Organic carbon determines nitrous oxide consumption activity of clade I and II nosZ bacteria: Genomic and biokinetic insights

Chuang Qi a,b, Yiwen Zhou b, Toshikazu Suenaga b,c,h, Kohei Oba b, Jilai Lu a, Guoxiang Wang d,e, Limin Zhang b,f, Sukhwan Yoon g, Akihiko Terada h

a College of Food Science and Engineering, Nanjing University of Finance and Economics, Nanjing 210023, China
b Department of Chemical Engineering, Tokyo University of Agriculture and Technology, 2-24-16 Naka Koganei, Tokyo 184-8588, Japan
c Department of Chemical Engineering, Hiroshima University, Hiroshima 739-8527, Japan
d School of Environment, Nanjing Normal University, Nanjing 210023, China
e Jiangsu Engineering Lab of Water and Soil Eco-remediation, Nanjing Normal University, Nanjing 210023, China
f Green Economy Development Institute, Nanjing University of Finance and Economics, Nanjing 210023, China
g Department of Civil and Environmental Engineering, Korea Advanced Institute of Science and Technology, Daejeon 34141, Korea
h Institute of Global Innovation Research, Tokyo University of Agriculture and Technology, 3-8-1 Harumi-cho, Fuchu, Tokyo, 185-8538, Japan

A R T I C L E   I N F O

Keywords:
Nitrous oxide reductase
Carbon sources
nosZ gene
Biokinetics
Genomic analysis

A B S T R A C T

Harnessing nitrous oxide (N\textsubscript{2}O)-reducing bacteria is a promising strategy to reduce the N\textsubscript{2}O footprint of engineered systems. Applying a preferred organic carbon source as an electron donor accelerates N\textsubscript{2}O consumption by these bacteria. However, their N\textsubscript{2}O consumption potential and activity when fed different organic carbon species remain unclear. Here, we systematically compared the effects of various organic carbon sources on the activity of N\textsubscript{2}O-reducing bacteria via investigation of their biokinetic properties and genomic potentials. Five organic carbon sources—acetate, succinate, glycerol, ethanol, and methanol—were fed to four N\textsubscript{2}O-reducing bacteria harboring either clade I or clade II nosZ gene. Respirometric analyses were performed with four N\textsubscript{2}O-reducing bacterial strains, identifying distinct shifts in DO- and N\textsubscript{2}O-consumption biokinetics in response to the different feeding schemes. Regardless of the N\textsubscript{2}O-reducing bacteria, higher N\textsubscript{2}O consumption rates, accompanied by higher biomass yields, were obtained with acetate and succinate. The biomass yield (15.45 ± 1.07 mg-biomass mmol-N\textsubscript{2}O\textsuperscript{-1}) of Azospira sp. strain I13 (clade II nosZ) observed under acetate-fed condition was significantly higher than those of Paracoccus denitrificans and Pseudomonas stutzeri, exhibiting greater metabolic efficiency. However, the spectrum of the organic carbon species utilizable to Azospira sp. strain I13 was limited, as demonstrated by the highly variable N\textsubscript{2}O consumption rates observed with different substrates. The potential to metabolize the supplemented carbon sources was investigated by genomic analysis, the results of which corroborated the N\textsubscript{2}O consumption biokinetics results. Moreover, electron donor selection had a substantial impact on how N\textsubscript{2}O consumption activities were recovered after oxygen exposure. Collectively, our findings highlight the importance of choosing appropriate electron donor additives for increasing the N\textsubscript{2}O sink capability of biological nitrogen removal systems.

1. Introduction

Nitrous oxide (N\textsubscript{2}O) is an important ozone-depleting substance and powerful greenhouse gas that is approximately 265 times more potent than CO\textsubscript{2} (Ravishankara et al., 2009; IPCC, 2014). The atmospheric N\textsubscript{2}O concentration has gradually increased by 0.73 ppb per year over the last three decades (Tian et al., 2020). Although N\textsubscript{2}O emissions are mainly derived from agricultural activities (Reay et al., 2012), the contribution from wastewater treatment plants (WWTPs) should be closely monitored. The necessity for this is indicated by recent reports claiming that the amount of N\textsubscript{2}O emitted during biological nitrogen removal in WWTPs accounts for 0–14.6% of a total nitrogen load and is more substantial than initially expected (Law et al., 2012; Yoon et al., 2017). N\textsubscript{2}O is produced during biological nitrogen removal in WWTPs, e.g., from ammonia oxidation, nitrifier denitrification, and incomplete heterotrophic denitrification (Rampschreur et al., 2009). Therefore, recent
studies have investigated strategies for N₂O emission mitigation, e.g., upgrading the process design to actively remove N₂O in the waste gas stream (Yoon et al., 2017), implementing a refined N₂O monitoring campaign (Gruber et al., 2019), and adjusting operating conditions of WWTPs to reduce N₂O production by activated sludge (Chen et al., 2020; Duan et al., 2021).

Biological mitigation of N₂O emissions is attributed to heterotrophic bacteria harboring nosZ gene, which encodes N₂O reductase (N₂O:OR); this enzyme catalyzes N₂O reduction to N₂ (Sanford et al., 2012; Thomson et al., 2012; Yoon et al., 2016). The nosZ gene phylogeny has been distinctively divided into two clades owing to differences in the amino acid sequences. In addition to the canonical clade I nosZ, a new lineage of nosZ gene (the atypical clade II nosZ gene) has been proposed and is found in diverse microbes with unique N₂O metabolism (Sanford et al., 2012; Jones et al., 2013). A recent survey indicates that the clade II nosZ gene is more abundant and widespread in engineered systems (Kim et al., 2020a), and clade II type bacteria likely contribute to N₂O consumption due to their higher affinities for N₂O (Zhao et al., 2019).

According to enrichment and isolation works (Gombe et al., 2018; Suenaga et al., 2019), clade II type N₂O-reducing bacteria, e.g., Dechloromonas and Azospira, are promising as a N₂O sink in engineered systems. These clade II N₂O-reducing bacteria likely have a higher growth yield (Yoon et al., 2016), higher affinities for N₂O (Suenaga et al., 2019; Yoon et al., 2016), and N₂O that is more resilient to oxygen exposure (Suenaga et al., 2018b). Thus, identifying factors that enhance the activities of these effective N₂O-reducing bacteria could lead to effective strategies to curb N₂O emissions.

Carbon sources externally added as electron donors, e.g., ethanol, methanol, and acetate, govern denitrification performances (Adouni et al., 2010; Song et al., 2015; Carlson et al., 2020) and the amount of N₂O emissions in a denitrifying bioreactor (Baytshtok et al., 2009; Song et al., 2015). Although methanol has been widely used for denitrification because it is a cost-effective electron donor (Hallin et al., 2006; Pan et al., 2013), alternatives can be potentially applied based on availability and accessibility. Glycerol, a byproduct of biodiesel production, exhibits promise to replace methanol and ethanol as an electron donor for mixed-culture biomass (Bodik et al., 2009; Schroeder et al., 2020). Despite the practical implementation of an external carbon supply for heterotrophic denitrifying bacteria, their N₂O consumption kinetics and performances fed with different carbon sources have not been systematically investigated. Therefore, further exploration of various carbon sources on the biokinetics of N₂O consumption as the final step of denitrification is necessary.

The long-term continuous supply of an external carbon source as an electron donor shapes denitrifying community compositions (Baytshtok et al., 2009; Carlson et al., 2020). For instance, Paracoccus, Methylphilus, and Methylloversatilis spp. thrive in methanol-fed conditions (Hallin et al., 2006; Kim et al., 2020b), while Acidovorax, Comamonas, and Thauera spp. thrive in acetate-fed conditions (Osaka et al., 2006; Lu et al., 2014). Such distinct community compositions suggest that the organic carbon source to improve denitrification (including N₂O consumption) should be carefully selected. Because attaining energy from a supplemented carbon source primarily depends on the genotypes of the denitrifying bacteria (Ribera-Guardia et al., 2014), the presence or absence of genes necessary for metabolizing particular carbon sources will determine the predominant and active denitrifying bacteria and may determine the amount of N₂O emissions. Genome-centric investigations have been performed in a pure culture of denitrifier (Silva et al., 2020) and denitrifying consortia (Oeschler et al., 2021), providing profound insights into the organic carbon metabolic potentials in denitrifiers. Nevertheless, no experimental validation has been reported regarding organic carbon effects on N₂O consumption activity, and the knowledge gap between genomic information and the physiological data pertaining to coupling of N₂O reduction and oxidation of organic electron donors remains to be bridged. Filling this gap is of significant importance because WWTPs receive highly mixed organic carbon sources with varying flow rates and concentrations (Kim et al., 2020b; Song et al., 2014). Therefore, as the first step forward, determining the biokinetics of N₂O-reducing bacteria when fed with various organic carbon species can improve our understanding of N₂O emissions from WWTPs supplemented with organic feed.

This study investigated the effects of different organic carbon sources on N₂O consumption biokinetics of canonical denitrifying clade I and clade II type N₂O-reducing bacteria. Given that availability of organic carbon sources is one of the factors that primarily determine N₂O consumption potentials, this study deciphered, revisited, and compared the genomes of the nosZ-containing denitrifying bacteria to acquire insights into their metabolic potentials. Organic carbon sources that are frequently used as an external electron donor for heterotrophic denitrification (acetate, succinate, glycerol, ethanol, and methanol) were added to bacterial cell suspensions. We hypothesized that the organic electron donor may be one of the factors that govern (1) the N₂O-associated bioactivities (e.g., maximum specific growth rate and half-saturation coefficient) and (2) the resilience of N₂O consumption activity to oxygen exposure, in a differential manner in different N₂O-reducers, depending on the energy metabolism-associated gene set they harbor. To verify these hypotheses, we performed online monitoring of N₂O and dissolved oxygen (DO) concentrations in microbial cultures incubated under anoxia with and without oxic intervals, using a microrespiration system. Furthermore, we queried the genomic libraries of the examined bacteria for genes with possible association with the observed biokinetic data. The findings of this study can contribute to the development of effective mitigation strategies for N₂O produced and emitted from heterotrophic denitrification in WWTPs.

2. Materials and methods

2.1. Bacterial strains and cultivation conditions

The two canonical denitrifiers (Paracoccus denitrificans NBRC102528 and Pseudomonas stutzeri JCM5965, harboring the clade I nosZ gene, were acquired from Biological Resource Center at National Institute of Technology and Evaluation in Japan and Japan Collection of Microorganisms, respectively. Two Azospira isolates (Azospira sp. strain 113 and Azospira sp. strain 109 [N2_AZP021844]) carrying the clade II nosZ gene had been enriched and isolated from N₂O-fed bioreactors (Suenaga et al., 2019). The rationale of the selection lies in that (i) both clade I and clade II N₂O reducers were represented, each with two strains, and that (ii) these bacteria are confirmed canonical denitrifying bacteria capable of converting NO₃⁻ to N₂ via nitrite (NO₂⁻), nitric oxide (NO), and N₂O (Yoon et al., 2016; Suenaga et al., 2018b).

The complex medium used for preparation of precultures (hereafter termed preincubation) contained per L: 5.0 g of NaCl, 5.0 g of Bacto™ Peptone (BD-Difco, NJ), and 3.0 g of Oxoid™ Lab-Lemco beef extract (Thermo Scientific, MA). The 50-mL precultures were aerobically incubated at 30 °C for 20–24 h in 100-ml Erlenmeyer flasks shaken at 150 rpm. The bacterial cultures were harvested at the early stationary phase and centrifuged at 4550 g for 5 min. The physiological experiments were performed with a previously described minimal salts medium containing per L: 100.0 mg L⁻¹ K₂HPO₄, 188.0 mg L⁻¹ NaHCO₃, 6.6 mg L⁻¹ NaCl, 115.0 mg L⁻¹ NH₄Cl, 8.2 mg L⁻¹ MgSO₄·7H₂O, 13.4 mg L⁻¹ KCl, and 1 mL L⁻¹ trace element solution (Suenaga et al., 2018b). The cell pellets from preincubation were washed with 0.05 × PBS (phosphate buffered saline) and resuspended in 25-ml of this medium for physiological examinations.

2.2. Acquisition and analyses of genome sequences

The genome sequences of P. denitrificans NBRC102528 and P. stutzeri JCM5965 were downloaded from the NCBI database (accession numbers: NZ_BJUR00000000, RRHFC00000000). The genome of Azospira sp. strain 113 was previously sequenced in house (Suenaga et al.,...
2018a; accession number: NZ_BFBP00000000), and that of the strain I09 was sequenced de novo. After aerobic incubation in the preculture medium, Azospira sp. strain I09 was harvested by centrifuging at 9100 g for 10 min. The cell pellet was lysed in 10% sodium dodecyl sulfate solution, and DNA was extracted with the phenol-chloroform method (Butler, 2012). RNA and polysaccharide-like contaminants were degraded by cetyltrimethylammonium bromide (10% w/v in 0.7 M sodium chloride solution) and stored at −20 °C. The genomic DNA was dissolved in Tris-EDTA buffer and stored at −30 °C.

Genome sequencing was performed as a hybrid of short-read and long-read sequencing, to ensure high completeness while maintaining high level of accuracy. Short-read Illumina sequencing was performed by Novogene (Beijing, China) on a NovaSeq platform (Illumina, San Diego, CA), generating 150-bp paired-end reads with a targeted throughput of 1 Gb. The adapter sequences and low-quality reads (Q < 30) were removed using Trim Galore ver. 0.6.5 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). The library for long-read sequencing was prepared with a 1D ligation sequencing kit (SQK-LSK-109; Oxford Nanopore Technologies, Oxford, UK) and sequenced on MinION Mk1B using an R.9.4.1 flow cell. Basecalling was performed using Guppy v3.3.2 using the high-accuracy model (Wick et al., 2019). The adaptor sequences, low-quality reads (Q < 7), header (75 bp), and short reads (<1000 bp) were removed using Porechop ver. 0.2.4 (https://github.com/rrwick/Porechop). The short paired-end sequences and the consensus sequences were assembled Unicycler ver. 0.4.7 (Wick et al., 2017).

The gene coding sequences were assigned Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology numbers using GhostKOALA v 2.1 (Kanehisa et al., 2000; 2016), which were subsequently used for prediction of metabolic potentials of the four organisms to utilize the organic species provided as electron donors.

2.3. Micro-respiration system setup and operation

A micro-respirometry system for simultaneous monitoring of DO and N,O was set up to examine the effect of different organic carbon sources on the biokinetics of N,O-reducing bacteria (Fig. 1). The cell cultures were incubated in a H-shaped vial (with an effective volume of 10 mL) that can accommodate two microelectrodes and a stir bar (Unisense, Aarhus, Denmark). Five batch tests were performed per bacterium, each with a different carbon source, i.e., acetate, succinate, glycerol, methanol, or ethanol. At the beginning of each batch test, 10 mL cell suspension was pipetted into the H-shaped vial to which DO and N,O microelectrodes (Unisense, Aarhus, Denmark) were inserted, leaving no headspace in the vial. Subsequently, 20 μL of concentrated N,O solution (24 mM at 25 °C) was injected into the vial to achieve an initial N,O concentration of approximately 50 μM L−1. The carbon and electron source was added as follows: acetate (320 μM L−1), methanol (420 μM L−1), ethanol (210 μM L−1), glycerol (180 μM L−1), or succinate (180 μM L−1). The organic compounds were added in excess to ensure that N,O reduction activity was not limited by electron donor shortage (Table S1). The micro-respiration system was operated in a water bath maintained at 30 ± 0.2 °C, to allow for comparison with the previous results performed at the same temperature, and stirred at 500 rpm (Yoon et al., 2016; Zhou et al., 2021). N,O and DO measurements were processed using the Sensor Trace Suite software (Unisense). DO concentration was monitored alongside N,O concentration, as it is a crucial factor affecting N,O consumption activity (Massara et al., 2017). After each batch experiment, the cell suspension was fixed by adding 2% glutaraldehyde solution, and the cell count was determined using a microscope (BZ-8100, Keyence, Japan). The details of this procedure are described elsewhere (Lunau et al., 2005). All batch tests were conducted in triplicate.

2.4. Net growth yield measurements

The net biomass yields of the bacterial strains on N,O were determined for each carbon substrate, as previously described (Sanford et al., 2012; Yoon et al., 2016). A 120-mL glass serum vial was filled with 50 mL of the minimal salts medium (pH 7.5), and the medium was then flushed with 99.99% helium gas for 10 min at 0.1 L min−1 to ensure anaerobic incubation condition. Each vial was immediately capped with a rubber plug and sealed with an aluminum crimp. Subsequently, 70 mL of mixed gas (5%/95% of N,O) replaced the helium gas in the vial. After sterilization, 2 mL of the preculture suspension and 1 mL of carbon source stock solution were injected into the vial. The carbon source was added in 25-fold excess of N,O in terms of electron equivalents. The cultures were incubated at 30 °C with shaking at 120 rpm. After the N,O was completely consumed, 40 mL of the cell suspension was filtered through a 0.22 μm pore size polyvinylidene fluoride membrane (Merk Millipore, Burlington, MA). The membrane was oven-dried at 105 °C for 24 h and then dried in a desiccator until no further decrease in the filter weight was observed. The weight of the salts on the membranes was measured after treating the membrane at 500 °C for 8 h as previously described by Sanford et al. (2012). The net growth yields were calculated with the following equation (Sanford et al., 2012):

\[
Y_{X/S} = \frac{[(m_2 - m_1) - (m_3 - m_0) - (m'_2 - m'_1) - (m'_3 - m'_0)]}{m_{NO}}
\]

Fig. 1. Diagram of a micro-respiration system.
m' are the weights of the membrane filters measured with the fully grown bacterial culture and the initial bacterial culture, respectively [mg]. The subscripts 1, 2, and 3 denote the pristine membrane [mg], the membrane with retentate after oven-drying (105 °C), and heat-treated (500 °C) membrane with retentate, respectively. \( n_{\text{N}_2\text{O}} \) is the amount of \( \text{N}_2\text{O} \) consumed during the incubation [mmol]. The measurement was performed in triplicate.

2.5. Biokinetic parameter calculations

Whole-cell kinetic parameters were estimated by fitting DO and \( \text{N}_2\text{O} \) concentration profiles to the whole-cell Michaelis-Menten equation (Martens-Habbena et al., 2009). As our previous work confirmed that the presence of \( \text{N}_2\text{O} \) had no significant effects on biokinetic parameters associated with DO (Zhou et al., 2021), the possibility of \( \text{N}_2\text{O} \)-to-anoxic transitions and vice versa, such transitional behavior has enabled observation of \( \text{N}_2\text{O} \) consumption in the course of recovery (fmol (cell h)\(^{-1}\)). In accordance with a previous study (Suena et al., 2018b), DO and \( \text{N}_2\text{O} \) concentrations were measured using DO and \( \text{N}_2\text{O} \) microelectrodes (Unisense), with detection limits below 0.5 μM and a response time shorter than 0.3 s. The data analysis was performed with Origin 9.0. Statistical analysis was performed using IBM SPSS Statistics version 19.0. The \( p \) values lower than 0.05 or 0.01 were considered statistically significant in ANOVA tests.

3. Results

3.1. Genotypes of the tested bacteria regarding organic carbon metabolism

The genome of \( \text{Azospira} \) sp. strain 109 consists of two contigs with 3.77 and 0.397 Mbp lengths, and the completeness is 100% by BUSCO ver. 1 (Simão et al., 2015). The GC content is 64.4%, and 3910 CDSs were detected. Interrogation of the genomes of the four examined \( \text{N}_2\text{O} \)-reducers predicted genomic potentials of these bacteria to utilize various organic electron donors (Fig. 2). All four bacteria possess the genes necessary for metabolizing acetate and succinate to carbon dioxide. \( \text{Azospira} \) sp. strain 113 lacks the gene encoding NAD-dependent alcohol dehydrogenase, which transforms ethanol to acetaldehyde and thus is essential for utilization of ethanol via the tricarboxylic acid (TCA) cycle. The other three strains possess a full suite of functional genes for metabolizing ethanol to \( \text{CO}_2 \). The two \( \text{Azospira} \) sp. strains lack the genes encoding enzymes that metabolize glycerol, while the genomes of \( \text{Pa. denitrificans} \) and \( \text{Ps. stutzeri} \) harbor the gene necessary for glycerol degradation into dihydroxyacetone phosphate via glycerol-3-phosphate (but not via dihydroxyacetone). The genes encoding glycerol-3-phosphate acyltransferase (EC 2.3.1.15) and acyl-phosphate glycerol-3-phosphate acyltransferase (EC 2.3.1.275) are found in the \( \text{Ps. stutzeri} \) genome, but only the latter is found in the \( \text{Pa. denitrificans} \) genome. Of the four bacteria, only \( \text{Pa. denitrificans} \) harbors a functional gene for methanol conversion into formaldehyde (EC 1.1.1.244).

3.2. Growth characteristics of the \( \text{N}_2\text{O} \)-reducing bacteria

The net biomass yields of the bacteria fed different organic substrates as organic electron donors and \( \text{N}_2\text{O} \) as the sole electron acceptor evaluated the metabolic efficiency of coupling \( \text{N}_2\text{O} \) reduction with oxidation of varying electron donors. Consistent with the findings from the genome analyses (Fig. 2), \( \text{Azospira} \) sp. strains 109, 113, and \( \text{Ps. stutzeri} \) did not consume a significant amount of either methanol or \( \text{N}_2\text{O} \) (Fig. S1). The biomass yield of \( \text{Azospira} \) sp. strain 113 was the highest when fed acetate (5.37 ± 0.48 mg-biomass-mmol-\( \text{N}_2\text{O}^-3 \)), and this value was 2.87 times higher than the yield observed with ethanol as the electron donor (Fig. 3, Table 1). Acetate generated the largest variance in biomass yields across the organisms. The clade II type \( \text{Azospira} \) strains favored acetate. Their net biomass yields on acetate (15.45 ± 1.07 mg-biomass-mmol-\( \text{N}_2\text{O}^-1 \)) for \( \text{Azospira} \) sp. strain 113 and 11.56 ± 0.62 mg-biomass-mmol-\( \text{N}_2\text{O}^-1 \) for \( \text{Azospira} \) sp. strain 109) were significantly higher than those of \( \text{Pa. denitrificans} \) (7.88 ± 0.95 mg-biomass-mmol-\( \text{N}_2\text{O}^-1 \)) and \( \text{Ps. stutzeri} \) (9.13 ± 0.53 mg-biomass-mmol-\( \text{N}_2\text{O}^-1 \)) \( (p < 0.05) \). The same trend was observed with succinate. Among the glycerol-fed cultures, \( \text{Ps. stutzeri} \) exhibited a significantly higher net growth yield than the other three organisms \( (p < 0.05) \). Net yields were statistically indistinct across the four organisms when ethanol was supplied as the electron donor \( (p > 0.05) \).

3.3. DO and \( \text{N}_2\text{O} \) respiration dynamics

The choice of organic electron donor significantly affected DO and \( \text{N}_2\text{O} \) respiration dynamics in the four bacterial strains examined. No significant decrease in DO and \( \text{N}_2\text{O} \) concentrations was observed without external organic carbon addition \( (\text{Pa. denitrificans} \) data shown in Fig. S2), indicating that DO and \( \text{N}_2\text{O} \) consumption was coupled with organic carbon oxidation and that the endogenous decay with \( \text{N}_2\text{O} \) was marginal. Regardless of the supplemented organic matter, all four
Fig. 2. Carbon source metabolic pathways of *Paracoccus denitrificans* NBRC102528 (orange), *Pseudomonas stutzeri* JCM5965 (yellow), *Azospira* sp. strain I09 (blue), and *Azospira* sp. strain I13 (green). The closed and open squares for each pathway represent the presence and absence of the corresponding functional genes, respectively. *Ps. stutzeri* harbors the genes for glycerol-3-phosphate acyltransferase (EC 2.3.1.15) and acyl-phosphate glycerol-3-phosphate acyltransferase (EC 2.3.1.275), whereas *Pa. denitrificans* harbors only EC 2.3.1.275.

Fig. 3. Net growth yield of the tested bacteria harboring the clade I or clade II *nosZ* gene with different carbon sources in batch culture experiments. The standard errors obtained from the triplicate experiments. Different lowercase and uppercase letters represent significant differences among the bacteria with each carbon source and among the carbon sources within each bacterium at the 0.05 probability level.
bacterial strains depleted DO prior to N\textsubscript{2}O (Fig. 4); the replicates are shown in Figs. S3 and S4). The repeated injections of N\textsubscript{2}O (vertical black arrows in Fig. 4) accelerated N\textsubscript{2}O consumption. The highest N\textsubscript{2}O consumption rate after each N\textsubscript{2}O injection differed and depended on the bacteria type and the supplemented organic carbon (summarized in Section 3.4). For Azospira sp. strain I09 fed with acetate (Panel a in Fig. 4), the maximum N\textsubscript{2}O consumption rates after the second and third N\textsubscript{2}O injections, obtained from the linear approximation of the experiment data, were 143 \pm 7\% (n = 3) and 201 \pm 18\% (n = 3) of the rate after the first injection, respectively.

3.4. Biokinetic parameters of N\textsubscript{2}O-reducing bacteria

Biokinetic parameters (V\textsubscript{max} and K\textsubscript{m}) of the tested bacteria were estimated by fitting the DO and N\textsubscript{2}O concentration profiles (Fig. 4) to the whole-cell Michaelis-Menten equation (Eq. (2)). The V\textsubscript{max} and K\textsubscript{m} values of each bacterium were largely dependent on the added carbon source (Table 1). Higher V\textsubscript{max,DO} and V\textsubscript{max, N2O} were obtained when acetate and succinate were added compared with the values obtained with ethanol or methanol as the electron donor (Table 1, Figs. 5 and 6). Azospira sp. strain I13 has the largest standard deviation [6.11 fmol (cell h)\textsuperscript{-1}] among the V\textsubscript{max,DO} values determined with five different organic electron donors (Table 1). For comparison, the standard deviations of Pa. denitrificans and Ps. stutzeri were 2.66 and 5.06 fmol (cell h)\textsuperscript{-1}, respectively.

The highest N\textsubscript{2}O consumption rates (V\textsubscript{max, N2O}) were markedly different across the tested bacteria when different carbon sources were injected. Pa. denitrificans exhibited substantially lower V\textsubscript{max, N2O} than the other bacterial strains. The V\textsubscript{max, N2O}/V\textsubscript{max, DO} values for Pa. denitrificans were 0.05 (acetate), 0.07 (succinate), 0.04 (glycerol), 0.03 (ethanol), and 0.06 (methanol). Even considering that the number of electrons required to reduce a mole of N\textsubscript{2}O is half of the number of electrons required for reducing a mole of oxygen, these values indicate a preference for oxygen as the electron acceptor (Table 1). The corresponding V\textsubscript{max, N2O}/V\textsubscript{max, DO} values for Azospira sp. strain I13 were 1.41 (acetate), 1.46 (succinate), 0.35 (glycerol), 0.91 (ethanol), and 0.38 (methanol), showcasing a preference for N\textsubscript{2}O as an electron acceptor (Table 1). The variance of V\textsubscript{max, N2O} values measured with organic carbon sources for Azospira sp. strain I13 using different organic carbon species was at least 157-fold higher than that of any other bacterial strain examined. Despite being phylogenetically closely affiliated with Azospira sp. strain I13, distinct V\textsubscript{max, N2O} values were obtained from microrespirometry experiments with Azospira sp. strain I09 with different organic electron donors (Table 1). Interestingly, only Ps. stutzeri exhibited a preference for glycerol as the electron donor. This bacterium exhibited 4–41-fold higher V\textsubscript{max, DO} [13.9 \pm 0.70 fmol (cell h)\textsuperscript{-1}] and 4–17 fold higher V\textsubscript{max, N2O} [2.03 \pm 0.11 fmol (cell h)\textsuperscript{-1}] than the other three bacteria when fed glycerol.

The whole-cell half-saturation constants (K\textsubscript{m}) and specific affinities (a\textsubscript{DO} and a\textsubscript{N2O}) of the tested bacteria also varied substantially depending on the organic carbon source (Table 1). The minimum and maximum K\textsubscript{m, DO} values ranged from 0.84 \pm 0.07 \mu mol L\textsuperscript{-1} (methanol) to 10.91 \pm 2.81 \mu mol L\textsuperscript{-1} (glycerol) for Ps. stutzeri (clade I nosZ) and 0.25 \pm 0.06 \mu mol L\textsuperscript{-1} (ethanol) to 5.73 \pm 0.50 \mu mol L\textsuperscript{-1} (acetate) for Azospira sp. strain I13 (clade II nosZ). Even with the same carbon source, the K\textsubscript{m} was significantly different across the tested bacteria. For acetate addition, the K\textsubscript{m, DO} ranged from 0.64 \pm 0.05 \mu mol L\textsuperscript{-1} for Azospira sp. strain I09 (the lowest) to 9.55 \pm 1.81 \mu mol L\textsuperscript{-1} for Ps. stutzeri (the highest).

The specific affinities for DO (a\textsubscript{DO}) of Pa. denitrificans were higher than those of the other tested bacteria, while those for N\textsubscript{2}O (a\textsubscript{N2O}) were much lower, suggesting once again that Pa. denitrificans favors DO over N\textsubscript{2}O (Table 1). Acetate-fed Azospira sp. strain I13 exhibited the highest a\textsubscript{N2O} value (8.88 \times 10\textsuperscript{-9} L (cell h)\textsuperscript{-1}), which was 19 times higher than the a\textsubscript{N2O} value of acetate-fed Pa. denitrificans.

3.5. Recovery of N\textsubscript{2}O consumption after DO exposure

Comparison of the relative N\textsubscript{2}O consumption activities, F (Eq. (3)), and the N\textsubscript{2}O reactivation rates, V\textsubscript{max} (h\textsuperscript{-1}), evaluated the effect of different electron donors on recovery of N\textsubscript{2}O consumption activity following the oxic-to-anoxic transition in the four bacterial strains. Regardless of the bacterium examined or the electron donor added, N\textsubscript{2}O activity increased linearly with time (Fig. 7). The four bacterial strains exhibited noticeably varied F values (Table S2). Intriguingly, the two Azospira strains had substantially differing F and V\textsubscript{max} values (Fig. 7). With acetate as the electron donor, the time required for complete recovery of N\textsubscript{2}O consumption activity (i.e., E = 1.0) was 5.46 h, 4.34 h, 2.73 h, and 1.37 h for Pa. denitrificans, Ps. stutzeri, Azospira sp. strain I09, and strain I13, respectively (Table S2). The recovery time differed depending on the supplemented organic carbon source. Also notable were the consistently high reactivation rates observed under the acetate-fed condition in all four bacteria. The V\textsubscript{max} values measured with acetate as the electron donor were 0.11 \pm 0.01, 0.23 \pm 0.02, 0.24 \pm 0.01, and 0.35 \pm 0.03 h\textsuperscript{-1} for Pa. denitrificans, Ps. stutzeri, Azospira sp. strain I09,
and strain I13, respectively, all significantly higher ($p < 0.05$) than the $V_{\text{nos}}$ values measured with any other electron donors for the respective bacteria. These results also demonstrated that *Azospira* sp. strain I13 was the most resilient bacterium against DO exposure among the four bacterial strains examined under the acetate-feeding scheme (Fig. 7). It should be noted that the $N_2O$-reducers invariably exhibited the highest $V_{\text{max, } N_2O}$ and $V_{\text{nos}}$ values when fed acetate.

**Fig. 4.** Dynamics of $N_2O$ (red) and DO (blue) concentration profiles in cultures of *Pa. denitrificans* (clade I), *Ps. stutzeri* (clade I), *Azospira* sp. strain I09 (clade II), and *Azospira* sp. strain I13 (clade II) using acetate (a), succinate (b), glycerol (c), ethanol (d), and methanol (e) as carbon sources. $N_2O$ injections are performed by black vertical arrows. Representative $N_2O$ and DO concentration profiles are shown, and the other replicates are presented in the SI (Figs. S1 and S2).
4. Discussion

4.1. Electron donor preference regulates physiological traits of \( N_2O \) consumption

Despite the significance of denitrification reducing reactive nitrogen oxides in wastewaters to nitrogen gas, one of the challenges lies in the accumulation and emission of a highly potent greenhouse gas, \( N_2O \). To limit \( N_2O \) emission, harnessing \( N_2O \)-reducing bacteria can contribute to \( N_2O \) mitigation during denitrification in engineered systems (Suenaga et al., 2019). However, more exploratory research on novel \( N_2O \)-reducing bacteria and ecophysiological characterizations is required (Hallin et al., 2018). Although the operating conditions, e.g., pH, DO level, co-existing \( NO_2^- \), and temperature, that facilitate \( N_2O \) consumption by these bacteria have been thoroughly investigated (Pan et al., 2012; Suenaga et al., 2018b; Wang et al., 2018; Zhou et al., 2021, 2022), their \( N_2O \) consumption associated with various organic carbon species is poorly understood. Given the context, our hypotheses regarded an organic carbon species as a vital factor in (1) \( N_2O \) consumption activity in an anoxia environment and (2) \( N_2O \)R recovery after DO exposure. The physiological assays carried out using a micro-respiration system illuminated the noticeable differences in their DO and \( N_2O \) respiration biokinetics of clade I and clade II nosZ canonical denitrifying bacteria and the dependencies upon the type of supplemented organic matter (Table 1). The different DO and \( N_2O \) biokinetics with the different electron donors likely provide significant implications for \( N_2O \) emissions from denitrification in anoxic tanks of WWTPs, when external organic carbon source is switched, for example, from ethanol to methanol (Baytshtok et al., 2009). Given that the external organic carbon source determines the microbial community composition and its resultant denitrification performances (Baytshtok et al., 2009; Lu et al., 2011, 2014; Carlson et al., 2020), our results underscore that the differences in \( N_2O \) consumption activity of the indigenous microbial consortium harboring \( N_2O \)-reducing bacteria are potentially caused by external organic carbon source, which plausibly determines a \( N_2O \) footprint of a WWTP.

4.2. Metabolic genotype affects the \( N_2O \)-related biokinetics of Azospira sp. strain I13

The genotype and phenotype investigation of Azospira sp. strain I13 elaborated its distinctive and unique \( N_2O \) consumption performance. The biokinetic analysis conducted with a micro-respiration system identified the advantage of this strain as a \( N_2O \) sink when acetate and succinate were supplied (Fig. 6 and Table 1). The far lower \( N_2O \) consumption activities with glycerol, ethanol, and methanol (Fig. 5) were...
contextualized by the absence of the key genes enabling their metabolisms (Fig. 2). These trends show why *Azospira* sp. strain I13 had the largest variance of $V_{\text{max}, \text{N}_2\text{O}}$ among the four tested bacteria when fed the five organic carbon sources (Table 1). The absence of certain genes may force $\text{N}_2\text{O}$-reducing bacteria to utilize alternative pathways to utilize electrons for $\text{N}_2\text{O}$ consumption, likely lowering the consumption rates (Carlson et al., 2020). Our follow-up experiment found that methanol consumption with $\text{N}_2\text{O}$ respiration was not observed for *Azospira* sp. strain I13 or *Ps. stutzeri*; this is because they lack the methanol dehydrogenase gene (Figs. S1 and 2). Either endogenous respiration or residual carbon from preculture media led to $\text{N}_2\text{O}$ consumption by bacteria that lack the genes necessary for metabolizing the supplemented organic carbon. Our result shed light on the significance of electron donor selection and expanded our understanding of $\text{N}_2\text{O}$-reducing bacterial physiology. This knowledge can support $\text{N}_2\text{O}$ mitigation in natural and engineered ecosystems.

The limited substrate range may be the general character of the genus *Azospira*. Mining of the *Azospira* genomes in the database revealed *Azospira* genotypes that are analogous to that of *A. oryzae* PS, which lacks genes essential for glycerol metabolism. Hitherto, no *Azospira* species has been found that harbors a complete set of genes needed for methanol metabolism. Although such genotypes may not be shared among all *Azospira* species (genomic information is indeed limited), it is plausible that *Azospira* may not effectively function as an effective $\text{N}_2\text{O}$ sink when fed with alcohol and polyol, which are cost-effective electron donors (Ribera-Guardia et al., 2014, Table S1). Therefore, effective strategies to control $\text{N}_2\text{O}$ emissions require further investigation on the physiologies pertinent to specific electron donors.

4.3. Genomic insights into the biokinetics of $\text{N}_2\text{O}$-reducing bacteria for suitable external carbon selection

Our experimental study demonstrated that the biokinetics of $\text{N}_2\text{O}$-reducing bacteria are largely dependent on the carbon source (Figs. 5 and 6). The acetate and succinate addition resulted in higher $V_{\text{max}, \text{DO}}$ and $V_{\text{max}, \text{N}_2\text{O}}$ values compared with ethanol and methanol addition (Figs. 5 and 6). These results are consistent with the electron donor preferences of the $\text{N}_2\text{O}$-reducing bacteria (Ribera-Guardia et al., 2014) that possess the complete set of genes necessary for the corresponding metabolic pathways (Fig. 2). Acetate and succinate are immediately metabolized in the TCA cycle, while ethanol degradation requires NAD-dependent alcohol dehydrogenase and aldehyde dehydrogenase to consecutively convert ethanol to acetate before entering the TCA cycle via acetyl-CoA (Adouani et al., 2010). Methanol degradation requires methanol dehydrogenase, utilizing a distinct pathway to fully oxidize it to $\text{CO}_2$ (Ribera-Guardia et al., 2014). Therefore, among the tested bacteria, the acetate and succinate additions led to higher DO and $\text{N}_2\text{O}$ consumption rates compared with the methanol and ethanol additions (Table 1). Interestingly, when glycerol was fed, the $V_{\text{max}, \text{DO}}$ and $V_{\text{max}, \text{N}_2\text{O}}$ for *Ps. stutzeri* were one to two orders of magnitude higher than those of the other $\text{N}_2\text{O}$-reducing bacteria (Figs. 5 and 6). This observation was especially interesting, as it is, to our knowledge, the first report of $\text{N}_2\text{O}$ reduction biokinetics data with glycerol as the electron donor. This remarkable capability of *Ps. stutzeri* to utilize glycerol as the electron acceptor for $\text{N}_2\text{O}$ reduction is likely because glycerol-based denitrification involves acyltransferase converting glycerol-3-phosphate into dihydroxyacetone phosphate (Fig. 2). Although *Ps. stutzeri* and *Pa. denitrificans* possess genes for glycerol metabolism, only *Ps. stutzeri* harbors the glycerol-3-phosphate acyltransferase genes for both EC 2.3.1.15 and EC 2.3.1.275 (Fig. 2), which require acyl-CoA and acyl-phosphate as substrates, respectively. *Pa. denitrificans* possesses
only the latter enzyme, but it may not be able to acquire sufficient acyl-phosphate substrate. Further investigation is necessary to elucidate why *Ps. stutzeri* can achieve high DO and N\textsubscript{2}O consumption rates when fed glycerol.

### 4.4. Effect of organic carbon source on N\textsubscript{2}O recovery after DO exposure

The presence or absence of DO drastically alters the N\textsubscript{2}O respiration capacity of bacteria (Lu and Chandran, 2010; Suenaga et al., 2018b) because DO exposure can alter gene and protein expressions. N\textsubscript{2}OR is reportedly vulnerable to DO exposure (Pomowski et al., 2011). A better understanding of N\textsubscript{2}OR activity recovery with different carbon sources is essential for mitigating N\textsubscript{2}O emissions in WWTPs where dynamic changes in redox conditions occur. Our investigation of N\textsubscript{2}O activity resilience in N\textsubscript{2}O-reducing bacteria found that the different organic carbon species caused different lag times in the recovery of N\textsubscript{2}O consumption (Fig. 7). At the same organic carbon source, the N\textsubscript{2}O resilience differed among the tested bacteria. When acetate and succinate were applied, *Azospira* sp. strain I13 showed higher N\textsubscript{2}O consumption and faster N\textsubscript{2}OR recovery than *Pa. denitrificans* and *Ps. stutzeri*, whereas, with glycerol, ethanol, and methanol, the N\textsubscript{2}OR recoveries were markedly impaired (Table S2). The recovery times are congruent with the presence or absence of the genes for the different carbon metabolisms.

Fig. 7. Recovery of N\textsubscript{2}O reduction activity under anoxic conditions. The horizontal axis indicates the time after DO depletion. The data points were derived from N\textsubscript{2}O respiration tests performed in triplicate. The solid lines represent the linear model of N\textsubscript{2}O reduction recovery in each carbon source, and the dashed lines represent the 95% confidence intervals.

A delayed N\textsubscript{2}OR recovery may boost N\textsubscript{2}O emissions in the transiently changing redox conditions often found in WWTPs. In stark contrast, *P. stutzeri* displayed relatively consistent N\textsubscript{2}OR resilience regardless of the supplemented carbon source, which may alleviate abrupt N\textsubscript{2}O emissions. The contrasting result implies that possessing diverse energy metabolisms genes could be an advantage for mitigating N\textsubscript{2}O emissions in engineered systems and agricultural croplands (Jonnasen et al., 2021), as the organic carbon constituents are varied. More N\textsubscript{2}O mitigation research is needed to identify and augment highly efficient bacteria amenable to various carbon sources as electron donors. In a MLE process, one of the mostly-widely implemented biological nitrogen removal (BNR) processes, activated sludge is recycled from an aerated tank to an anoxic tank. Our experimental setup mirrors a realistic condition where the redox condition is shifted from aerobic to anoxic conditions as the aerobic activated sludge is recycled to the anoxic tank, to which an organic electron donor is added. Therefore, our biokinetic results regarding the retardation of N\textsubscript{2}OR activity recovery after oxygen exposure would prove crucial for enhancing the understanding of N\textsubscript{2}O dynamics in BNR systems.
4.5. Carbon sources likely shape different microbial community structures: implications

According to the whole-cell Michaelis-Menten profiles with N2O as an electron acceptor (Fig. S5), Azospira sp. strain 113 could dominate in a denitrifying reactor fed with acetate and succinate, whereas the others tested bacteria with glycerol, ethanol, and methanol. The distinct N2O biokinetic data suggest that a carbon source likely shapes different predominant species as a N2O reducer. Microbial community compositions receiving different carbon sources, e.g., methanol or acetate, have been reported (Lu et al., 2014; Song et al., 2015; Carlson et al., 2020) and have shown the predominance of different genera as denitrifiers (Bayshtok et al., 2009; Osaka et al., 2006; (Ma et al., 2015). Acetate-fed biomass reportedly emits less N2O than methanol-fed biomass (Song et al., 2015). As shown in the current study, given the unique metabolic genotypes and phenotypes of each N2O-reducing bacterium, a microbial community will have a distinct N2O consumption activity. This notion is supported by previous work in which an acetate-fed biomass consisting of Rubrivivax and Dechloromonas (N2O reducers) exhibited greater N2O consumption than a methanol-fed biomass that mainly consisted of Methylotenera (Song et al., 2015). This is likely because, from the bio-kinetic point of view, highly efficient N2O-reducing bacteria, e.g., Dechloromonas and Azospira, can be dominant in a biomass (Song et al., 2014; Suenaga et al., 2019; Kim et al., 2020). Further studies should investigate the link between the organic carbon source and the N2O emission in wastewater bioreactors.

Given the efficiency and cost-effectiveness of nitrogen removal in WWTPs, it is imperative to select an appropriate carbon source for N2O reduction (Riberia-Guardia et al., 2014; Song et al., 2015). It is perceived that a growth yield is a measure of the effectiveness of energy transfer from catabolism to anabolism (Russel and Cook, 1995). The high net growth yield found for the Azospira spp. is in keeping with the high metabolic efficiency of Azospira when fed acetate and succinate; by contrast, this trend was not observed with glycerol and ethanol (Fig. 3). From a practical point of view, acetate may not be a suitable external organic electron donor for denitrification because it costs seven times more than methanol (Mokhayeri et al., 2009). Glycerol is a good alternate due to its relatively higher N2O reduction efficiency and lower cost (when crude glycerol is used) than the other carbon sources (Song et al., 2015, Table S1). When glycerol was applied, Ps. stutzeri exhibited the highest Vmax, DO and Vmax, N2O, which were nearly one order of magnitude higher than those of the other tested bacteria (Figs. 5 and 6). The Vnos of Ps. stutzeri with glycerol addition was also high, indicating its fast recovery of N2O consumption activity when transitioning from aerobic to anoxic conditions (Table S2). Based on this short recovery time, biomass harboring Ps. stutzeri in a biological nitrogen removal system would rapidly adapt to glycerol when applied as an alternative carbon source. However, not all strains of Ps. stutzeri necessarily possess the complete gene set for glycerol metabolism, and more research is needed to clarify this. Additionally, further studies are required to verify a suitable external organic carbon source that is inexpensive and will result in a high N2O consumption rate in WWTPs.

5. Conclusion

This study systematically compared the N2O consumption and N2O OR recovery of four N2O-reducing bacteria harboring either clade I or clade II nosZ when fed five different carbon sources. The conclusions are as follows:

- Organic carbon source remarkably affected the performance, biokinetics, and biomass yield of heterotrophic N2O-reducing bacteria when N2O served as the sole electron acceptor.
- Of the tested carbon sources, acetate and succinate resulted in the highest DO and N2O consumption rates, and clade II type Azospira sp. strain 113 had a higher consumption rate than clade I type Ps. stutzeri and Pa. denitrificans.
- The N2O resiliency after transitioning from aerobic to anoxic conditions heavily depended on the type of N2O-reducing bacteria and the supplemented carbon source.
- When acetate and succinate were added, the clade II nosZ bacteria had higher biomass yields than the clade I nosZ bacteria.

Collectively, the performances and biokinetics of N2O-reducing bacteria are closely associated with their physiological traits and the metabolic potential of the electron donor. Identifying highly efficient N2O reducers and supplementing them with their preferable organic carbon source would improve N2O mitigation in WWTPs.

CRediT authorship contribution statement

Chuang Qi: Investigation, Data curation, Writing – review & editing, Software. Yiwen Zhou: Investigation, Data curation. Toshikazu Sue-naga: Formal analysis, Writing – review & editing, Software. Kohei ObA: Investigation, Data curation. Jial Li: Writing – review & editing, Software. Guoxiang Wang: Writing – review & editing, Software. Limin Zhang: Writing – review & editing, Software. Sukhwan Yoon: Writing – review & editing, Software. Akihiko Terada: Visualization, Writing – review & editing, Software.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We acknowledge Prof. Shohei Riya and Ms. Kanako Mori at Tokyo University of Agriculture and Technology for technical advice on microsensor and molecular analyses. This research was supported by the Japanese Society for the Promotion of Science (17H01893 and 20H04362 to A. T.), by the Research Project Program of the Institute for Fermentation, Osaka (G-2019-3-040 to A. T.), by the Chinese Scholarship Council (201906860008 to C. Q.), by Natural Science Foundation of Jiangsu Province (BK20210676 to C. Q.), by Jiangsu Agricultural Science and Technology Innovation Fund (CX(21)3069 to C. Q.), by the National Natural Science Foundation of China (41877336 to L. Z.), and by National Research Foundation of Korea (2020R1C1C1007970 to S. Y.).

Supplementary materials


References
