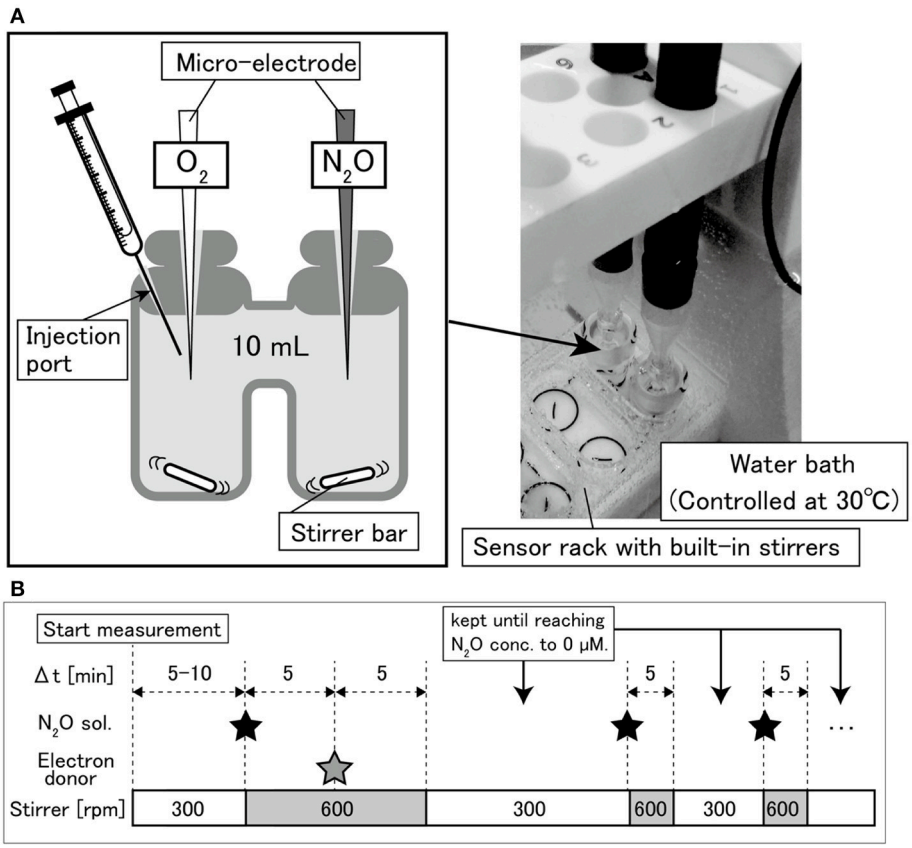

FIGURE 1 | (A) Diagram of double-port chamber and **(B)** experimental sequence of N_2O and O_2 respiration measurements.

the filter were stained with Moviol-SYBR Green I (Thermo Fisher Scientific, MA) and enumerated under a fluorescence microscope (BZ-8100, Keyence, Osaka, Japan).

Kinetic Parameter Estimation

The O_2 and N_2O concentration profiles were smoothed with the function of Sigma Plot 13.0 (Systat software, CA) to remove high-frequency noise and instantaneous reduction rate ($\Delta = 3$–5 s) was calculated and normalized with cell number (Vs [pmol/h/cell]). The maximum O_2 and N_2O uptake rates ($V_{m,O2}$ and $V_{m,N2O}$ [pmol/h/cell]) and the half-saturation constants for O_2 and N_2O ($K_{m,O2}$ and $K_{m,N2O}$ [μM]) were determined by fitting the profiles to the Michaelis–Menten equation (Equation 1) (Martens-Habbena et al., 2009). The $V_{m,N2O}$ value was calculated from the last measurement of the N_2O profiles.

$$V_s = \frac{V_{m,S} \cdot S}{K_{m,S} + S} \quad (1)$$

where S [μM] is the concentration of either O_2 or N_2O. To compare the N_2O reduction activities among the strains, the specific affinity for N_2O ($a_{0,N2O}$ [L/cell/h]) was calculated as follows (Equation 2):

$$a_{0,N2O} = \frac{V_{m,N2O}}{K_{m,N2O}} \times 10^{-6} \quad (2)$$

Statistical analysis was performed with ANOVA (Tukey HSD) in SPSS Statistics (IBM, NY), and statistical significance was evaluated by p-value below 0.05 as a threshold.

The effect of O_2 on the N_2O-reducing activity was investigated by fitting the experimental profiles to a mathematical model. The heterotrophic denitrification model, incorporating the O_2 inhibition (Ni et al., 2011), was used to estimate the degree of O_2 inhibition to N_2O uptake rates. The terms of an electron donor, nitric oxide (NO) and ammonium (NH_4^+) were excluded in the model proposed by Ni et al. (2011) (Equation 3) because theoretically NO is not produced and organic carbon and NH_4^+ were in excess in the medium (i.e., addition of NH_4^+, organic carbon, and N_2O under anoxic conditions).

$$\frac{dS_{N2O}}{dt} = V_{m,N2O} \frac{S_{N2O}}{K_{m,N2O} + S_{N2O}} \frac{K_{I,O2}}{K_{I,O2} + S_{O2}} X \times 10^{-6} \quad (3)$$

where X [cells/L] is the concentration of bacterial cells in the chamber, and $K_{I,O2}$ [μM] is the O_2 inhibition coefficient. For *Azospira* sp. strains I09, I13, and *Ps. stutzeri*, $K_{I,O2}$ values were estimated by fitting the 1st-spiked N_2O profile to the model based on the least-squares method using the solver function of Microsoft Excel ver. 15.26. For *Pa. denitrificans*, determination of $K_{I,O2}$ was not feasible because the trend of N_2O consumption is not explainable by the model. The lowest $K_{I,O2}$ value detected in this study was 0.1 μM due to the detection limit of an O_2 microelectrode.

The relative activity of N_2O reduction rate E [dimensionless] under an anoxic condition was calculated by Equation (4).

$$E = \frac{V_{N2O}}{V_{m,N2O}} \quad (4)$$

V_{N2O} was attained from an N_2O profile with the concentration range from 10 to 40 μM under an anoxic condition. The Nos activation rate (V_{Nos} [h^{-1}]) was defined as a degree of activity recovery of N_2O reduction after changing an aerobic to anoxic condition. V_{Nos} value was acquired by linear approximation of E as a function of elapsed time after DO concentration becomes zero (t_{anoxic}). The analysis was performed with Sigma Plot 13.0.

Quantifying Transcripts of *nosZ* Gene

Dynamics of *nosZ* gene transcripts of *Azospira* sp. strain I09 were quantified by reverse-transcription quantitative PCR (RT-qPCR). A medium, adding (per liter of distilled water) 0.20 g of sodium acetate and 0.050 g of NH_4Cl to the synthetic medium for the respirometric test, was autoclaved, followed by pH adjustment at 7.5 with 1 M HCl. *Azospira* sp. strain I09 was inoculated and aerobically pre-grown at 30°C in a 500 mL bottle until the late-exponential growth phase (OD600 = 0.121 after incubation for 23 h). After the aerobic pre-growth, different gases were supplied via a sterilized filter (HEPA-VENT, GE Healthcare, UK) at three different phases: 1 L/min of air in Phase 1, 0.5 L/min of N_2 in Phase 2, and 0.5 L/min of 100 ppm N_2O/N_2 in Phase 3. The gas flow rate was controlled using a mass flow controller (HORIBA, Kyoto, Japan). DO concentration in the medium was monitored using a DO meter (FDO Multi3410, WTW, Weilheim in Oberbayern, Germany). At each sampling point, cell suspension (10 mL) was transferred to a 15 mL tube and centrifuged at 10,000 rpm for 5 min at 4°C, and the supernatant was subsequently decanted. One milliliter of RNApro solution (FastRNA Pro Blue Kit, MP Biomedicals, CA) as a retardant of RNA degradation was immediately added to completely re-suspend the pellet according to the manufacture's protocol, followed by storage at 4°C until RNA extraction. RNA was extracted with a FastRNA Pro Blue Kit (MP Biomedicals, CA) and quantified by a UV-Vis spectrophotometer (Nanodrop 2000c, Thermo Fisher Scientific, MA). The extracted RNA was reverse-transcribed to complementary DNA with a QuantiTect Reverse Transcription Kit (QIAGEN, Hilden, Germany). The clade II type *nosZ* and 16S rRNA genes were quantified by real-time qPCR using a CFX96 Real-Time PCR Detection System (BioRad Laboratories, CA). The primer sets for clade II type *nosZ* and 16S rRNA genes were nosZ-II-F (5′-CTIGGICCIYTKCAYAC-3′)—nosZ-II-R (5′-GCIGARCARAAITCBGTRC-3′) (Jones et al., 2013) and 341f (5′-CCTACGGGAGGCAGCAG-3′)−517r (5′-ATTACCGCGGCTGCTGG-3′) (Muyzer et al., 1993), respectively. PCR for 16S rRNA gene amplification was initiated with initial denaturation at 95°C for 2 min, followed by 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. PCR for the clade II type *nosZ* gene was initiated with denaturation at 95°C for 1 min, followed by 50 cycles at 95°C for 15 s, 54°C for 30 s, 72°C for 30 s, and 80°C for 30 s. The reaction buffer for the 16S rRNA gene consisted of 10 μL of SsoFast™ EvaGreen® Supermix (BioRad Laboratories, Hercules, CA), 1 μL of 10 mM forward and reverse primers, 5 μL of template, and 3 μL of distilled water. The PCR buffer for the clade II type *nosZ* gene consisted of 10 μL of SYBR Premix Ex Taq II (Tli RNaseH Plus, Takara Bio, Shiga, Japan), 2 μL of 10 mM forward and reverse primers, 1 μL of distilled water, and 5 μL of template.

Plasmid DNA (pGEM-T Easy Vector, Promega, WI) containing each gene was transformed into *Escherichia coli* competent cells (Competent High DH5α, Toyobo, Osaka, Japan) and the plasmids were isolated for each standard solution with a plasmid extraction kit (MagExtractor -Plasmid-, Toyobo, Osaka, Japan). After confirmation of insertion of each functional gene by Sp6 and T7 primers (Huang et al., 2005), the plasmid DNA as a positive control was diluted to obtain standard solutions containing 1.0×10^8 to 1.0×10^2 copies per 5 μL in series. To increase the PCR efficiency, the plasmid containing the *nosZ* clade II amplicon was linearized with the restriction enzyme *Eco*RI (Takara bio, Kyoto, Japan) and used as a standard solution. The gene transcripts of *nosZ* were normalized with the amount of total RNA and gene transcripts of 16S rRNA to trace dynamics of *nosZ* gene expression. The transcripts of 16S rRNA gene was in the same order of magnitude during the tested three phases (Data not shown).

RESULTS

Activity Measurements and Biokinetic Comparison

Activity measurement of each strain was conducted in triplicate. The representative O_2 and N_2O concentrations profiles are shown in **Figure 2** and the two other replicates in SI (Figures S1–S4). All the bacterial strains showed facultative N_2O-reducing activities, consuming O_2 prior to N_2O. However, the O_2 concentration at which N_2O-reducing activity was initiated was not consistent for each strain. Except in the case of *Pa. denitrificans*, the first-spiked N_2O was completely consumed, followed by injection of N_2O-concentrated liquid. For *Azospira* sp. strains I09 and I13, the maximum N_2O reduction rate reached a plateau at the second or third additional N_2O injection. The maximum N_2O reduction rate of *Azospira* sp. I09 after the second injection was normalized to 114 ± 8% ($n = 3$) higher than that after the first injection. The analogous trend was attained for *Azospira* sp. I13, displaying that the maximum N_2O reduction rates after the second and third injections were 132 ± 14% ($n = 3$) and 132 ± 8% ($n = 2$) of the rate after the first one, respectively. For *Ps. stutzeri*, the maximum N_2O reduction rates after the additional injections further increased: 224 ± 69% ($n = 3$) for the second, 267 ± 62% ($n = 3$) for the third, 280 ± 40% ($n = 2$) for the fourth, and 294 ± 29% ($n = 2$) for the fifth, higher than the rate after the first injection. *Pa. denitrificans* was spiked with N_2O once because the reduction rate slowed down below 50 μM N_2O, entailing 10 h to consume the first-spiked N_2O (Figure S4). The initial N_2O concentration hampered accurate measurement for the N_2O reduction activity of *Pa. denitrificans*; therefore, the volume of injected N_2O solution was adjusted to ensure a higher N_2O concentration (100–150 μM) (**Figure 2D** and Figure S4). The maximum N_2O uptake rate ($V_{m,N2O}$) and half saturation constant for N_2O ($K_{m,N2O}$) were estimated by fitting the Michaelis–Menten equation to the N_2O profile at the final injection in each run (**Table 1**). *Azospira* sp. I13 showed the highest $V_{m,N2O,}$ i.e., $5.80 \pm 1.78 \times 10^{-3}$ pmol/h/cell, among all the strains, followed by *Ps. stutzeri* ($1.64 \pm 0.34 \times 10^{-3}$ pmol/h/cell), *Azospira* sp. I09 ($6.34 \pm 0.8 \times 10^{-4}$ pmol/h/cell), and *Pa. denitrificans* ($5.01 \pm 1.0 \times 10^{-4}$ pmol/h/cell). The highest and lowest $K_{m,N2O}$ values were 34.8 ± 10.2 μM for *Pa. denitirificans* and 0.866 ± 0.29 μM for *Azospira* sp. I09, respectively. The other two strains displayed comparable $K_{m,N2O}$

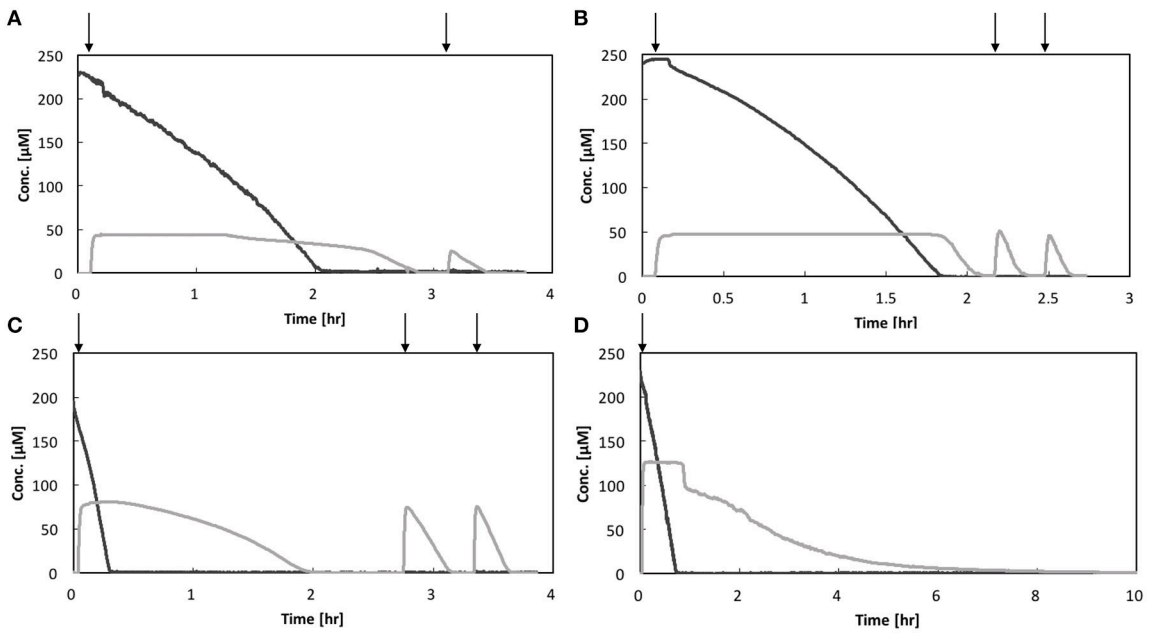

FIGURE 2 | N_2O and O_2 respiration profiles of isolates: **(A)** *Azospira* sp. strain I09, **(B)** *Azospira* sp. strain I13, **(C)** *Pseudomonas stutzeri* JCM5965, and **(D)** *Paracoccus denitrificans* strain NBRC102528. Black line: O_2, gray line: N_2O. Vertical arrows indicate N_2O spikes.

TABLE 1 | Biokinetic parameters for the tested strains.

Strains	*nosZ* type	$V_{m,O2}$ [$\times 10^{-3}$ pmol/h/cell]	$K_{m,O2}$ [μM]	$a_{0,O2}$ [$\times 10^{-9}$ L/cell/h]	$V_{m,N2O}$ [$\times 10^{-3}$ pmol/h/cell]	$K_{m,N2O}$ [μM]	$a_{0,N2O}$ [$\times 10^{-9}$ L/cell/h]	V_{Nos} [h^{-1}]	$K_{I,O2}$ [μM]
Azospira sp. I09	II	1.13 (0.43)[a]	2.90 (1.69)[a]	0.391	0.634 (0.08)[a]	0.866 (0.29)[a]	0.732	0.319 (0.028)	2.33 (1.7)
Azospira sp. I13	II	3.70 (0.45)[b]	1.68 (0.33)[a]	2.21	5.80 (1.78)[b]	3.76 (1.99)[a]	1.54	0.397 (0.064)	0.330 (0.204)
Ps. stutzeri JCM5965	I	3.03 (0.60)[b]	5.94 (1.05)[a]	0.510	1.64 (0.34)[a]	4.01 (0.77)[a]	0.408	0.200 (0.013)	0.164 (0.042)
Pa. denitrificans NBRC102528	I	4.07 (1.11)[b]	5.39 (3.21)[a]	0.756	0.501 (0.10)[a]	34.8 (10.2)[b]	0.0144	*n.a.***	*n.a.***

*These values were acquired from N_2O activity measurements in triplicate. Values in parentheses represent standard deviations. *Statistically different values ($p < 0.05$) are distinguished by superscripts a and b. **Not applicable.*

values of about 4 μM. The N_2O affinities ($a_{0,N2O}$) of *Azospira* sp. strains I13 and I09 were higher than those of *Ps. stutzeri* and *Pa. denitirificans* because of the inherently higher $V_{m,N2O}$ (I13) or lower $K_{m,N2O}$ (I09).

Effects of O_2 on N_2O Reduction

The effects of O_2 on N_2O reduction were compared on the basis of the relative activities of N_2O reduction, E (Equation 4). E as a function of O_2 concentration is shown in **Figure 3**. The effect of the DO concentration on E differed among the tested strains. Although the activity was lower (10% of $V_{m,N2O}$) than that in the absence of oxygen, *Azospira* sp. I09 showed N_2O consumption activity under microaerophilic conditions at DO concentrations of 100–110 μM. In contrast, the DO concentration needed to initiate N_2O consumption by *Azospira* sp. I13 and *Pa. denitrificans* was much lower (25 μM O_2). The N_2O consumptions of these three strains recovered significantly after complete consumption of O_2. *Ps. stutzeri* did not consume N_2O until the O_2 was completely depleted.

The time series for the N_2O relative reduction rates under anoxic conditions are shown in **Figure 4**. *Pa. denitrificans* was excluded from the analysis because the inherently low N_2O affinity of *Pa. denitrificans* hampered accurate measurement of the N_2O reduction rate (**Table 1**). Relative activity of N_2O reduction rate increased linearly in the three strains. The two *Azospira* sp. strains showed the same trend in E-values, and regained 0.8 of the initial value in 1.93 h (I09) and 1.38 h (I13), respectively. In contrast, *Ps. stutzeri* required 4.32 h for 80% recovery of E (**Figure 4**). The Nos activation rates (V_{Nos}), i.e., the slopes in **Figure 4**, were estimated to be 0.319 ± 0.028, 0.397 ± 0.064, and 0.200 ± 0.013 h^{-1} for *Azospira* sp. strain I09, I13, and *Ps. stutzeri*, respectively, indicating the highest V_{Nos} value for *Azospira* sp. strain I13.

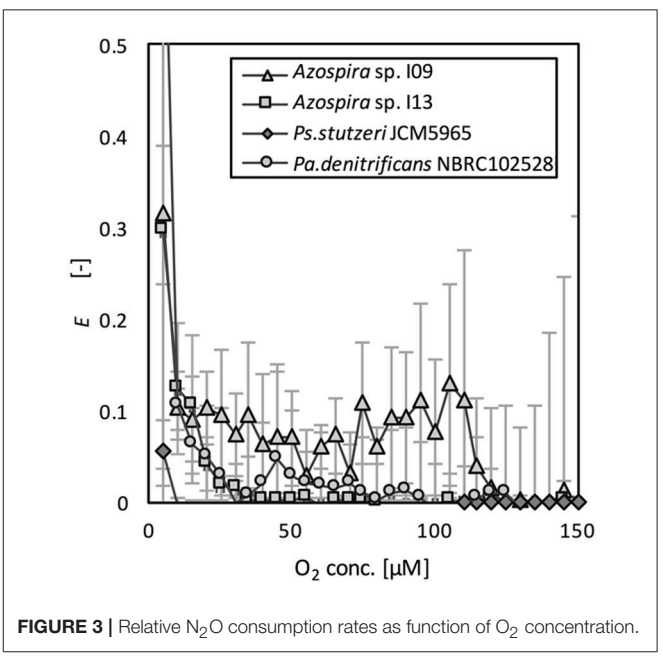

FIGURE 3 | Relative N_2O consumption rates as function of O_2 concentration.

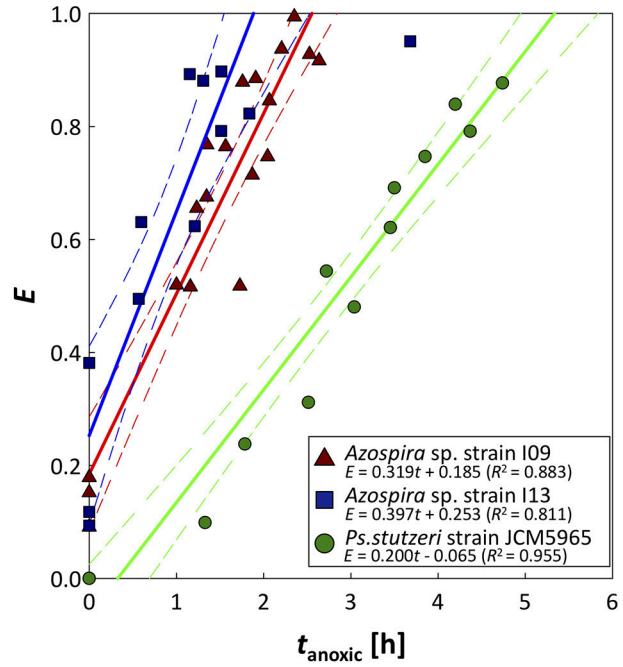

FIGURE 4 | Recovery of N_2O reduction activity after O_2 exposure. Plots are derived from N_2O consumption tests, performed in duplicate or triplicate: $t_{anoxic} = 0$ is elapsed time after DO is completely depleted. Solid and broken lines represent approximated activity recovery for N_2O reduction in each strain and the 95% confidence interval of each approximated line, respectively. For the approximation, triplicate and duplicate data were used for the two *Azospira* sp. strains and *Ps. stutzeri*, respectively.

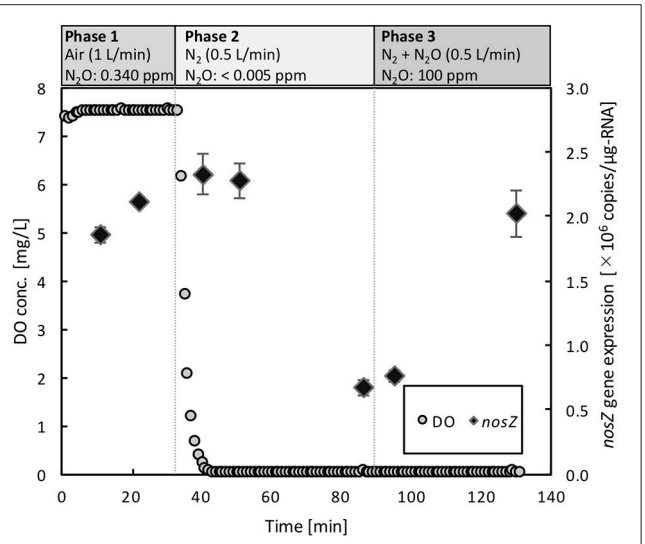

FIGURE 5 | Dynamics of *nosZ* gene expression in *Azospira* sp. I09 under three different redox conditions. The gaseous compositions were ambient air (N_2O: 0.34 ppm) in Phase 1, N_2 (N_2O: <0.005 ppm) in Phase 2, and N_2 + N_2O (N_2O: 100 ppm) in Phase 3, respectively. The gas flow rate was consistently 0.5 L/min.

The effect of O_2 on the N_2O reduction activity was quantitatively compared by determination of biokinetic parameters (Figure S5). $K_{I,O2}$ was estimated by the model fitting for N_2O profiles (Figure S5). *Azospira* sp. strain I09 showed the highest $K_{I,O2}$ value of 2.33 ± 1.7 μM while those of *Azospira* sp. strain I13 and *Ps. stutzeri* were below 0.5 μM as summarized in **Table 1**.

nosZ Gene Transcription of *Azospira* sp. Strain I09

Dynamics of *nosZ* transcription of *Azospira* sp. strain I09, displaying N_2O uptake even at above 100 μM O_2, was monitored under different redox conditions (**Figure 5**). During Phase 1 for aerobic pre-incubation by air bubbling, *nosZ* transcription level slightly increased. Switching to the anoxic condition by pure N_2 bubbling decreased the *nosZ* transcription level after 40 min by 74%. Subsequently, mixing N_2O with N_2 gas to keep N_2O concentration of 100 ppmv (equivalent dissolved N_2O of 2.16 μM at 30°C) in Phase 3 stimulated *nosZ* transcription, reaching the comparable level in Phase 1.

DISCUSSION

This study, for the first time, showed the effects of oxygen on the N_2O reduction activities of *nosZ* clade I and II type N_2O-reducing bacteria in a comparative manner. Monitoring N_2O consumption dynamics has always been challenging because of the difficulty in setting up conditions under which the N_2O and O_2 concentrations are always transitioning. A common batch experiment, measuring the headspace gas in a vial (Bueno et al., 2015), tackled the difficulty of monitoring N_2O and O_2 concentrations within short intervals. The micro-respirometric system using DO and N_2O microsensors used in this study enabled real-time monitoring of these dissolved gases at a much higher resolution (second level) than in conventional methods. The highly resolved profiles detect N_2O-reducing activity even under aerobic conditions. We found that physiological characteristics do not clearly distinguish clade I and II type N_2O-reducing bacteria.

There has been open discussion on whether clade II type N_2O-reducing bacteria have inherently high N_2O affinities compared with those of their clade I type counterparts (Yoon et al., 2016; Hallin et al., 2018). Current research suggested the existence of unique electron transport module in clade II type microorganisms, i.e., *Wolinella succinogenes* (Hein et al., 2017). The feature is advantageous for low-energy translocation pathways and effective energy conversion within the N_2O respiration compared to *nosZ* clade I type bacteria. Our biokinetic analyses of the clade II type N_2O-reducing bacterium, i.e., two *Azospira* sp. strains, show that their half-saturation constants ($K_{m,N2O}$) are lower than those of the two clade I type denitrifying bacteria (**Table 1**). This is in agreement with previous studies, in which clade II type strains, namely *Dechloromonas aromatica* strain RCB and *Anaeromyxobacter dehalogenans* strain 2CP-C, gave lower $K_{m,N2O}$ and higher biomass yields than did clade I type strains (Yoon et al., 2016).

Further comparison, with the substrate affinity $a_{0,N2O}$ as an indicator of N_2O-reducing activity, shows that clade II type *Azospira* sp. strain I13 is a promising N_2O sink (**Table 1**). However, even for the same *Azospira* sp., the two isolates had distinct $a_{0,N2O}$ values. Furthermore, the recent report demonstrated that a chemostat system, supplying N_2O as a sole electron acceptor, enriched clade I type N_2O reducing bacteria with high $a_{0,N2O}$ (Conthe et al., 2018). Given that taxonomic proximity is not necessarily linked to physiology, N_2O-reducing bacteria having very high affinity to N_2O could be broadly distributed irrespective *nosZ* clade type. Comprehensive biokinetic analysis focusing on not only clade II type but also clade I type is needed.

The N_2O reduction activities in the presence of O_2 differed among the four bacterial strains, irrespective of their *nosZ* clade types. *Azospira* sp. strain I09 exhibited N_2O-reducing activity at a DO concentration of 110 μM (3.52 mg/L) and higher O_2 inhibition coefficient (2.33 μM), confirming the participation of aerobic N_2O reduction. Previous physiological studies using isolates or mixed cultures implied that some bacteria are capable of denitrifying and respiring N_2O under aerobic or microaerobic conditions, but the level of DO concentration needed for detectable N_2O-reducing activity depends on the bacterial strain (Liu et al., 2013). *Thauera* sp. strain 63 expresses *nosZ* mRNA under microaerobic conditions (<10 μM), although *T. terpenica* strain DSM12139 does so under obligate anaerobic conditions (Liu et al., 2013). The Nos of *Ps. stutzeri* strain ATCC14405 is expressed at a DO concentration of 119 μM (Körner and Zumft, 1989) and *Ps. stutzeri* strain TR2 showed N_2O-reducing activity at a DO concentration of 35 μM (Miyahara et al., 2010). In a mixed culture, NO_2^- reduction activity by microorganisms in soils appears at a DO concentration of 80 μM (Morley et al., 2008), and denitrification gene transcription is continuously detected under O_2-remaining conditions in coastal sediments (Marchant et al., 2017). On the basis of previous reports, the DO level for the emergence of N_2O consumption by *Azospira* sp. strain I09 (110 μM) is higher than those of *Thauera* sp. strain 63 and *Ps. stutzeri* strain TR2, and comparable to that of *Ps. stutzeri* strain ATCC14405. Thorough, holistic investigations are needed, but our results suggest that *Azospira* sp. strain I09 has promise as a N_2O consumer under microaerophilic conditions.

The activity tolerance of O_2 inhibition was reasonably described applying the Nos activation rate, V_{Nos} (**Figure 4**). The higher values of V_{Nos} for the *Azospira* sp. strains (0.319 ± 0.028h^{-1} for I09 and 0.397 ± 0.064 h^{-1} for I13) than that for *Ps. stutzeri* (0.200 ± 0.013 h^{-1}) indicate that the recovery of N_2O reduction activity by the *Azospira* sp. strains is faster than that by *Ps. stutzeri*. In addition, *E*-values for the *Azospira* sp. strains higher than 0 at $t_{anoxic} = 0$ suggests their superior N_2O uptake resilience to O_2 exposure, which was not observed for *Ps. stutzeri* (**Figure 4**). In previous reports, two mechanisms of O_2 inhibition to N_2O reduction activity have been hypothesized, namely regulation of the RNA transcription level (Arai, 2011; Bergaust et al., 2012) and Nos inactivation (Otte et al., 1996; Miyahara et al., 2010). The quantification of *nosZ* clade II type mRNA transcripts for *Azospira* sp. strain I09 under different redox conditions likely provided Nos reactivation as a limiting factor to determine N_2O uptake rate. The application of RT-qPCR demonstrated that expression of the *nosZ* mRNA by *Azospira* sp. strain I09 was suppressed in the absence of O_2 and N_2O but not in the presence of N_2O and O_2 (Phase 1 and Phase 3 in **Figure 5**). This agrees with the trend that *nosZ* gene is expressed even in the presence of O_2 in coastal sediments (Marchant et al., 2017). The consistent trend corroborates that Nos is probably synthesized even under aerobic or microaerobic conditions. Studies on extracted and purified Nos support Nos recovery after switching from aerobic to anoxic conditions (Ghosh et al., 2003; Chan et al., 2004). On the basis of these observations, it is likely that recovery of N_2O consumption depends on the rate of Nos reactivation.

Toward N_2O mitigation in engineered systems, the degree of Nos reactivation after O_2 exposure should be quantitatively estimated. Combination of a respirometric analysis with a mechanistic modeling potentially accelerates selection of highly efficient N_2O-reducing bacteria able to respire N_2O under microaerophilic conditions. The heterotrophic denitrification model, incorporating the O_2 inhibition (Equation 3) (Ni et al., 2011), likely necessitates the extension due to some discrepancies with the experimental results (Figure S5). Validation and verification of the extended models, e.g., an enzyme-explicit denitrification model, where a ratio of inactive Nos to total Nos and its recovery rate determine N_2O uptake rate (Zheng and Doskey, 2015) and an integrated model incorporating the O_2 inhibition and Nos recovery, warrant future intensive studies.

This study shows that strains of the genus *Azospira* are promising N_2O reducers because of their O_2 inhibition tolerance and high affinity for N_2O. Use of the proposed kinetic parameters, V_{Nos} and $K_{I,O2}$, provides, for the first time, quantitative information on Nos recovery from O_2 inhibition. Application of respirometric biokinetic analysis will be useful in developing N_2O mitigation strategies using N_2O reducers in engineered systems. Comprehensive screening of N_2O-reducing bacteria displaying high N_2O affinities ($a_{0,N2O}$) and high resilience against O_2 exposure (V_{Nos}, $K_{I,O2}$) is required.

CONCLUSION

This study investigated the effects of O_2 on the N_2O consumption biokinetics of bacterial isolates affiliated to *nosZ* clade I and II types. Respirometric assays showed that the N_2O affinities of two *Azospira* sp. strains of *nosZ* clade II type were higher than those of *Pa. denitrificans* and *Ps. stutzeri* of *nosZ* clade I type. However, the clade type does not completely explain the different N_2O affinities, and this suggests that the physiological traits of N_2O-reducing bacteria differ at the species and strain levels. The N_2O consumption activities are also significantly affected by the O_2 levels but the degree of O_2 inhibition differs depending on the species or strain rather than the *nosZ* type. *Azospira* sp. strain I09 showed aerobic N_2O-reducing activity and *nosZ* gene transcription at a DO concentration of 110 μM. Recoveries of the N_2O-reducing activities of *Azospira* sp. strains I09 and I13 after O_2 exposure are faster than that of *Ps.*

stutzeri of *nosZ* clade I type. This underscores that the Nos of *Azospira* spp. is reactivated after switching from aerobic to anoxic conditions. Our results provide new information on the physiological characteristics of N_2O-reducing bacteria, and also a valuable investigative parameter involving a respirometric analysis. This comprehensive and thorough analyses based on a respirometric approach will accelerate the discovery of novel N_2O reducers and contribute to N_2O mitigation in engineering applications.

AUTHOR CONTRIBUTIONS

AT: designed and led the study. TS: performed the biokinetic estimation and oxygen effect on nitrous oxide reduction of the isolates. TS: wrote the paper with major edits and inputs from SR, MH, and AT.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00697/full#supplementary-material

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