Genetic analysis of active N_2O-emitting bacteria from tropical peat soils for their nitrous oxide reductase gene

Y. Hashidoko[A,D], H. Takeda[A], U. Darung[B], L. Melling[C] and R.Hatano[A]

[A]Research Faculty of Agriculture, Hokkaido University, Sapporo, Japan.
[B]Palangka Raya University, Palangka Raya, Central Kalimantan, Indonesia.
[C]Tropical Peat Research Laboratory Unit, Chief Minister's Department, Kuching, Sarawak, Malaysia.
[D]Corresponding author. Email yasu-h@abs.agr.hokudai.ac.jp

Abstract
Tropical peat swamp forest is an important carbon deposit in terrestrial ecosystems, in which carbon is accumulated as a large mass of woody peat soil. Reclamation of the peat swamp forest into plantation led to degradation of peat soil to release a massive nitrous oxide (N_2O) emission along with CO_2, both known as important greenhouse gases. Active N_2O emitting bacteria isolated from the reclaimed peat land soils and identified as *Burkholderia* spp. of β-proteobacteria were saprophytic denitrifiers more adaptable to nutrient-poor, medium-strongly acidic medium, than reference denitrifying bacteria, such as *Paracoccus denitrificans* NBRC 12442. N_2O emission by *Burkholderia* spp. from acidic peat soils was highly pH-dependent to be active at acidic regions, ranging from pH 4.0 to 4.5. In a nitrous oxide reductase (N_2OR) inhibition assay using 10% acetylene gas for a specific N_2OR inhibitor, these active N_2O emitters showed no increase of N_2O emission under exposure to 10% acetylene gas, while normal denitrifiers possessing N_2OR produced a large amount of N_2O after injection of 10% acetylene gas. In genetic analysis, PCR using specific and degenerate primers for *NosZ* for the N_2O emitting *Burkholderia* spp. did not give any *NosZ* amplicons, while *P. denitrificans* NBRC 12442 and *Pseudomonas nitroreducens* NBRC 12694 clearly gave amplicons for *NosZ*. N_2O emission from reclaimed tropical peat soil is thus caused by the N_2OR gene-missing denitrifying bacteria of Burkholderiales.

Key Words
Nitrous oxide reductase, NosZ, nitrous oxide, N_2O emission, tropical peat soil, *Burkholderia* spp.

Introduction
N_2O is an important greenhouse gas in global warming, mainly produced from agriculture farmland and sewage-treating plant, both of which are fundamental in human life (Crutzen 1981). Moreover, N_2O gas is chemically stable, with its half-life period of 114 years, and therefore this stability led to 298-fold higher greenhouse effect than CO_2 (IPCC 2007). It was also warned that N_2O might be the worst gaseous principle to destroy O_3 layer in stratosphere during 21st century (Ravishankara *et al.* 2009). To date, however, no effective manner to quench N_2O gas has been developed. Our recent studies revealed that active N_2O emitters inhabit in tropical peat soil of reclaimed fertilized farmlands including vegetable fields and oil palm plantation lands (Takakai *et al.* 2006, Hashidoko *et al.* 2009a). Major N_2O emitters from peat soil in Central Kalimantan and Sarawak were characterized as a group of *Burkholderia*.

Methods
Soil sampling and site description
Land use of each sampling site is as follows: *Acasia* pulp plantation site in peat soil (Riau, Indonesia), peat soil farmland reclaimed 2 y ago (Central Kalimantan, Indonesia), and oil palm plantation, 5 y old (Sarawak, Malaysia). Land soils were collected in mid-September 2008 from the sampling sites at depths of 5, 10 and 20 cm.

N_2O emission assay in the absence of soil
Basic ingredients of medium for culture assay (Hashidoko *et al.* 2008) are as follows: 5 mL of Winogradsky's mineral mixture (100 mg $CaCO_3$, 3.7 g KNO_3 as 500 ppm N and 3 g gellan gum, all dissolved in 1 L of milli-Q water and pre-heated). To a 30-mL glass vial plugged with a butyl rubber and a screw cap (Nichiden-Rika Glass Co., Kobe, Japan), 10 mL of the medium afforded 22.6 mL of headspace volume. The medium autoclaved at 120^{o}C for 15 min and cooled was inoculated with supernatant of the soil suspension and cultured for 5-7 days. N_2O produced in headspace of the vials was analysed by a Porapak N (Varian, 1 m) column-connected gas-chromatography equipped with micro-electron ion capture detector. From the headspace gas, 1 mL of the gas was sampled and injected to the GC.

Bacterial isolation and identification from soil
Soil samples that had emitted remarkable amounts of N_2O in the headspace were re-suspended in sterilized water (10 mg/10 mL) and the upper solution (100 μL) was plated on Winogradsky's agar medium supplemented with 0.005% (w/v) yeast extract. Majorly emergent colonies were isolated (4-5 distinguishable colonies per plate), and these soil bacterial isolates were again tested in the N_2O emission culture assay.

Bacteria that have been characterized as active N_2O emitters were shaking-cultured in nutrient broth (NB) medium at 25°C to over OD_{550} 0.4, and the bacterial cells centrifuged, washed several times with sterilized water and subjected to extraction of chromosomal DNA using a DNA extraction kit, Isoplant II (Nippon Gene, Toyama, Japan), and PCR for 16S rRNA gene region were done using EX Taq HS (Takara, Kyoto, Japan) with universal 27F and 1525R primers. Resulting PCR amplicons (1.5 kbp) were each subjected to direct PCR sequence analysis, using an ABI Prism 310 genetic analyzer with a BigDye Terminator version 3.1 cycle sequencing ready-reaction kit (Applied Biosystems, Foster City, CA).

Appropriate conditions for N_2O emission
Using the N_2O emission assay, activity of the N_2O emitting soil bacteria isolated from the peat soil was all maximal in acidic pH. Moreover, these acidic soil-adapting N_2O emitters accelerated N_2O emission as 20-570 fold increase in the medium supplemented with 0.5% sucrose (Hashidoko *et al.* 2010). Optimum conditions for active N_2O production were acidic at pH 4.2-4.5. Supplemental 0.5 % sucrose in the medium led to maximal 560-fold N_2O emission in some N_2O emitting *Burkholderia* spp. in the N_2O emission assay.

Inhibition Assay for N_2O reductase (N_2OR) using acetylene gas as its specific inhibitor
N_2O reductase is thoroughly inhibited by 10% acetylene gas in headspace. Hence, 10% acetylene gas was injected to the headspace immediately after the inoculation of bacterial cell suspension. After 7-day-incubation, N_2O in headspace was measured by ECD-GC and compared to that without acetylene injection (n=3 or 5). All the incubation was done at 25°C.

PCR for detection of nosZ region from chromosomal DNA of denitrifiers
As N_2OR gene, *nosZ* is involved in denitrifying gene cluster of bacterial denitrifiers. Therefore, using specific *nosZ* gene primers (nosZ-661F, nosZ-1111F, nosZ-1527R, nosZ-1773R) (Scala and Kerkhof 1998), N_2OR gene for bacterial denitrifiers were searched by PCR technique. PCR amplicons using the specific primers were purified by agarose gel electrophoresis, and sequenced for searching on Blastox program of DDBJ database. As reference denitrifiers, *nosZ* region of *Paracoccus denitrificans* NBRC 12442 and *Pseudomonas nitroreducens* NBRC 12694 were also amplified for database analysis.

Results
Eight active N_2O emitters were isolated from three different sites of peat soils, and were all identified as genus *Burkholderia*, sub-division of □-proteobacteria. These N_2O emitting *Burkholderia* were grouped into three different clusters in phylogenetic classification using a partial 16S rRNA gene region. All these N_2O emitting soil bacteria showed the highest N_2O production in the soilless N_2O emission assay in acidic region (pH 4.2-4.6). We therefore tested N_2OR inhibition assay in the soilless culture medium of pH 4.5, of which pH values is close to of acidic peat soil and active in N_2O emission. In parallel, we did the same inhibition test at pH 6.0. At both pH 4.5 and 6.0, N_2O emission by the *Burkholderia* spp. was less affected. At pH 4.5, N_2O emissions were increased 15-30% under the presence of 10% acetylene from those without acetylene gas, while at pH 6.0, N_2O were increased 50-70% compared to those without acetylene. However, absolute amounts of N_2O that were increased by N_2OR inhibition at pH 6.0 were rather smaller than those at pH 4.5 (Hashidoko *et al.* 2009).

PCR detection of *nosZ* was unsuccessful. Two amplicons obtained from an active N_2O emitter isolated from Central Kalimantan were not *nosZ* gene but matched to that of function-unknown membrane-binding protein. In contrast, PCR amplicons obtained from complete denitrifiers (*P. denitrificans* NBRC 12442 and *P. nitroreducens* NBRC 12694, both used as the reference bacteria) were both characterized as those of *nosZ*.

Conclusion

In the N_2O emission assay for soil suspension and isolated bacteria from several sampling sites, N_2O emitting bacteria of highly pH-dependent (appropriate pH value for the most active N_2O emission was pH 4.2-4.5) activate nitrate respiration under acidic conditions. In neutral conditions, N_2OR recovery was not so clear, suggesting that both nitrate respiration and nitrous oxide reduction are repressed. Searching for *nosZ* in chromosomal DNA of *Burkholderia* sp. using PCR and DNA sequencing techniques led to temporal conclusion that N_2OR gene of these active N_2O emitters is missing or replaced with gene for another membrane protein.

In this study, all the active N_2O emitters were identified as *Burkholderia* spp. by 16S rRNA gene sequence determination. Phylogenetic cluster analysis showed that these active N_2O emitting *Burkholderia* spp. are composing three major groups, and these *Burkholderia* spp. probably most contribute to active N_2O emission in reclaimed peat soil. As our other study also suggest positive correlation between nitrogen contents in soil and distribution of active N_2O emitting bacteria. *Burkholderia* spp. also showed clear responses to supplemented sugar to activate N_2O emission. Regarding it as organic carbon sources in soil, peat decomposition reasonably resulted in active N_2O emission.

Taken together, active N_2O emitters in tropical peat soil are adapted to acidic peat soil, and during nitrate respiration in the acidic soil, they probably lost functionality of N_2OR encoding *nosZ* gene. From this genetic point of view, it is necessary to investigate gene structure in denitrification gene cluster in N_2O emitting bacteria, and should be further discussed genetic evolution of *nosZ* gene functionality in soil.

References

Crutzen PJ (1981) Atmospheric chemical processes of the oxides of nitrogen including nitrous oxide. In 'Denitrification, Nitrification and Atmospheric Nitrous Oxide'. (Ed CC Delwiche) (Wiley: Chichester, England).

Hashidoko Y, Takakai F, Toma Y, Darung U, Melling L, Tahara S, Hatano R (2008) Emergence and behaviors of acid-tolerant *Janthinobacterium* sp. that evolves N_2O from deforested tropical peatland. *Soil Biology and Biochemistry* **40,** 116-125.

Hashidoko Y, Takeda H, Hasegawa S, Hara S, Wijaya HC, Darung U, Melling L, Hatano R (2009) Screening of N_2O emitting bacteria from acidic peat soils and their characteristics under acidic conditions. In 'Asfas 2009 in Seoul, 27-28 October 2009, Abstract CD'.

Hashidoko Y, Takeda H, Hasegawa S, Hara S, Wijaya HC, Darung U, Melling L, Hatano R (2010) Screening of N_2O-emitting bacteria from acidic soils and their characteristics under acidic conditions. In 'Proceedings of Bogor Symposium and Workshop on Tropical Peatland Management, Bogor, Indonesia, 14-15 July 2009'. pp. 52-56. (IFES-GCOE Indonesian Liaison Office).

IPCC 2007 (2007). Mitigation of Climate Change In 'Climate Change 2007'. Working group III Contribution to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. (Eds B Metz, O Davidson, P Bosch, R Dave, L Meyer). (Cambridge University Press, Cambridge, UK).

Ravishankara AR, Daniel JS, Portmann RW (2009) Nitrous oxide (N_2O): the dominant ozone-depleting substance emitted in the 21st century. *Science* **326,** 123–125.

Scala DJ, Kerkhof LJ (1998) Nitrous oxide reductase (*nosZ*) gene- specific PCR primers for detection of denitrifiers and three *nosZ* genes from marine sediments. *FEMS Microbiol. Lett.* **162,** 61–68.

Takakai F, Morishita T, Hashidoko Y, Darung U, Kuramochi K, Dohong S, Limin SH, Hatano R (2006) Effects of agricultural land-use change and forest fire on N_2O emission from tropical peatlands, Central Kalimantan, Indonesia. *Soil Science and Plant Nutrition* **52,** 662-674.