An Unknown Non-denitrifier Bacterium Isolated from Soil Actively Reduces Nitrous Oxide under High pH Conditions

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A nitrous oxide (N2O)-consuming bacterium isolated from farmland soil actively consumed N2O under high pH conditions. An acetylene inhibition assay did not show the denitrification of N2 to N2O by this bacterium. When N2O was injected as the only nitrogen source, this bacterium did not assimilate N2O. A polymerase chain reaction demonstrated that this bacterium did not have the typical nosZ gene. This bacterium belonged to Chitinophagaceae, but did not belong to known families that include bacteria with the atypical nosZ. This is the first study to show that a non-denitrifier actively reduces N2O, even under high pH conditions.

Key words: bacteria, farmland soil, N2O, N2O reduction, nosZ

Although carbon dioxide is a well-known greenhouse gas (GHG), other GHG also influence climate change (Montzka et al., 2011). Among these GHG, nitrous oxide (N2O) has a major impact on global warming. N2O absorbs infrared radiation, and its potential to cause global warming is 298-fold that of carbon dioxide and, thus, is regarded as the most important ozone-depleting substance in this century (Ravishankara et al., 2009; Montzka et al., 2011). The emission of N2O from agricultural soil is accelerated by the addition of large amounts of nitrogen-containing fertilizers to farmlands, and accounts for 60% of the atmosphere (Mosier et al., 1998; Zhou et al., 2015). Various methods have been attempted to mitigate N2O emissions. Recent studies reported that N2O emissions may be suppressed by the addition of a substance used as an agrochemical (Obia et al., 2015; Abbruzzini et al., 2019; Takatsu et al., 2019). However, the use of agrochemicals is associated with a number of issues, such as the loss of soil biodiversity and the persistence of soil chemicals (Stolte et al., 2016; Silva et al., 2018), thereby necessitating other methods. Therefore, N2O-reducing microorganisms have been attracting increasing attention (Hallin et al., 2018).

N2O is emitted from soil into the atmosphere through the processes of nitrification and denitrification by soil microorganisms, which are major sources of N2O in soil (Skiba and Rees, 2014). In the denitrification pathway, complete denitrifiers (NO3−/NO2− → NO → N2O → N2) and incomplete denitrifiers (NO3−/NO2− → NO → N2O) contribute to the emission of N2O. Complete denitrifiers possess the nosZ gene, which encodes N2O reductase (Zumft, 1997; Wunsch and Zumft, 2005). Furthermore, some non-denitrifying N2O-reducing microorganisms lack the pathway for the conversion of NO3 to N2O, but have the capacity to convert N2O to N2 (Payne et al., 1982; Simon et al., 2004). Therefore, non-denitrifying N2O-reducing microorganisms have the potential to be true N2O sinks without contributing to N2O production (Hallin et al., 2018). NosZ protein phylogeny has two distinct groups, clade I and II nosZ (Hallin et al., 2018). These clades have been reported as typical and atypical nosZ (Sanford et al., 2012). Clade I nosZ comprises alpha-, beta-, or gamma-proteobacteria, while clade II nosZ consists of a large range of archaeal and bacterial phyla (Jones et al., 2013). Non-denitrifying N2O-reducing microorganisms belong to clade II and possess abundant diversity in all ecosystems (Hallin et al., 2018).

Complete denitrifiers utilize the N2O present in soil gas as the final electron acceptor in the nitrate respiratory system and emit N2 as the final product into the atmosphere (Hutchins, 1991; Wunsch and Zumft, 2005). N2O is used to promote cell survival, even in the absence of oxygen (Park et al., 2017). Based on the assimilation of N as a nutrient, when N2O is abundant, from the perspective of activation energy, it is more efficient in the assimilation of N2O than N2 fixation (Kryachko et al., 2001). Available N (NO3− and NH4+) in soil is limited, even in relatively fertile soils because these nitrogen sources are competitively assimilated by plants and other microorganisms (Kaye and Hart, 1997). In terms of a survival strategy for bacteria, the assimilation of N2O is advantageous when N2O is abundant. Therefore, some bacteria that positively absorb N2O for assimilation may exist; however, this has not yet been demonstrated.

Therefore, the purpose of the present study was to search for a bacterium in soil that consumes N2O. We hypothesized that some bacteria among N2O-consuming microorganisms in farmland soil may assimilate N2O when it is abundant through the denitrification process. By detecting changes in N2O concentrations in gas chromatography vials injected with N2O before incubations, strains with the potential to consume N2O were screened among bacteria isolated from...
farmland soil. Furthermore, an acetylene inhibition assay was conducted to establish whether the decrease in N\textsubscript{2}O concentrations was due to assimilation or reduction. We herein report the taxonomic affiliation and optimal pH conditions required for N\textsubscript{2}O reduction by this isolated N\textsubscript{2}O-reducing bacterium.

Andisol was collected on April 14, 2016 from a pasture farmland and the maize field at the Hokkaido University Shizunai Experimental Livestock Farm (Shinhidaka, Hokkaido, Japan [42°25'9"N, 142°29'1"E]) (Katayanagi et al., 2008). Soil samples were collected at a depth of 0–10 cm and used in the N\textsubscript{2}O reduction assay and the isolation of microorganisms. We used soil from the maize field. The soil suspension was prepared as described previously (Hashidoko et al., 2008).

Winograsky’s mineral solution containing 0.5% (w/v) sucrose and 5 mM KNO\textsubscript{3} (0.52 g L\textsuperscript{-1}) was used as the medium in the culture-based N\textsubscript{2}O reduction assay (Hara et al., 2009; Nie et al., 2015). Since pH plays a key role in the emission of N\textsubscript{2}O (Nie et al., 2015), the pH of the solution was adjusted to various values (4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0) using 2 M H\textsubscript{2}SO\textsubscript{4} and 1 M KOH that was gelled with 0.5% (w/v) gellan gum and then autoclaved. The same medium was used in subsequent experiments. N\textsubscript{2}O levels were measured as described in a previous study (Nie et al., 2015). N\textsubscript{2}O was emitted in the culture at pH 4.5–7.5 (Fig. 1). However, N\textsubscript{2}O emissions decreased in the culture at pH 8.5. This decrease in N\textsubscript{2}O emissions indicated the presence of N\textsubscript{2}O-consuming microorganisms. Therefore, we focused on this culture and isolated the bacterium from N\textsubscript{2}O-consuming microorganisms.

To screen for N\textsubscript{2}O-consuming microorganisms, colonies were isolated as described in a previous study (Nie et al., 2015). Fifteen distinguishable bacterial colonies, marked A to O, were identified. Standard N\textsubscript{2}O gas (GL Sciences) was injected using a gas-tight syringe into the headspace of gas chromatography vials to a final concentration of 2,000 ppmv. After incubations for 0, 1, and 4 days at pH 8.5, N\textsubscript{2}O concentrations in the headspace gas were measured. The results obtained showed that strain A (Sac-f1) exhibited the greatest consumption of N\textsubscript{2}O (Fig. 2).

To examine the potential of N\textsubscript{2}O reducers to reduce N\textsubscript{2}O to N\textsubscript{2}, 10% volume (2.25 mL) acetylene gas and N\textsubscript{2}O (12,000 ppmv) were injected into the headspace of the assay vials immediately after the inoculation of the isolated bacterium, and media were then incubated at 25\textdegree C for 0, 1, 2, 3, and 6 weeks. The concentration of N\textsubscript{2}O was measured after these incubation periods. N\textsubscript{2}O concentrations did not decrease with the acetylene gas treatment, which confirmed that the bacterium reduced N\textsubscript{2}O (Fig. 3). Based on the results of OD\textsubscript{600} measurements in the medium, when N\textsubscript{2}O was injected into gas chromatography vials as the only nitrogen source, this bacterium displayed no growth. This result indicated that this bacterium did not use N\textsubscript{2}O as a nutrient. Furthermore, N\textsubscript{2}O concentrations did not increase during the incubation with the acetylene gas treatment (Fig. 3). Therefore, the bacterium reduced, but did not assimilate, N\textsubscript{2}O and did not denitrify NO\textsubscript{3} to N\textsubscript{2}O.

The DNA of this bacterium was extracted using an Iso‐plant II DNA Extraction kit (Nippon Gene), and the nosZ gene was subjected to a polymerase chain reaction (PCR) using nosZ gene-specific primers (nosZ-1111F and nosZ-1773R) (Scala and Kerkhof, 1998). The 16S rRNA region was amplified with PCR using the primers 27F and 1525R (Lane, 1991; Weisburg et al., 1991). PCR amplicons using the specific primers were purified by agarose gel electrophoresis. Pseudomonas denitrificans NBRC 12442 was used as the positive control. This bacterium did not have a nosZ gene (Fig. 4), and the region of 16S rRNA was successfully amplified from the DNA template.

The 16S rRNA sequence of the isolated bacterium was highly homologous to those of the species belonging to...
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**Fig. 2.** N₂O-consuming activities of the isolated bacterium in the N₂O reduction assay. The N₂O reduction activities of 15 isolates were tested together with a blank in the soft gel medium for the culture-based N₂O reduction assay (pH 8.5). Among those tested, one isolate (marked as A) reduced the concentration of N₂O from the background levels of N₂O (2,000 ppmv) and media were incubated at 25°C for 4 days before N₂O (approximately 2,000 ppmv) was injected into gas chromatography vials. Blank indicates no bacterial inoculation treatment. The arrow indicates the greatest consumption of N₂O (A=Sac-f1).

**Fig. 3.** Responses of N₂O reducers to 10% acetylene gas. The soft gel medium for the culture-based N₂O reduction assay was adjusted to pH 8.5, and media were incubated at 25°C for 6 weeks before N₂O (about 10,000 ppmv) was injected into gas chromatography vials. Control indicates no acetylene treatment, and +10% acetylene indicates the acetylene treatment. Blank indicates no bacterial inoculation treatment. Error bars indicate SE (n=3).

**Fig. 4.** Agarose gel showing the species-specific amplification of the 662-bp fragment. Fluorescence and related species obtained using the primers nosZ 1111 F and nosZ 1773 R. Lane 1: marker gene, lane 2: *Pseudomonas denitrificans* NBRC 12442 (positive control), lane 3: isolated bacterium, and lane 4: Blank.

Chitinophagaceae. The closest species to the isolated bacterium was *Chitinophaga eiseniae* (96.35% similarity). A phylogenetic analysis was performed based on the neighboring method using MEGA X (Kumar et al., 2018). The sequences of the species belonging to Chitinophagaceae were retrieved from the GenBank database. A similar phylogenetic analysis was performed using the 16S rRNA sequence data of previously characterized bacteria showing an atypical nosZ gene (Liu et al., 2008; Sanford et al., 2012; Jones et al., 2013; Park et al., 2017; Hallin et al., 2018) to identify the taxonomic group of the isolated bacterium. We reviewed these studies for species with an atypical nosZ gene in cases where 16S rRNA sequence data were not available. Consequently, this isolated bacterium belonged to the genus Chitinophaga (Fig. 5A). However, it was not reported whether the bacteria from this family belonged to clade II nosZ (Fig. 5B).

To assess the effects of pH on N₂O reduction by the iso-
A) Phylogenetic tree with references from Chitinophagaceae. Similarity and distance matrices were calculated using MEGA X. The phylogenetic tree was constructed based on available 16S rRNA sequences using the neighbor-joining method with 1,000 bootstrap replicates. The scale bar represents the expected number of changes per sequence position.

B) Phylogenetic tree with references from the atypical nosZ clade. We used the neighbor-joining method with 1,000 bootstrap replicates. The scale bar represents the expected number of changes per sequence position.

Fig. 5. The neighbor-joining tree shows phylogenetic relationships of the isolated bacterium.
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![Diagram](image)

Fig. 6. Responses of the isolated bacterium, a N₂O reducer, to optimal pH. The soft gel medium for the culture-based N₂O reduction assay was adjusted to alternative pH values (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0), and media were incubated at 25°C for 3 weeks before N₂O (approximately 10,000 ppmv) was injected into gas chromatography vials. Blank indicates no bacterial inoculation treatment. Error bars indicate SE (n=3).

lated bacterium, the pH of the media was adjusted to various values (4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0), followed by incubations for 0, 1, 2, and 3 weeks. N₂O was injected as described earlier. This isolated bacterium reduced N₂O at pH in the range of 4.5 to 9.0, with the optimum pH being 8.5 (Fig. 6). Previous studies reported that soil microorganisms belonging to clade II reduce N₂O at pH 7.0–7.5 (Liu et al., 2008; Sanford et al., 2012; Jones et al., 2013; Park et al., 2017; Hallin et al., 2018), whereas the isolated bacterium in the present study reduced N₂O under alkaline rather than neutral conditions (Fig. 6).

The present results clearly demonstrated that the isolated bacterium did not assimilate N₂O, but reduced N₂O to N₂. The results of the phylogenetic tree analysis revealed that this bacterium was an unknown species belonging to Chitinophagaceae and reduced N₂O at high pH (8.5). Since the application of nitrogen fertilizers, such as urea, to farmlands results in the largest increase in pH (Black et al., 1985) and accelerates N₂O emissions (Zhou et al., 2018), a fertilizer inoculated with this isolated bacterium may be used to suppress the N₂O flux from agricultural soil. Further investigations, draft genome analyses, and measurements of enzyme activity are needed to clarify the genetic background of this isolated bacterium.

Nucleotide sequence accession number

The 16S rRNA sequence obtained in the present study has been deposited under the following GenBank/ENE/DDBJ accession number: LC554186.

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