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N_2O EMITTERS FROM DIFFERENT HABITATS, BOREAL PEATS AND TROPICAL PEATS AND COMPARISON OF THEIR PHYSIOLOGICAL TRAITS

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SUMMARY

Eubacterial nitrous oxide (N_2O) emitters inhabiting (a) boreal peat in North Europe, (b) an Andisol corn farm in northern Japan, and (c) tropical peat in South East Asia were investigated. After isolation of active N_2O emitters, their response to pH (range 3-8), supplemental sucrose, and 10% acetylene were compared. Active N_2O emitting bacteria, *Burkholderia phenazinium* SF-E2 and *Pseudomonas* sp. SC-H2 isolated from *Sphagnum fuscum* and *S. capillifolium* respectively in degrading palsa mires, showed an optimum pH for N_2O emissions around neutral rather than at acidic levels. *B. phenazinium* SF-E2 showed almost no response to supplemented sucrose. In contrast, *Pseudomonas* sp. SC-H2 clearly accelerated N_2O emissions under supplementation with 0.02% sucrose. *Pseudomonas* sp. SC-H2 possess the *nosZ* gene and displayed a 4-times increase in N_2O production upon exposure to 10% acetylene gas. Conversely, eubacterial N_2O emitters isolated from reclaimed tropical peat soils (e.g. *Janthinobacterium* sp. A1-13 and *Burkholderia* sp. 112-30B-5S) showed significantly high responses to supplemented sucrose (50-80 fold increase in N_2O production), but their optimal pH for N_2O emissions was acidic (pH 4.0-5.0). They did not show a positive response to the 10% acetylene due to the missing *nosZ* gene. Similarly, *Pseudomonas* spp. from the Andisol corn farm soils in the cold-temperate zone showed intermediate behaviors between emitters that originated from the boreal and tropical peatlands. It is most likely that the emergence of active N_2O emitters in the boreal and tropical peat ecosystems is due to high disturbance of the ecosystems by human activity and climate change.

***Keywords**: N_2O emitter; N_2O emitting hotspot; denitrifier; N_2O reductase; nosZ gene*

INTRODUCTION

Nitrous oxide (N_2O) is a greenhouse gas that is 300 times active per mole equivalent of carbon dioxide (CO_2) (Metz *et al*., 2007). Almost 60% of global N_2O emissions are related to agriculture including farming soils and cattle manure, and the widespread use of nitrogenous fertilizers in agricultural soil is a major cause of in N_2O emission (Galloway *et al*., 2004; Reay *et al*., 2012). A recent report demonstrated that nearly 70% of the N_2O emissions from agricultural soils are due to the process of denitrification by soil microorganisms (Metz *et al*., 2007). Biological denitrification known as nitrate respiration uses nitrate (NO_3^-) and other oxygenated nitrogen molecules (NO_2^-, NO, and N_2O) as electron acceptors for degradation of organic compounds under anaerobic conditions. In the process of denitrification, N_2O is the final electron acceptor to be reduced into nitrogen (N_2) gas by N_2O reductase. Hence, some N_2O reductase-malfunctional or *nosZ* gene-missing denitrifiers often emit N_2O actively (Philippot *et al*., 2011; Shiina *et al*., 2014).

Some hotspot areas of N_2O emission have been found, in which reclaimed tropical peatlands, boreal peatlands disturbed by cryoturbation, and volcanic Andisol farmlands are recognized as particular active ones (Takakai *et al*., 2006; Repo *et al*., 2009; Takeda *et al*., 2012). Using culture-based N_2O emission assay in a gellan gum soft gel medium, hyper-active N_2O-emitting eubacteria have been identified from those hotspot areas. Thus, saprophytic and facultatively anaerobic eubacteria of denitrifiers were characterized as hyper-active N_2O emitters. In this paper, we show the physiological and metabolic traits of the N_2O emitting eubacteria isolated from three different N_2O emission hotspots sites, compare their N_2O emitting potential and behaviors, and further discuss their similarity and unique characteristics in these different ecosystems.

MATERIALS AND METHODS

Samples

Sphagnum mosses (approximately 50 g as living tissues) were collected from mires in Joensuu (62°47′N, 30°58′E) and Kirpisjarvi (60°26′N, 8°28′E), Finland, in early September 2010 and 2014. Soil samples

(approximately 10–50 g as raw soil) were collected at Hokkaido University Shizunai Livestock Farm in Hokkaido, Japan (42°26′N, 142°28′E) in November 2011 and April 2012. Tropical peat soils were collected in 2008-2010 from Riau (0°10′S, 102°9′E) and Central Kalimantan (0°09′N, 101°34′E) in Indonesia, and Sarawak (02°08′N, 111°54′E), Malaysia. Screening and identification of N_2O emitters were performed by means of culture-based N_2O emission assay and homology search on 16S rRNA gene sequence as described previously (Nie *et al.,* 2016).

N_2O emission assay for N_2O emitters

To investigate the main causative microorganisms of N_2O production, culture-based N_2O emission assays were used as previously reported (Nie *et al.*, 2016). As mineral N for the substrate of N_2O production, an excessive concentration of $(NH_4)_2SO_4$ (2.4 g L^{-1}) or KNO_3 (3.6 g L^{-1}) was added to Winogradsky's mineral solution separately with 0.01% (w/v) $CaCO_3$ (Takeda *et al*., 2012) followed by adjustment to pH 5.0 with H_2SO_4. To the resulting solution previously filtered on a 0.45-µm pore membrane, 0.3% gellan gum powders were added and heated over 100 °C for 15 min to liquefy it. After cooling, 10.0 mL medium was placed in a 30-mL gas chromatographic vial (Nichiden-Rika Glass Co., Kobe, Japan) with a sealed butyl rubber plug and a screw cap septum and autoclaved at 121 °C for 15 min. The headspace volume of the vial was 22.6 mL (Hara *et al.*, 2009). The medium used in the preliminary culture-based N_2O emission assay contained no additional sugar (N_2O emitters from tropical peat), or contained 0.05-1.0% sucrose.

A 100 µL aliquot of bacterial cell suspension was inoculated to 10.0 ml soft gel medium and vortexed for 1 min. The incubation condition was generally at 20-25 °C for 7 days in the dark. A 1 mL volume of the sampled headspace gas was injected to a gas chromatograph (GC) (Shimadzu GC-14B, Kyoto, Japan) equipped with an electron capture detector (ECD) (Shimadzu ECD-2014) kept at 340 °C using a 1 m Porapak N column (Waters, Milford, MS, USA) at 60 °C, with a carrier gas of Argon with 5% methane (CH_4).

Optimal conditions for N_2O production

To evaluate the effect of supplemented sucrose on N_2O emissions, sucrose at different concentration (0.03-1.0% w/v) was added to the medium for the N_2O emission assay. After a 7-day-incubation at 20 or 25°C in the dark, the headspace gas was analyzed by ECD-GC as described above. In addition, pH values of the media were altered in the range f3 to 8 with 1 M H_2SO_4 or 1 M KOH solution, and the amount of N_2O in the headspace gas was also measured in the same manner.

Acetylene blocking assay

To investigate the effect of acetylene on N_2O emitters, pure acetylene gas (2.25 mL) was injected into the headspace. After incubation for 5-7 days, the amount of N_2O in the headspace gas was measured.

Detection of nosZ gene by PCR

Among denitrifying N_2O emitters, the *nosZ* gene was searched by PCR, using a primer set *nosZ-1111F* (5′-STA CAA CWC GGA RAA SG-3′), *nosZ-661F* (5′-CGG CTG GGG GCT GAC CAA-3′), *nosZ-1527R* (5′-CTG RCT GTC GAD GAA CAG-3′), and *nosZ-1773R* (5′-ATR TCG ATC ARC TGB TCG TT-3′) (Scala and Kerkhof, 2006). *NosZ912F* (5'-CGT CCC CGG CCT CGT GTA-3') and *NosZ1853R* (5'-GAG CAG AAG TTC GTG CAG TAG TAG GG-3') (Sanford *et al*., 2012) were as described in our previous paper (Nie *et al*., 2016). PCR amplicons assignable as *nosZ* fragments were sequenced followed by the homology search on the DNA database (NCBI).

RESULTS

N_2O emitters from disturbed boreal peat mosses

Boreal peatlands that have been disturbed by cryoturbation and accelerated permafrost defrosting due to current global warming are known to be one of the most active hotspots of N_2O emissions (Repo *et al*., 2012). In our investigation in a Finnish boreal boreal peat ecosystem, *Burkholderia* spp. and *Pseudomonas* spp. were isolated as leaf epiphytes from *Sphagnum fuscum* and *Sphagnum capillifolium* of upland-adaptable sphagnum mosses, and some of the eubacteria in the North European subarctic tundra showed a potent dominant N_2O emitting capability. These eubacterial N_2O emitters of phylum *Proteobacteria,* widely inhabiting soil, rhizosphere, phyllosphere of agriculture crops, and farm soils in tropical, subtropical, temperate, and cold-temperate zones, acted as potent denitrifiers. The denitrifiers did not have the ability to reduce N_2O into N_2 gas, known as the final step of the denitrification process. In comparison with eubacterial N_2O emitters isolated from different ecosystems other than boreal peatlands, the eubacterial N_2O emitters in disturbed boreal peatlands showed relatively unique traits for N_2O emissions in responses to several environmental factors.

The active N_2O emitting bacteria from the boreal peat; *Burkholderia phenazinium* SF-E2 and *Pseudomonas* sp. SC-H2, isolated from *Sphagnum fuscum* and *S. capillifolium* in degraded palsa mires respectively, showed an optimum pH for N_2O emissions around neutral rather than at acidic levels. *B. phenazinium* SF-E2 showed almost no response to sucrose supplementation, in which N_2O emissions increased by only 2.5 fold, while supplementation of

100 mg L^{-1} caffeic acid led to an obvious acceleration of N_2O emissions (0.45 μg $vial^{-1}$ day^{-1}, 30 fold higher than the control). Both increased pH and phenolic compounds stimulated N_2O in the culture-based N_2O emission assay, and these conditions are probably provided in nature by the disruption of boreal peat ecosystems along with sphagnum peat degradation (Nie *et al*., 2015).

In contrast, while unresponsive to caffeic acid, N_2O emissions by *Pseudomonas* sp. SC-H2 were accelerated (16 fold higher than the control) under supplementation with 0.02% sucrose. This N_2O emitter possessed the *nosZ* gene and showed 4-times increase in N_2O production upon exposure to 10% acetylene gas (known as acetylene blocking assay to measure the activity of N_2O reductase). Effective blockage of N_2O reduction by acetylene gas suggests that *Pseudomonas* sp. SC-H2 is a hyper-active denitrifier at neutral pH. As a large amount of minerals and ammonia are released during degradation of sphagnum peat, and the ammonium cation (NH_4^+) is biologically oxidized to nitrate anion (NO_3^-), *Pseudomonas* sp. SC-H2 may act as remediators for an ecosystem by maintaining the C:N balance in the soil (Mu *et al*., 2012; Lau *et al*., 2014).

N_2O emitting Burkholderia spp. from tropical peatlands reclaimed for agriculture

A powerful eubacterial N_2O emitter, *Burkholderia* spp. were commonly found in tropical woody peatland ecosystems distributed throughout South East Asia, particularly those reclaimed without proper soil and water table management (Hashidoko *et al*., 2010; Jauhiainen *et al.*, 2012). These dominant N_2O emitters inhabit the medium to strongly acidic tropical peat soils and show an acid-tolerant trait, and more importantly, are often denitrifiers that actively emit N_2O as the final electron acceptor. In tropical peatlands utilized for crop production, substrate (NO_3^- or NH_4^+) and some essential minerals (Fe, Mo, and S) necessary for denitrification and/or nitrification processes are provided by several types of fertilizers (ash, chemical fertilizers, manure, river sediments, etc.).

From one of the most active N_2O emission hotspots in the world (Takakai *et al*., 2006), order *Burkholderiales* of class *Betaproteobacteria* (e.g. genera *Burkholderia* and *Janthinobacterium*) were commonly isolated and characterized as major eubacterial N_2O emitters. All the N_2O emitters of *Burkholderia* isolated from the tropical peat soils showed hyper-active N_2O emissions (maximum 643 μg d^{-1} $vial^{-1}$) in the culture medium containing 0.3-1.0% sucrose and 0.03-0.2% NO_3^- (Hashidoko *et al*., 2008). This clear response of the N_2O emitters to supplemented sucrose are due to their oligotrophic nature; an important metabolic trait of denitrifiers.

Many N_2O emitters of *Burkholderiales* isolated from tropical peat soils are acid-tolerant eubacteria, and adaptable to nutrient-poor, medium to strongly acidic tropical peat soil (pH 3.0~4.5). Some *Burkholderia* spp. could survive in an acidic medium of less than pH 3.0. Therefore, we have speculated that frequent isolation of *Burkholderia* spp. was probably due to malfunctionality of N_2O reductase or complete loss of the enzyme in the acidic environment. N_2O reductase is highly susceptible to acidic conditions and loses its enzymatic activity in acidic conditions (Bergaust *et al*., 2010). Hence, denitrifiers in acidic soil often cause active N_2O emissions when the substrate (NO_3^-) was sufficient in the soil.

Two active N_2O emitting *Burkholderia* spp. selected from Riau, Indonesia and from Sarawak, Malaysia were subjected to genome sequencing using a second generation DNA sequencer, and it was directly evidenced that both *Burkholderia* spp. lost the N_2O reductase gene (*nosZ*). Thus, tropical peat soil under medium to strongly acidic conditions led to a defect of *nosZ* gene encoding the large subunit of N_2O reductase because N_2O reduction in acidic environment is neither effective nor costless. N_2O used as the final electron acceptor in the denitrification process is not an effective substrate because the catalytic enzyme N_2O reductase is highly susceptible to acidic conditions, and does not function in acidic soils. Hence, *nosZ*-missing denitrifiers are frequently isolated as powerful N_2O emitters.

N_2O emitting Pseudomonas spp. from vitric Andisol corn farmland

Ten strains of *Pseudomonas* denitrifiers were isolated from pre-tilled and post-harvest vitric Andisol corn farm soil (Nie *et al*., 2016). Using a combination of several universal primers (2 forward and 2 reverse primers, and a pair for atypical *nosZ* gene), PCR assay for detection of *nosZ* gene revealed that four out of ten strains harbored the *nosZ* gene. However, acetylene-blocking assay showed relatively weak N_2O reductase activities in the *nosZ*-harboring *Pseudomonas* denitrifiers. The remaining six *nosZ*-missing strains of *Pseudomonas* denitrifiers showed negative responses in the acetylene-blocking assay. This suggested that freeze-thawing conditions of the pre-tilled Andisol corn farm soils led to a suppression of N_2O reductase activity.

In a report by Qin *et al*. (2014), the optimal temperature of N_2O reductase ranged from 35-45 °C, relatively high compared to other enzymes associated with denitrification (e.g. nitrate reductase), and activity was drastically decreased below 20 °C (Qin *et al*., 2014). Thus, relatively low temperatures in the Andisol corn farm (Hokkaido, Japan, 42°25′N, 139°29′E) in winter may have caused a loss of the N_2O reductase activity in the *Pseudomonas* denitrifiers. They were typical oligotrophs and actively emitted N_2O in the 0.5% sucrose-supplemented medium for N_2O emission assay.

The response of *Pseudomonas* N_2O emitters to pH (range 3.2 to 7.6) was opposite to those isolated from tropical peat soils. In the acidic soils (3.2-5.5), N_2O emission was almost zero, but at more neutral conditions (5.5-7.6), N_2O constantly accumulated in the headspace, and maintained high levels of concentration (Nie *et al*., 2016).

CONCLUSION

In comparison of N_2O emitting potentials among the soils that we have examined, the tropical peat soils from the lands reclaimed for agricultural use and managed by fertilization of nitrogen and minerals showed relatively high N_2O emitting potentials (Hashidoko *et al*., 2010). Similarly, the vitric Andisol from the corn farmland of sufficiently fertilized showed relatively active N_2O emission potential than the soils from the corn farmland without fertilization and from the pasture without tillage (Nie *et al*., 2016). In the case of the boreal peatlands, *Sphagnum* mosses sampled from the degrading palsa mires along with increasing pH of the stagnant water tended to show relatively high N_2O emitting potentials. Thus, it is most likely that the emergence of active N_2O emitters in the terrestrial ecosystems is due to high disturbance of soil conditions mainly by human activity and global warming impact. Almost all of the denitrifying N_2O emitters that we have isolated from the global N_2O emission hotspots were mostly incomplete denitrifiers, which are missing the enzymatic activity of N_2O reductase or the *nosZ* gene itself.

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