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Physiological and genetic traits of the N\textsubscript{2}O-emitting 
*Proteobacteria* isolated from latent hot spots for N\textsubscript{2}O 
emission, and their response to environmental factors 
including plant polyphenols

(潜在的 N\textsubscript{2}O 放出ホットスポットから分離した N\textsubscript{2}O 
放出能をもつグラム陰性細菌の細菌生理学的および 
分子遺伝学的性状と，植物ポリフェノールを含めた 
環境諸因子に対するそれら分離細菌株の応答)

Ph. D. Dissertation

(The Special Postgraduate Program in Biosphere Sustainability Science)

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ABBREVIATIONS

AA  amino acids
AMO  ammonia monooxygenase
AOA  ammonia-oxidizing archaea
AOB  ammonia-oxidizing bacteria
BLASTn  nucleotide basic local alignment search tool
BLASTp  protein basic local alignment search tool
bp  base pairs
CYP  cytochrome P450
DDBJ  DNA databank of Japan
DNA  deoxyribonucleic acid
DGGE  denaturing gradient gel electrophoresis
DNRA  dissimilatory nitrate reduction to ammonium
ECD-GC  electron capture detector gas chromatograph
GHG  greenhouse gas
HAO  hydroxylamine oxidoreductase
IPCC  Intergovernmental Panel on Climate Change
MWA  modified Winogradsky’s agar
MWG  modified Winogradsky’s gellan gum
N₂O  nitrous oxide
N₂OR  nitrous oxide reductase
NAP  periplasmic nitrate reductase
NAR  membrane-bound nitrate reductase
NCBI  National Centre for Biotechnology Information
NH₂OH  hydroxylamine
NIR  nitrite reductase
NOR  nitric oxide reductase
NrfA  periplasmic cytochrome c nitrite reductase
OD  optical density
PCR polymerase chain reaction
PDA potato-dextrose agar
rpm rotations per minute
rRNA ribosomal ribonucleic acid
SD standard deviation

STRUCTURES OF COMPOUNDS USED

![Gallic acid structure](image1.png)

![Caffeic acid structure](image2.png)

Gallic acid  (E)-Caffeic acid
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SUMMARY

Although acidic lands all over the world are known to have potentials to be an active spot of nitrous oxide (N$_2$O) emission, major microbial contributors to the N$_2$O efflux and mechanisms behind the frequent emergence of active N$_2$O emitters from the boreal acidic soils still remain unclear. Screening of several active N$_2$O emitters from soil or Sphagnum mosses associated epiphytic/endophytic bacterium and analyses of their response to environmental factors including plant polyphenols and functional genes associated with N$_2$O production were attempted to give an answer to this fundamental query.

1. N$_2$O emission potentials of Burkholderia species isolated from the leaves of a boreal peat moss Sphagnum fuscum

Using a culture-based N$_2$O emission assay, three active N$_2$O emitters were isolated from Sphagnum fuscum leaves and all identified as members of Burkholderia. These isolates showed N$_2$O emission in the medium supplemented with NO$_3^-$ but not with NH$_4^+$, and Burkholderia sp. SF-E2 showed the most efficient N$_2$O emission (0.20 µg vial$^{-1}$ day$^{-1}$) at 1.0 mM KNO$_3$. In Burkholderia sp. SF-E2, the optimum pH for N$_2$O production was 5.0, close to that of the phyllosphere of Sphagnum mosses, while the optimum temperature was uniquely over 30 °C. The stimulating effect of additional 1.5 mM sucrose on N$_2$O emission was ignorable, but Burkholderia sp. SF-E2 upon exposure to 100 mg L$^{-1}$ (E)-caffeic acid showed clear acceleration of its N$_2$O emission. All of three N$_2$O emitters were negative in both acetylene inhibition assay and PCR assay for nosZ-detection, suggesting that N$_2$O reductase or the gene
itself is missing in the N\textsubscript{2}O-emitting \textit{Burkholderia}.

2. Isolation of hyper-active N\textsubscript{2}O emitting \textit{Pseudomonas} sp. SC-H2 from \textit{Sphagnum capillifolium} in palsa bog

As \textit{Sphagnum} moss-dominant palsa bogs mainly composed of \textit{Sphagnum capillifolium} and \textit{S. fuscum}, and degrading palsa bog often becomes a hot spot for N\textsubscript{2}O emission. Some gammaproteobacteria isolated from the \textit{S. capillifolium} showed hyper active N\textsubscript{2}O emitting capability in the culturing systems, and the most active N\textsubscript{2}O emitter from the culturable community was identifiable as \textit{Pseudomonas} sp. by 16S rRNA gene-targeted homology search. The N\textsubscript{2}O emitting \textit{Pseudomonas} sp. SC-H2 showed over 20 µg vial\textsuperscript{-1} day\textsuperscript{-1} of N\textsubscript{2}O production in 10 mL culture medium containing 0.05% sucrose only at neutral pH (6.8-7.3) but not at acidic regions. As its unique characteristic, \textit{nosZ} gene-harboring \textit{Pseudomonas} sp. SC-H2 skipped reduction process for N\textsubscript{2}O in the neutral to alkaline regions to produce high level of N\textsubscript{2}O.

3. Effect of gallic acid or (E)-caffeic acid on N\textsubscript{2}O emission by N\textsubscript{2}O emitters

All three \textit{Burkholderia} isolates from leaves of \textit{S. fuscum} were responsive to gallic acid and (E)-caffeic acid, and the most activated \textit{Burkholderia} sp. SF-E2 showed 67-fold higher N\textsubscript{2}O emission upon exposure to 100 mg L\textsuperscript{-1} (E)-caffeic acid. Relatively low concentration of (E)-caffeic acid (≤ 0.1 g L\textsuperscript{-1}) also obviously accelerated N\textsubscript{2}O emission by an \textit{Enterobacteriaceae} bacterium SC-L1 and \textit{Serratia} sp. SC-K1. Among them, \textit{Serratia} sp. SC-K1 was 13-fold higher of N\textsubscript{2}O production with 0.1 g L\textsuperscript{-1} than that without (E)-caffeic acid. On \textit{Pseudomonas} sp. SC-H2, N\textsubscript{2}O emission significantly decreased when concentration of (E)-caffeic acid was supplemented more than 0.01 g L\textsuperscript{-1}. In addition, N\textsubscript{2}O production in \textit{S. capillifolium}
with 0.1 g L$^{-1}$ (E)-caffeic acid was higher than those of *S. fuscum* during the incubation periods. According to their response to (E)-caffeic acid, two types of *Pseudomonas* denitrifiers isolated from Andisol were found. One group of the strains showed that N$_2$O production decreased when the concentration of (E)-caffeic acid over 0.05 g L$^{-1}$. Conversely, other group of the strains produced lower N$_2$O than control under lower concentration of (E)-caffeic acid ($\leq 0.01$ g L$^{-1}$). However, the latter group produced much more N$_2$O with supplemental 0.1 g L$^{-1}$ (E)-caffeic acid. For these *Pseudomonas* denitrifiers, phylogenetic tree of *nirS* and *narG* genes had a close relationship with phylogeny of 16S rRNA genes. In contrast, there is no similarity between *nosZ* and 16S rRNA gene phylogenetic tree.
GENERAL INTRODUCTION

This chapter briefly describes the major greenhouse gas of N₂O, N₂O emitting microorganisms particularly some eubacteria, the different pathways of their N₂O production, including microbial aspects associated with N₂O flux, the related enzymes and functional genes involved in the process of N₂O production. As molecular basis, phylogeny relationship between 16S rRNA gene and functional genes of N₂O emission, the environmental and chemical factors for N₂O gas efflux in soil, the characteristics of boreal palsa peat bogs in sub-arctic region including Finland, and the important secondary metabolites of *Sphagnum* mosses affecting microbial N₂O emission. At last, the main aims of this study were also displayed.

1.1 Nitrous oxide (N₂O), a potent greenhouse gas

1.1.1 N₂O gas

N₂O is a potent greenhouse gas (GHG) that contributes to about 7.9% of global warming (IPCC, 2011) and also depletion of stratospheric ozone (Crutzen, 1981; Ravishankara et al., 2009). The global warming potential (GWP) of N₂O is approximately 298-fold higher than that of carbon dioxide (CO₂) during a 100-year horizon (IPCC, 2007), due to its longer half-life in the atmosphere and higher radiation absorption efficiency than other greenhouse gases (Juszczak and Augustin, 2013).
1.1.2 Available pathways of N\textsubscript{2}O production

By microorganisms, N\textsubscript{2}O gas is biologically produced by pathways of nitrification, nitrifier denitrification, and denitrification and nitrate ammonification (DNRA) (Wrage et al., 2001; Baggs, 2008).

![Diagram of microbial pathways of N\textsubscript{2}O emission in soil](image)

(Baggs, 2008)

Fig. 0.1 Microbial pathways of N\textsubscript{2}O emission in soil

*Nitrification*

The pathway of hydroxylamine oxidation: nitrifiers oxidize ammonia (NH\textsubscript{4}\textsuperscript{+} or NH\textsubscript{3}) to NO\textsubscript{2} via hydroxylamine (NH\textsubscript{2}OH), and further the intermediary product NH\textsubscript{2}OH reduce to NO followed by N\textsubscript{2}O (Hooper and Terry, 1979). The pathway of nitrifier denitrification: after oxidization of ammonia oxidized to NO\textsubscript{2} via
hydroxylamine, and then reduced to N$_2$O via NO under the conditions of less concentration of O$_2$ (Goreau et al., 1980; Wrage et al., 2001).

*Denitrification*

This pathway is known as denitrification process that includes the reduction of NO$_3^-$ or NO$_2^-$ to N$_2$ via NO and N$_2$O gases mainly by aerobic or anaerobic denitrifiers under anoxic conditions (Robertson and Tiedje, 1987). Incomplete denitrification means that the reduction of N$_2$O to N$_2$ was blocked, resulting in active production of N$_2$O. Some are missing N$_2$O reductase or the enzyme does not function due to the lower pH (pH $\leq$ 6.1) in acidic soil (Philippot et al., 2011; Liu et al., 2014).

*Nitrate ammonification (DNRA)*

Another important pathway for N$_2$O emission is dissimilatory nitrate reduction to ammonium (DNRA) by microorganisms under the anaerobic conditions (Welsh et al., 2001). In this pathway, atypical nitrous oxide reductase genes (atypical nosZ) were often found instead of normal type nosZ. The atypical nosZ product may contribute to the reduction of N$_2$O, because DNRA eubacteria show potent activity to consume N$_2$O (Sanford et al., 2012). Among these N$_2$O emission pathways, denitrification is the most important source for N$_2$O emission in soils (Mosier, 1998).
1.2 Microbiological aspects of N$_2$O production

Nitrifiers, denitrifiers, and also including some nondenitrifiers drive the N$_2$O emission (Bleakley and Tiedje, 1982). N$_2$O-emitting nitrifiers are widely distributed in the genera of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB). A previous study reported that N$_2$O gas was produced by marine ammonia-oxidizing archaea CN25 and CN75 (Santoro et al., 2011), and AOA *Nitrosopumilus maritimus* SCM1 was also found as a N$_2$O emitter in a pure culture (Löscher et al., 2012). In addition, pure cultures of autotrophic AOB *Nitrosomonas* spp. were found to emit N$_2$O (Goreau et al., 1980; Lipschultz et al., 1981).

Denitrifiers are commonly found in the genera of archaea, bacteria, and fungi. Among them, denitrifying bacterium are widely observed, and they belong to several phyla including *Firmicutes* (genus *Bacillus*), *Actinobacteria* (genera *Propionibacteria*, *Corynebacterium*), *Bacteroidetes* (genus *Cytophaga*), and *Proteobacteria* including class *Alphaproteobacteria* (genera *Paracoccus*, *Rhizobium*, *Hyphomicrobium*, *Agrobacterium*), *Betaproteobacteria* (genera *Thiobacillus*, *Janthinobacterium*, *Burkholderia*, *Spirillum*, *Alcaligenes*), and *Gammaproteobacteria* (genera *Pseudomonas*, *Acinetobacter*). These microorganisms are commonly found in soils.

In the tropical peatlands, isolates of *Burkholderia* spp. as N$_2$O emitters were presented (Hashidoko et al., 2010b). In the early studies, pure cultures of denitrifying *Pseudomonas* species (*P. denitrificans*, *P. perfectomarinus*, *P. fluorescens* *P. stutzeri*, *P. aeruginosa*, *P. nautica*) were also investigated in their N$_2$O emission potentials (Delwiche, 1959; Payne et al., 1971; Balderston et al., 1976; Sørensen et al., 1980;
Dooley et al., 1987; Viebrock and Zumft, 1988; SooHoo and Hollocher, 1991; Prudêncio et al., 2000).

Nondenitrifiers of Enterobacter are also the common genus that contains some species to emit N$_2$O (Smith and Zimmerman, 1981). Furthermore, Anderson and Levine (1986) offered the evidence that a denitrifier Serratia sp. that can perform an effective nitrate respiration can produce N$_2$O gas.

1.3 Enzymes and functional genes involved in the process of N$_2$O production

The process for oxidation of NH$_3$ or NH$_4^+$ to NH$_2$OH is catalyzed by membrane-bound, multi-subunit enzyme, ammonia monooxygenase (AMO), encoded by amoABC (Hyman et al., 1988; Klotz et al., 1997). Abundance of amoA genes of AOB and AOA in soil was often investigated for the copy numbers and composition and diversity of ammonia oxidizing bacteria and archae by using real time PCR with amoA-specific primers amoA–1F (GGG GTT TCT ACT GGT GGT) and amoA–2R (CCC CTC KGS AAA GCC TTC TTC) (Rotthauwe et al., 1997; Araki et al., 2004).

(Wrage et al., 2001)
Scheme 1 Enzymes and their functional genes involved in nitrification

Oxidation of NH$_2$OH to NO by hydroxylamine oxidoreductase (HAO) encoded by hao gene (Hooper and Terry, 1979; Bergmann et al., 1994). In addition, electrons provided by HAO are accepted by a cytochrome P450 (CYP) family protein, cytochrome C554 encoded by cycA gene (McTavish et al., 1993).

![Scheme 1 Enzymes and their functional genes involved in nitrification](image)

(Philippot, 2002)

Scheme 2 Enzymes and functional genes involved in denitrification process

The process for denitrification of NO$_3^-$ to N$_2$ is catalyzed by nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase, which are encoded by napA/narG, nirS/nirK, norB, and nosZ genes respectively in complete denitrifying eubacteria.

Two types of dissimilatory nitrate reductase in different locations exist in denitrifiers. Membrane-bound nitrate reductase (NAR) encoded by narGHJI is the key enzyme in the first step of denitrification process (Zumft, 1997), and the narG gene that codes α-subunit of NAR is often used to assess the presence of environmental denitrifiers (Henry et al., 2006; Palmer et al., 2009). In addition, the periplasmic nitrate reductase (NAP) encoded by napEDABC (nap-α) and napDAGHB (nap-β) is also present (Simpson et al., 2010). Among them, napA gene codes the large catalytic subunit of NAP (Siddiqui et al., 1993). Both of them reduce NO$_3^-$ to...
The process of NO\textsuperscript{2−} reduction to NO is catalyzed by two types of nitrite reductases (NIR). One containing copper is encoded by \textit{nirK} gene, while the other containing cytochrome-cd\textsubscript{1} is encoded by \textit{nirS} gene, and these two genes are never found in the same strain (Zumft, 1997). Furthermore, \textit{nirS}-harboring denitrifiers have a larger abundance than those of \textit{nirK}-harboring denitrifiers. However, the latter is found in diverse microbial communities throughout prokaryotes (Coyne et al., 1989).

Nitric oxide reductase (NOR) encoded by \textit{norBC} gene catalyzes the reduction of NO to N\textsubscript{2}O, and \textit{norB} gene codes NOR larger subunit, which is classified into two types, cytochrome bc-cnor\textsubscript{B} and quinol-oxidizing single-subunit qnor\textsubscript{B} (Braker and Tiedje, 2003).

Nitrous oxide reductase (N\textsubscript{2}OR), encoded by \textalpha-subunit gene \textit{nosZ} and other \textit{nos}-related genes and located in the periplasm of Gram-negative bacteria, is the key enzyme (Cuypers et al., 1992; Pauleta et al., 2013). If functionality of N\textsubscript{2}OR as the catalyst, N\textsubscript{2}O emission becomes much more active than usual, while over expression of \textit{nos}-genes leads to mitigation of N\textsubscript{2}O emission. Thus, incomplete denitrifiers that have lost the \textit{nosZ} gene are active N\textsubscript{2}O emitters. Previous studies have presented that some \textit{Burkholderia} spp. isolated from tropical peatlands (Hashidoko et al., 2010a) and several \textit{Pseudomonas} isolates from Andisol corn farmland (Li et al., 2014) lack or have a malfunctional \textit{nosZ} gene. Incomplete denitrifiers missing the \textit{nosZ} gene, such as \textit{Agrobacterium tumefaciens} C58, have been reported in previous studies (Wood et al., 2001; Philippot et al., 2011); However, an atypical \textit{nosZ} gene was in
contrast, found in a non-denitrifying *Anaeromyxobacter* sp. (Sanford et al., 2012). The detailed mechanism of N₂O reduction and its repression at the gene level still remains unclear.

![Scheme 3 Enzymes and functional genes involved in DNRA](image)

(Stolz and Basu, 2002)

1.4 Phylogeny relationship between 16S rRNA gene and functional genes of N₂O production

Phylogeny analysis of *amoA* gene appears to be consistent with that based on 16S rRNA gene sequences (Purkhold et al., 2003). Several studies comparing the phylogenies of *nar, nir, nor,* and *nos* gene sequences for denitrification in bacterial
isolates showed that phylogenies of the functional genes are more or less inconsistent with that of 16S rRNA gene (Delorme et al., 2003; Heylen et al., 2007).

Jones et al. (2008) offered the evidence that a higher level of similarity was found in gene phylogeny between nirS and 16S rRNA gene sequences. While there is no similarity between phylogeny patterns of 16S rRNA gene and nosZ among a group of closely related denitrifying Pseudomonas (Delorme et al., 2003).

1.5 \( \text{N}_2\text{O} \) emissions in soils

Soils are a major source of \( \text{N}_2\text{O} \) emission during microbial N cycle (Röver et al., 1998). Some previous studies showed that \( \text{N}_2\text{O} \) emission rate was determined by different soil types, and \( \text{N}_2\text{O} \) flux rate is in the order of agricultural soils > drained and rewetted peatlands > pristine mires > pristine bogs (Novak et al., 2015b). Agricultural soils are important \( \text{N}_2\text{O} \) sources owing to nitrogen fertilizer and manure inputs (Smith et al., 1998), and Kusa et al. (2006) reported that maize farmland Andisol displayed a high \( \text{N}_2\text{O} \) emission.

\( \text{N}_2\text{O} \) emissions in boreal peatlands

Peatlands store one-third of global soil carbon and cover nearly one third of Finnish land area (Eurola et al., 1984; Savolainen et al., 1994). The larger \( \text{N}_2\text{O} \) fluxes were found in the farmed peat soil in southern area of Finland (Regina et al., 2004), while in the natural northern peatlands, lowering of water table increased \( \text{N}_2\text{O} \) emission (Regina et al., 1999). In arctic tundra, large \( \text{N}_2\text{O} \) emission from cryoturbated
peat soil was observed (Repo et al., 2009). Thus, *Sphagnum*-dominated boreal peat soils have shown relatively high eubacterial diversity, in which acid-tolerable bacteria of *Burkholderia* occupy predominant population in the community (Sun et al., 2014). 

$\text{N}_2\text{O}$ emitting *Burkholderia* spp. were also isolated from deforested acidic tropical peatlands (Hashidoko et al., 2010a). In addition, Palmer and Horn (2012) reported that palsa peatlands in northwestern Finnish Lapland showed variable *in situ* $\text{N}_2\text{O}$ emission.

![The development of mires in Finland: Palsa bogs (Northern Lapland)](image)

(Palsa bogs is above on the red line)

(Virtanen and Valpola, 2011)

**Fig. 0.2 Distribution of peatlands in Finland**
1.6 Characteristics of palsa peatlands

In Finland, palsa peat bogs formed due to the ice core under the sphagnum peat layer are frequent in the northern region with the lower temperature in the winter (Seppala, 1982).

Fig. 0.3 Palsa bog and its core ice

Seppälä (2011) described the general model of the formation of frozen palsa peat with the seasonal change.

Once the ice core melted, the drained palsa bogs start to show ground subsidence, collapses, and becomes to the catchment areas (Seppälä, 2011). Furthermore, degraded palsa bogs often have relatively low C/N ratios than intact sites (Krüger et al., 2014). So the degraded peat bogs may be potential hotspots for N₂O emission.

Fig. 0.4 Degradation of palsa bog due to global warming effect
1.7 Sphagnum mosses in boreal peat bogs

*Sphagnum* mosses are the dominant genus of vegetation in northern peatland ecosystem, which harbor a high diversity of microbial communities (Dedysh et al., 2006; Gilbert et al., 2006; Opelt et al., 2007b). Thus, *Sphagnum* mosses are regarded as the keystone species in northern peatlands (Rochefort, 2000).

![Sphagnum fuscum](image1) ![Sphagnum capillifolium](image2)

**Fig. 0.5 Sphagnum fuscum and Sphagnum capillifolium**

*Sphagnum fuscum* and *Sphagnum capillifolium* are the typical upland *Sphagnum* species in northern peatlands (Novak et al., 2015a). *S. fuscum* is brown, while *S. capillifolium* is a wine-red color, both widely distributed throughout peat bogs of European and North America. Both contain unique pigments, and inhabited upland to develop peat bogs. In 1985, the greater nitrate reductase activity was measured in *S. fuscum*-dominated peat bogs due to wet deposition (Woodin et al., 1985). Increased
atmospheric N deposition can reduce the growth of some *Sphagnum* species, such as *Sphagnum magellanicum* (Limpens and Berendse, 2003). In contrast, an increased production of *S. fuscum* along with the elevated N deposition was reported by Vitt et al. (2003), and *S. capillifolium* is also tolerant to high N supply (Bonnett et al., 2010).

1.8 The impact factors of N$_2$O emission

Many factors effect on N$_2$O emission in soil systems, such as pH, water-table, temperature, nitrogen and carbon availabilities, and C/N ratio (Regina et al., 1996; Pfenning and McMahon, 1997; Klemedtsson et al., 2005; Liu et al., 2014). Numerous of reports on effect of pH on N$_2$O emission in soil are available. Soil pH $>$ 6.1 is necessary for the functionalities of N$_2$OR (nitrous oxide reductase) to reduce N$_2$O into N$_2$ (Liu et al., 2014). *Sphagnum* mosses as the predominant plant that formed peat bogs create an acidic environment (Verhoeven and Liefveld, 1997) and decompose slowly under an anoxic condition (Dorrepaal et al., 2005). Wetlands covered with acidic *Sphagnum* peat are global carbon sink while simultaneously a source of greenhouse gases, methane (CH$_4$) and N$_2$O (Kolb and Horn, 2012). In fact, an anoxic environment can accelerate the process of denitrification, while low pH generally inhibits reduction of N$_2$O to N$_2$ gas by interfering in the assembling of N$_2$OR. Thus, both environmental factors lead to the promotion of an N$_2$O flux (Liu et al., 2014).

The pristine peat bogs are often water-logged, leading to anoxic conditions of the peat soil, and their capacity of N$_2$O emission is negligible owing to less accumulation of NO$_3^-$ in the soil (Martikainen et al., 1995; Novak et al., 2015b). Some reports
showed that lowering water table enhanced \( \text{N}_2\text{O} \) emission in nutrient-rich peatlands than those in nutrient-poor bogs (Martikainen et al., 1993; Regina et al., 1996). Water-drained peat soils also can enhance the emissions of \( \text{N}_2\text{O} \) possibly due to increased nitrification activity (Goodroad and Keeney, 1984; Regina et al., 1996).

Temperature is another key factor for denitrification activity. \( \text{N}_2\text{O} \) production had a 77% increase from 4 to 22 \(^\circ\)C, indicating that \( \text{N}_2\text{O} \) emission increased along with progressed global warming (Pfenning and McMahon, 1997). Indeed, the optimal temperature of denitrifiers \textit{Pseudomonas denitrificans} for denitrification is 38 \(^\circ\)C as reported by Wang et al. (1995).

The C/N ratio is also an important factor to give a potent impact to \( \text{N}_2\text{O} \) emission. Several studies showed that the lower C/N ratio \( \leq 20 \), in which peat soil decomposition is progressed, stimulated \( \text{N}_2\text{O} \) emission (Huang et al., 2004; Klemetsson et al., 2005; Maljanen et al., 2012), while lower \( \text{N}_2\text{O} \) emission in the virgin or ombrotrophic peat bogs with a higher C/N ratio was also found (Regina et al., 1996).

1.9 N and C sources in northern peat bogs for \( \text{N}_2\text{O} \) emission

Certain parts of boreal peatlands have been recognized as an important source of atmospheric \( \text{N}_2\text{O} \) (Regina et al., 1999; Repo et al., 2009). However, boreal peat bogs are generally known to emit low level of \( \text{N}_2\text{O} \) due to a high C/N ratio, so they were thought as the negligible region in source of \( \text{N}_2\text{O} \) (Pendea and Chmura, 2012). Nevertheless, an increase in N deposition from 15 Tg N·yr\(^{-1}\) to 187 Tg N·yr\(^{-1}\) from the mid-19\(^{th}\) century to early 21\(^{st}\) century (Galloway et al., 2008) enhanced the potential for
carbon loss from boreal peat bogs (Bragazza et al., 2006; Kivimäki et al., 2013), and might have enhanced the N\textsubscript{2}O emission potential of the *Sphagnum*-dominated ombrotrophic peat system. In addition, deposition of ammonia-form N (NH\textsubscript{4}\textsuperscript{+} or NH\textsubscript{3}) by nitrogen-fixers results in the dominance of certain species of *Sphagnum* mosses (Sheppard et al., 2013), even though uptake of negligible amount NH\textsubscript{4}\textsuperscript{+} by *Sphagnum* mosses in the interstitial water was observed (Limpens and Berendse, 2003).

The excess NH\textsubscript{4}\textsuperscript{+} that is accumulated in the topsoil is actively oxidized into NO\textsubscript{3}\textsuperscript{−} by nitrifiers, and eventually reduced by denitrifiers to N\textsubscript{2}O in the peat bog system. In a previous study, addition of ammonium/nitrate (NH\textsubscript{4}\textsuperscript{+}:NO\textsubscript{3}\textsuperscript{−}, 1:1 mixture) solution in boreal *Sphagnum magellanicum*-dominated peat triggered *de novo* synthesis of denitrification-associated enzymes in denitrifiers, leading to active N\textsubscript{2}O emission (Francez et al., 2011). At the same time, the available NO\textsubscript{3}\textsuperscript{−}-N from N deposition or organic N mineralization can be directly used by denitrifiers as electron accepter for nitrate respiration.

### 1.10 Polyphenols of *Sphagnum* mosses

*S. fuscum* which exists in acidic peat bogs as the dominated species is rich in polyphenols, which play an important role in protecting *Sphagnum* moss from biological decomposition via inhibition of cell hydrolysis by saprophytic microorganisms associated with peat moss (Hättenschwiler and Vitousek, 2000). It is unclear how polyphenols influence on the N\textsubscript{2}O emitters, especially of incomplete N\textsubscript{2}O
emitters (which is missing nosZ gene or N₂OR functionalities). However, such chemicals probably act mitigation of N₂O emission.

The major aim of this study is as follows: 1) isolation and investigation of N₂O emitters isolated from sphagnum peat bogs, 2) influence of different concentration of plant polyphenols on N₂O production by the N₂O-emitting bacteria, and 3) phylogenetic analyses of denitrification associating genes (narG, nirS, nosZ) and 16S rRNA gene of the available N₂O emitters isolated from Sphagnum mosses and acidic soils.
CHAPTER 1. Isolation of N\textsubscript{2}O emitting-bacteria from \textit{Sphagnum fuscum}

Materials and Methods

1.1 Soil and \textit{Sphagnum} moss sampling in 2009 in Finland

Peat soils and \textit{Sphagnum} mosses (tentatively identified as \textit{Sphagnum angustifolium} (pale brownish green, large), \textit{S. girgensohnii} (green, large), and \textit{S. fuscum} (brown, small) were collected from Kilpisjärvi (69°0′N; 20°82′E), and one sample of \textit{S. fuscum} was collected from a mire near Joensuu (66°19′N; 29°29′E) in Finland in August, 2009 as shown in Table 1.1. Six Histosol samples under \textit{Empetrum nigrum} and \textit{Vaccinium vitis-idaea} vegetation were collected from two different sites, a wet bog (at the surface [0 cm], and 10 cm and 30 cm in depth) and a relatively dry bog (at 10 cm, 30 cm, and 50 cm in depth). These ten samples, stored in zippered plastic containers at 4 °C, were subjected to culture-based N\textsubscript{2}O emission assay. From the cultures that showed active N\textsubscript{2}O emission, N\textsubscript{2}O emitters were also isolated. All necessary permits were obtained for the described field study at Kilpisjärvi Biological Research Station, Finland. Permission for the soil sampling was obtained from the Metshallitus (the State Forest Enterprise of Finland). The sampling process did not disturb the Strict Nature Reserve Areas or disturb endangered or protected species.
Fig. 1.1 Sampling sites were located in Kilpisjärvi and Joensuu, Finland

Table 1.1 Sampling conditions of 10 soil and *Sphagnum* moss samples.

<table>
<thead>
<tr>
<th>Number</th>
<th>Sampling Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Relatively dry bog, <em>Sphagnum fuscum</em> (brown)</td>
</tr>
<tr>
<td>2</td>
<td>Relatively dry bog, <em>Sphagnum angustifolium</em> (green/large)</td>
</tr>
<tr>
<td>3</td>
<td>Relatively wet bog, <em>Sphagnum girgensohnii</em> (green/large)</td>
</tr>
<tr>
<td>4</td>
<td>Relatively wet bog, <em>Sphagnum fuscum</em> (brown)</td>
</tr>
<tr>
<td>5</td>
<td>Relatively dry bog under <em>Empetrum</em> (10 cm)</td>
</tr>
<tr>
<td>6</td>
<td>Relatively dry bog under <em>Empetrum</em> (30 cm)</td>
</tr>
<tr>
<td>7</td>
<td>Relatively dry bog under <em>Empetrum</em> (50 cm)</td>
</tr>
<tr>
<td>8</td>
<td>Relatively dry bog under <em>Empetrum</em> (top soil, 0 cm)</td>
</tr>
<tr>
<td>9</td>
<td>Relatively dry bog under <em>Empetrum</em> (10 cm)</td>
</tr>
<tr>
<td>10</td>
<td>Relatively dry bog with <em>Empetrum</em> (30 cm)</td>
</tr>
</tbody>
</table>

All samples were collected from Kilpisjärvi, except for sample 4, which was collected at mire near Joensuu.
For the culture-based N\textsubscript{2}O emission assay, the medium used was Winogradsky’s mineral solution with 6.25 mM NH\textsubscript{4}NO\textsubscript{3} (0.5 mg·mL\textsuperscript{-1}) without a sugar supplement. The solution was adjusted to pH 5.0 with a 2 M solution of H\textsubscript{2}SO\textsubscript{4} that was gelled with 0.3% gellan gum to mimic phyllosphere of water-filled peat moss, and then autoclaved. A 10 mL portion of the medium was poured into a 30-mL gas–chromatographic vial with a butyl-rubber plug (Nichiden-Rika Glass Co., Kobe, Japan) for the culture-based N\textsubscript{2}O emission assay.

The headspace volume of the culture medium was 22.5 mL, after the addition of 100 µL of inoculants. For preparation of the inoculants, a suspension of \textit{Sphagnum} moss tissue was generated by adding 100 mg as air-dried tissue into 10 mL of sterilized Milli-Q water, vortexing for 30 s to mix thoroughly, and then leaving the mixture to stand for 10 min. An aliquot (100 µL) of the resulting supernatant was added to the medium as the inoculants for the N\textsubscript{2}O assay. The medium, inoculated with the microbial communities from the phyllosphere, was vortexed for 30 s to mix thoroughly, followed by incubation at 15 °C in the dark. After 6-, 16-, and 32-days of incubation, the N\textsubscript{2}O concentration in each headspace gas was measured using an ECD-gas-chromatograph (Shimadzu GC-14B, Kyoto, Japan) connected to a Porapak N column (1-m long, Waters, Milford, MS, USA).
1.2 Extraction of DNA from the cultured medium inoculated with the suspension of *Sphagnum* mosses tissue and PCR-DGGE analysis for the dominant eubacteria

The DNA of bacterial communities in the vial inoculated with *Sphagnum* mosses tissues, which displayed the most active N$_2$O emission among all samples incubated for 16 days, was extracted using an Isoplant DNA Extraction kit (Nippon Gene, Toyama, Japan). To determine the dominant bacterial community structure in the culture, denaturing gradient gel electrophoresis (DGGE) was performed using a DCode™ Universal Mutation Detection System according to the manufacturer’s instructions (Bio-Rad, Hercules, California, USA). PCR amplification of the 16S rRNA gene was performed using a universal primer pair, 341F with a GC clamp (341gcF)/907R (5'-CGC CCG CCG CGC CCC GGG GTC CCG CCG CCC CCG CCC GCC TAC GGG AGG CAG CAG-3'/5'-CCG TCA ATT CCT TTG AGT TT-3'), and AmpliTaq Gold ® DNA Polymerase as follows: for initial activation of the DNA polymerase, 95 °C for 5 min, and then 30 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, then at 72 °C for 10 min, and finally maintained at 4 °C. The PCR products were run on a polyacrylamide gel (6% w/v) with a gradient ranging from 30 to 70% of denaturant.

1.3 Screening and identification of N$_2$O emitting bacteria

To screen N$_2$O emitting microorganisms, 100 µL aliquots of the culture media that produced significant amounts of N$_2$O were $1 \times 10^4$- and $10^5$-fold diluted, spread
over plates of modified Winogradsky’s mineral solution containing 0.005% yeast extract and 0.5% sucrose and solidified with 3% gellan gum (MWG), and incubated for 5 days at 15 °C in the dark. Eight distinguishable bacterial colonies, A, B, C, D, E, F, G, and I, characterized by size, color, shape, gloss, and other characteristics such as transparency, were isolated from the MWG plates and transferred to potato-dextrose agar (PDA) plates for future purification. For each bacterium, 8 similar colonies were isolated (marked as A1–A8, B1–B8, C1–C8, D1–D8, E1–E8, F1–F8, G1–G8, and I1–I8). A total of 64 isolates, with 100 µL of each bacterial cell suspension (OD660 nm 0.9~1.0) were subjected to the culture-based N2O emission assay.

For identification of the top three most active isolates (SF-A1, SF-C1, and SF-E2), 16S rRNA genes were sequenced, and the resulting sequences were compared with sequences in the BLASTN (nucleotide basic local alignment search tool) database program provide by NCBI (National Center of Biotechnology Information, Bethesda, MD, USA; http://blast.ncbi.nlm.nih.gov/Blast.cgi). The target gene first amplified by PCR using the universal primer pair of 27F/1525R (5’-AGA GTT TGA TCC TGG CTC AG-3’/5’-AAA GGA GGT GAT CCA GCC-3’) were sequenced using a ABI prism™ 310 Genetic Analyzer (Applied Biosystems, USA) with several universal primers for eubacterial 16S rRNA gene, 338R, 341F, 907R, 1080R, 1380R, 1492R, and 1112F.
1.4 Response of N$_2$O emitters to NO$_3^-$ and NH$_4^+$

For culture-based N$_2$O emission assay with each bacterial pure isolate, the bacterial colonies pre-cultured on PDA for 3-4 days at 15 °C were collected with a nichrome wire loop and suspended into 1.5 mL MilliQ water. A 20 µL portion of the inoculant that showed optical density of 0.9–1.0 was added to the medium and then briefly vortexed, followed by incubation at 15 °C for 5-10 days in the dark. To examine the optimum substrate for N$_2$O emission of the three isolates of *Burkholderia* (SF-A1, SF-C1, and SF-E2), 1 mM of KNO$_3$, NH$_4$NO$_3$, and NH$_4$Cl were alternatively added to 0.3% gellan gum soft gel of the sugar-free Winogradsky’s mineral solution. For the culture-based N$_2$O emission assay, each isolate was incubated at 15 °C for 7 days in the dark in triplicate. To confirm the optimum concentration of the most acceptable substrate, KNO$_3$, for N$_2$O production, the N$_2$O
emission assay was also performed on each active isolate of *Burkholderia* in a series of concentrations (0.1, 0.5, 1.0, 2.0, and 5.0 mM KNO$_3$) in triplicate, and incubated under the same conditions as described above.

### 1.5 Response of the N$_2$O emitters to supplemented sucrose

To observe the impact of a supplemented carbon source, the N$_2$O emission assay for each N$_2$O emitter isolated from the *Sphagnum* moss was performed at sucrose concentrations of 0.0, 0.1, 0.5, 1.0, 2.0, and 5.0 g L$^{-1}$. At each concentration, the N$_2$O emission test was done in triplicate. Incubation conditions were the same as described above.

### 1.6 Optimum pH and temperature for the N$_2$O emitters

In order to determine the effect of pH on N$_2$O production by N$_2$O emitters, media were adjusted to various pH values (pH 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0, before autoclaving) with a 2 M solution of H$_2$SO$_4$ or KOH added to the N$_2$O emission assay. All of the N$_2$O emitters tested in triplicate were incubated at 15 °C for 7 days in the dark for the culture-based N$_2$O assay. In parallel with pH, the response of N$_2$O emitters to alternative temperatures (at 4, 10, 15, 20, 25, and 30 °C) was examined in triplicate. In the investigation of the optimal temperature for N$_2$O emission by the active isolates, we used the same culture and assay conditions except for the incubation temperature.
1.7 C$_2$H$_2$ blocking assay

To examine the potential of the N$_2$O emitters to reduce N$_2$O to N$_2$, 10% volume (2.25 mL) acetylene gas (C$_2$H$_2$) was injected into the headspace of the assay vials immediately after the inoculation of each N$_2$O emitter, and incubated at 15 °C for 7 days. For controls, the same N$_2$O emitter was incubated without injection of 10% C$_2$H$_2$.

1.8 Cell growth under different pH and temperatures

To examine their growth characteristics, each bacterium was static-cultured in 5 mL MW liquid medium in a 18-cm test tube in triplicate. The ingredients of the liquid medium was the same as that used for the culture-based N$_2$O emission assay, except for gellan gum powders removed from the liquid medium. We examined cell growth performance of each bacterium in triplicate at 15 °C and 25 °C (both at pH 5), and also at pH 5.0 and 7.0 (both at 15 °C) respectively. After vortexed briefly at day 5, a 200 µL portion of the culture medium was poured into a well of a 90-well plate (Costar 3599, Corning Inc., New York, USA) and OD values was measured at 660 nm by using a Microplate Reader, Sunrise™ (Tecan, Männedorf, Switzerland).

1.9 PCR assay for detection of denitrification-associated genes, nosZ and narG

Two different primer pairs for nosZ gene, nosZ1111F/nosZ1773R (5'-STA CAA CWC GGA RAA SG-3'/5'-ATR TCG GAT CAR CTG BTC GTT-3') and nosZ661F/nosZ1527R (5'-CGG CTG GGG GCT GAC CAA-3'/5'-RCT GRC TGT
CGA DGA ACA G-3') (Scala and Kerkhof, 1998), were used to examine the presence or absence of a functional nosZ gene in N₂O emitters. The PCR was performed at alternative annealing temperatures (40–60 °C), using a thermal gradient PCR (Takara PCR Thermal Cycler Dice TP600, Takara, Otsu, Japan) under the conditions as follows: 95 °C for 10 min, and then 30 cycles of denaturation at 95 °C for 1 min, a gradient of annealing temperatures from 48 to 55 °C for 1 min, and extension at 72 °C for 1.5 min, followed by the final re-assembling at 72 °C for 10 min, and then maintained at 4 °C. Under the same PCR conditions, a primer pair, NosZ912F/NosZ1853R (5'-CGT CCC CGG CCT CGT GTA-3'/5'-GAG CAG AAG TTC GTG CAG TAG TAG GG-3') for an atypical nosZ gene, was also used).

For the comparisons, the narG gene was also targeted for PCR assay with a pair of specific primers (narG-2168F, 5'-TCG GGC AAG GGC CAC GAA TAC-3' for forward and narG-2391R, 5'-TTC TCG TAC CAC GTC GCG GTC-3' for reverse) for the genus Burkholderia were used. The PCR conditions were as follows: 95 °C for 10 min, 30 cycles (95 °C for 1 min, gradient of annealing temperatures from 48 to 55 °C for 1 min, and 72 °C for 1.5 min), final reassembly at 72 °C for 10 min, and then maintenance at 4 °C.

### 1.10 Sequencing of amplified nosZ and narG products and DGGE-cutting bands

Amplicons of target genes were purified using a Sephadex G-25 column. A sequencing reaction was performed in a total volume of 20 µL as a mixture of 2 µL
purified primary amplicons, 3 µL 5× sequencing buffer, 3.2 µL of 1 µM primers, and 2 µL of Big Dye™ pre-mixture (Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems) and up to 20 µL with Milli-Q water. The sequencing PCR conditions were 25 cycles (95 °C for 15 s, 50 °C for 15 s, and 60 °C for 4 min).

Results

1.1 N₂O emitting activity of the samples examined and dominant bacteria in the most active culture inoculated with *Sphagnum fuscum*

According to N₂O assay, microbial communities in sample 4 (*Sphagnum fuscum* from Joensuu) showed a remarkably high N₂O production potential among the samples tested at 15 °C (Fig. 1.3). The culture medium inoculated with a 100 mg tissue of *S. fusum* produced 185.5 ng N₂O vial⁻¹ day⁻¹ by 16-day incubation and 171.2 ng N₂O vial⁻¹ day⁻¹ by 32-day incubation. During the incubation periods, N₂O production in this culturing system was more likely constant and sustainable, and total amount of N₂O accumulated in the headspace was 5.48 µg after incubation for 32 days. In contrast, the other samples, particularly peat soils, did not show any significant N₂O emission in the culture-based N₂O emission assay, suggesting that highly moist and heath-dominant vegetative conditions result in the repression of denitrification and N₂O production.
Fig. 1.3 Culture-based N$_2$O assay of 10 peat soils and *Sphagnum* mosses.

(A) *Sphagnum fuscum* used for inoculants. (B) Culture-based N$_2$O assay medium with sucrose was inoculated with portion of each peat soil or tissue fragment of *Sphagnum* moss and the headspace gas of each vial were sampled at 6$^{th}$, 16$^{th}$, and 32$^{nd}$ day of incubation at 15 °C in the dark for the following N$_2$O analysis. The serial number of samples is shown in Table 1.1

Profiles of 16S rRNA gene-targeted PCR-DGGE in the bacterial community cultured *S. fuscum*-associating microorganisms indicated that the culturable bacterial community from *S. fuscum* leaves contained *Burkholderia* sp. and *Mucilaginibacter* sp. as the major components (Fig. 1.4). Many species of genus *Mucilaginibacter* are known to produce extracellular polysaccharides and also plant growth–promoting rhizobacteria (Madhaiyan et al., 2010). Some are also characterized as xylanolytic and laminarinolytic bacteria highly associated with degradation of peat bogs to provide carbon sources to other heterotrophs (Timonen and Hurek, 2006; Shcherbakov et al., 2013).
Fig. 1.4 PCR-DGGE profile of bacterial community from *Sphagnum fuscum* leaves

Culture-based N₂O assay for bacterial communities in phyllosphere of *Sphagnum* mosses. (A) DNA extracted from medium incubated for 16 days with epiphytic/endophytic bacteria from *S. fuscum* leaves was subjected to 16S rRNA gene-targeted DGGE in a denaturing gradient polyacrylamide gel containing 30–70% formamide/urea mixture. (B) Community structures of culturable bacteria revealed by the PCR-DGGE profile were subjected to phylogenetic analysis using DNA sequences of the separated DGGE bands (from 499 to 788 for *Escherichia coli*)
Fig. 1.5 Identification of N₂O emitting *Burkholderia* isolated

Nearly full sequences (1358 pb) of 16S rRNA gene targeted phylogenetic analysis for the N₂O emitting *Burkholderia* species isolated from the culture and some reference strains within the same genus. Outgroup is *Escherichia coli* (accession no. AF233451.1).

N₂O emission potentials of 64 colonies were also examined using a culture-based N₂O emission assay for mainly four distinguishable bacteria with 8 replicate. Every eight similar colonies were collected for N₂O assay from the master plate as bacteria A, B, C, D, E, F, G and I. (note: data of B, D, F, and I did not show because of their very low N₂O emission). Controls 1 and 2 are without inoculants.
1.2 Isolation and identification of the N$_2$O emitters

Identification was carried out by using 16S rRNA gene sequence. Most aligned DNA sequence was searched from NCBI database. Among the bacteria isolated from the culture, three isolates of *Burkholderia*, SF-A1, SF-C1 and SF-E2, were relatively active in N$_2$O emission (Fig. 1.6). Two isolates, SF-A1 and SF-E2, were in the same clade of a *Burkholderia* with the highest sequence homology to *Burkholderia* sp. DF4EH4 (accession no. AJ884797.1) that had been isolated from *Pinus sylvestris*-Suillus bovinus mycorrhizosphere in Canadian boreal forest (Timonen and Hurek, 2006), two uncultured *Burkholderia* from Lake Motykino in Northern Russia (accession nos. JN825266.1 and JN825267.1) (Fedotova et al., 2012), and another uncultured *Burkholderia* sp. from a PCB-polluted soil in Germany (AJ292641.1) (Nogales et al., 2001), all of which are without giving species name (Fig. 1.5). *Burkholderia* sp. DF4EH4 is described as a probable helper of the conifer trees for nitrogen acquisition, but no information for metabolic properties of inorganic nitrogen was shown in the other *Burkholderia* bacteria.

![Fig. 1.6 Screening of N$_2$O emitters using a culture-based N$_2$O emission assay](image-url)
Table 1.2 Identification of N$_2$O emitters isolated from the surface of Sphagnum fuscum leaves using 16S rRNA gene sequence

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Length (bp)</th>
<th>Accession no.</th>
<th>Most aligned DNA (accession no.)</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF-A1</td>
<td>1528</td>
<td>LC008480</td>
<td>Uncultured bacterium MB108 (JN825266.1)</td>
<td>1493/1496</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Burkholderia</em> sp. DF4EH4 (AJ884797.1)</td>
<td>1494/1498</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Uncultured bacterium WD263 (AJ292641.1)</td>
<td>1491/1497</td>
</tr>
<tr>
<td>SF-C1</td>
<td>1522</td>
<td>LC008479</td>
<td><em>Burkholderia phenazinium</em> ATCC 33666 (NR112068.1)</td>
<td>1495/1515</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Burkholderia phenazinium</em> A1 (NR029212.1)</td>
<td>1470/1472</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Burkholderia fungorum</em> KN-02 (AB091188.1)</td>
<td>1501/1524</td>
</tr>
<tr>
<td>SF-E2</td>
<td>1526</td>
<td>LC008478</td>
<td>Uncultured bacterium MB108 (JN825266.1)</td>
<td>1492/1497</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Burkholderia</em> sp. DF4EH4 (AJ884797.1)</td>
<td>1493/1499</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Uncultured bacterium WD263 (AJ292641.1)</td>
<td>1490/1498</td>
</tr>
</tbody>
</table>

In contrast, SF-C1 was identifiable as *Burkholderia phenazinium* due to a high matching with *B. phenazinium* A1 (NR029212.1) originally isolated from soil (Viallard et al., 1998). Also, 16S rRNA gene-targeted phylogenetic analysis among genus *Burkholderia* (Fig. 1.5) clearly showed that SF-C1, *B. phenazinium* A1, and *B. phenazinium* LMG2247$^T$ (U96931) are involved in the same clade different from that of SF-A1 and SF-E2. Sequence data from three 16S rRNA genes of the N$_2$O emitters were deposited in the DNA Data Bank of Japan (DDBJ) (Table 1.2).

1.3 Optimum substrate and its concentration

Among the mineral nitrogen tested for the substrates of N$_2$O emission, 1 mM KNO$_3$ is the most acceptable substrate for these N$_2$O emitting *Burkholderia* spp. (Fig. 1.7A). In comparison, 1 mM NH$_4$Cl or NH$_4$NO$_3$ induced less N$_2$O emission than KNO$_3$. NH$_4$NO$_3$ as the mineral N gave induced higher N$_2$O production by the N$_2$O emitters than that in the NH$_4$Cl-supplemented culturing system. The optimal concentration of KNO$_3$ was observed at 1 mM, but N$_2$O emissions were maintained at higher concentrations (2 to 5 mM) of KNO$_3$ (Fig. 1.7B).
Fig. 1.7 Response of N$_2$O emitting *Burkholderia* to mineral nitrogen and dose-response of N$_2$O emitters to KNO$_3$

(A) Alternative substrates (1 mM of KNO$_3$, NH$_4$Cl, or NH$_4$NO$_3$) were added to the N$_2$O assay medium to test the optimum substrate for N$_2$O emission by *Burkholderia* sp. SF-A1 and SF-E2 and *B. phenazinium* SF-C1. (B) To the N$_2$O assay medium, different concentrations of KNO$_3$ were added to elucidate the optimum concentration of the substrate for N$_2$O emission by the *Burkholderia* isolates. pH was adjusted to 5.0.

1.4 Optimum pH and temperature for N$_2$O emission

The optimum pH for N$_2$O emission was pH 5.0 for all the strains examined (Fig. 1.8A). In contrast, at a pH below or above 5.0, N$_2$O emission was significantly repressed. For the optimum temperature, *Burkholderia* sp. SF-E2 showed a positive linear relationship between N$_2$O emission and temperature from 4 to 30 °C: the total amount of N$_2$O emitted from the culture at 30 °C was approximately 4-fold higher than that at 15 °C. In contrast, the other two isolates (*B. phenazinium* SF-C1 and *Burkholderia* sp. SF-A1) decreased their N$_2$O emission at temperatures above 25 °C (Fig. 1.8B).
Fig. 1.8 Optimal pH value and incubation temperature for N₂O emitting *Burkholderia* species

(A) The soft gel medium for the culture-based N₂O assay was adjusted to alternative pH values (pH 4.0, 5.0, 6.0, 7.0, or 8.0) and incubated at 15 °C for 7 days. (B) *Burkholderia* sp. SF-A1 and SF-E2 and *Burkholderia phenazinium* SF-C1 were cultured at different temperatures (4, 10, 15, 20, 25, and 30 °C) to examine optimal temperature for N₂O emission. The medium contained 0.5 mM KNO₃ and was adjusted to pH 5.0.
1.5 Trends in response to supplemental sucrose and acetylene gas

Addition of sucrose in the culturing medium for the N\textsubscript{2}O emission assay of these three N\textsubscript{2}O emitters showed a linear relationship between N\textsubscript{2}O emissions and sugar concentration from 0.1 to 5 g L\textsuperscript{-1}. However, the accelerating effect of the additional sucrose on N\textsubscript{2}O emission was not drastic, unlike \textit{Burkholderia} N\textsubscript{2}O emitters in tropical peat soils and pseudomonads in Andisol corn farm soils. N\textsubscript{2}O emission of the three emitters in this study at a sucrose concentration of 5 g L\textsuperscript{-1} was 3.5-fold higher than that without sucrose (Fig. 1.9A). This low response suggests that these three isolates of \textit{Burkholderia} are oligotrophs rather than saprophytic heterotrophs.

When exposed to 10% acetylene (C\textsubscript{2}H\textsubscript{2}) gas, three \textit{Burkholderia} N\textsubscript{2}O emitters, including \textit{Burkholderia} sp. SF-E2, did not show any significant increase or decrease in N\textsubscript{2}O emission in the C\textsubscript{2}H\textsubscript{2} inhibition assay when compared with the control (without C\textsubscript{2}H\textsubscript{2}) (Fig. 1.9B).
Fig. 1.9 Response of N$_2$O emitters to supplemental sucrose and 10% acetylene gas

(A) Sucrose (0.05, 0.1, 0.2, 0.3, and 0.5% w/v) was added to the N$_2$O assay medium to examine the N$_2$O emissions by *Burkholderia* sp. SF-A1 and SF-E2 and *B. phenazinium* SF-C1. Incubation was done at 15 °C for 7 days. (B) Their N$_2$O emissions upon exposure to 10% acetylene, which inhibits N$_2$O reductase to release N$_2$O as the final product of nitrate-respiration, were tested with treatments but without injection of acetylene gas as a control.
1.6 Cell growth

After the 5-day-incubation, *Burkholderia* sp. sfA1 and *B. phenazinium* C1 showed similar cell growth performances without any statistic significance between those incubated at 15 °C and 25 °C. Conversely, *Burkholderia* sp. SF-E2 exhibited 1.3 times higher turbidity at 25 °C than that cultured at 15 °C at day 5. At day 7, however, all the N₂O emitters cultured at 25 °C showed better cell growth than those cultured at 15 °C (Fig. 1.10). Consequently, all the bacteria of *Burkholderia* are mesophile, and *Burkholderia* sp. SF-E2 is particularly adaptable for relatively high temperature.

![Fig. 1.10 Adaptable pH and temperature for cell growth of *Burkholderia* N₂O emitters](image)

(* **P < 0.01, ***P < 0.001 toward pH 5.0*)
In addition, all the N\textsubscript{2}O emitters of *Burkholderia* showed 1.5-2.0 times higher cell growth at pH 5.0 compare to those cultured at pH 7.0, suggesting that all are also adapted for weakly acidic conditions of sphagnum moss tissues. Although the turbidity of the culture was almost the same level at pH 7.0 (OD\textsubscript{660} 0.183-0.189 for three isolates at day 7), *Burkholderia* sp. SF-E2 at pH 5.0 showed the best cell growth performance that is 2.0 times higher than that cultured at pH 7.0. In parallel with this cell growth promotion under a weakly acidic condition, 7-day-incubated *Burkholderia* sp. SF-E2 showed 1.7 times higher N\textsubscript{2}O emission at pH 5.0 than that cultured at pH 7.0 (note Fig. 1.8A). Taken together, all the N\textsubscript{2}O emitters isolated from *S. fuscum* are likely to show an acidophilic property with high adaptation for weakly acidic environments of *Sphagnum* bogs.

1.7 PCR assay for detection of *narG* and *nosZ* genes

The PCR assay showed that all of the *Burkholderia* N\textsubscript{2}O emitters possessed the *narG* gene (Fig. 1.8A), but *nosZ* amplicons were undetected with the specific primer set (\textit{nosZ}-661\textit{F} and \textit{nosZ}-1111\textit{F}, and \textit{nosZ}-1527\textit{R} and \textit{nosZ}-1773\textit{R}) (Fig. 1.8B), under alternative annealing temperatures (48–55 °C), using a thermal gradient PCR. Thus, it is most likely that the three N\textsubscript{2}O-emitting *Burkholderia* spp. are denitrifiers that are missing the *nosZ* gene, suggesting that the final reduction process of N\textsubscript{2}O into N\textsubscript{2} does not function in these *Sphagnum* moss-epiphytic/endophytic denitrifiers.
Fig. 1.11 PCR assay for the detection of narG and nosZ gene from Burkholderia N₂O emitters

(A) Agarose gel profile for the detection of narG gene by the PCR assays. Amino acid sequences of NAR large subunit protein translated from the nucleotide sequences amplified from narG are also shown here. PCR assay for detection of narG gene was carried out by using the special primer. Translated protein sequences were listed by http://www.ebi.ac.uk/Tools/st/emboss_transeq/. The red square region is conserved region of NarG protein sequence. As a molecular size marker, 2-kbp DNA ladder was used. (B) PCR assay was performed to detect nosZ gene using two pairs of nosZ primers (1111F and 1773R; 661F and 1527R). A nosZ-possessing Pseudomonas sp. 05CF15-5C (accession no. LC007966) was used for the positive control. As a molecular size marker, 2-kbp DNA ladder was used. The PCR assays for nosZ gene were negative in all of three Burkholderia N₂O emitters.
Discussion

*Sphagnum* mosses play dominant roles as primary producers and carbon depositors in peatland ecosystems, and their tissues decompose very slowly (Dorrepaal et al., 2005). In this study, 16S rRNA gene-targeted PCR-DGGE (Fig. 1.4A) profiles showed that *Mucilaginibacter* sp. and *Burkholderia* sp. are the major bacterial constituents in the tissues of *S. fuscum*. Indeed, *Burkholderia* spp., widely distributed throughout acidic soils in the tropics, subtropics, arctic, and subarctic zones, are the predominant bacteria in ombrotrophic peatland ecosystems (Belova et al., 2006). Both *Mucilaginibacter* and *Burkholderia*, endophytic and epiphytic bacteria in *Sphagnum magellanicum* and *Sphagnum fallax*, have been reported as the major genera comprising microbial communities in peat bogs from surveys based on direct isolation and 16S rRNA gene-targeted pyrosequencing (Opelt et al., 2007b).

This study revealed that *Burkholderia* spp. are the predominant bacterial group on *Sphagnum* moss that contribute to N\textsubscript{2}O emissions, and that many environmental factors, such as nitrogen source (Fig. 1.9A), pH (Fig. 1.8A) and temperature (Fig. 1.8B), determine their N\textsubscript{2}O-emitting capacity. As shown in cell growth test for three *Burkholderia* N\textsubscript{2}O emitters (Fig. 1.10), it is most likely that all the N\textsubscript{2}O emitters are mesophile and weakly acidophile. Their high cell growth under the optimum conditions partly result in much active N\textsubscript{2}O emission in the culture as shown in Fig. 1.8.

It was found that 0.5 mM KNO\textsubscript{3} is the substrate most effective in the conversion to N\textsubscript{2}O by the three isolates of N\textsubscript{2}O-emitting *Burkholderia*, while 0.5 mM NH\textsubscript{4}Cl
induced almost no \( \text{N}_2\text{O} \) emission (Fig. 1.7A). This substrate specificity and the intermediate level of \( \text{N}_2\text{O} \) production upon addition of \( \text{NH}_4\text{NO}_3 \) suggested that all three isolates of \( \text{N}_2\text{O} \)-emitting *Burkholderia* identified in this study are denitrifiers (Fig. 1.5). These results suggested that \( \text{N}_2\text{O} \) emission in the culture-based \( \text{N}_2\text{O} \) emission assay is mainly due to the process of denitrification by the \( \text{N}_2\text{O} \) emitters. The constant \( \text{N}_2\text{O} \) emission by the \( \text{N}_2\text{O} \) emitters at higher concentrations (1 to 5 mM) of \( \text{KNO}_3 \), beyond the optimum concentration (1 mM), strongly suggests that the accumulation of \( \text{NO}_3^- \) in aerobic bogs leads to stable \( \text{N}_2\text{O} \) emissions. The result of this experiment was inconsistent with results reported by Nykänen et al. (2002), in which a short time increase of \( \text{N}_2\text{O} \) fluxes in a boreal *Sphagnum*-dominant peatland was observed after addition of \( \text{NH}_4\text{NO}_3 \)-N followed by a 13-day-incubation. The diversity of the microbial community in *Sphagnum* moss species is probably one of the reasons for the different potentials of boreal *Sphagnum*-peatland systems with global or local environmental variations, such as changes in rainfall due to global warming or an increased N deposition by human impacts.

The weakly acidic environment (pH 5.0) of a *Sphagnum*-predominant bog system agrees with the experimentally-determined optimal pH for active \( \text{N}_2\text{O} \) emission as demonstrated in the three \( \text{N}_2\text{O} \) emitters of *Burkholderia* (Fig. 1.8A). Environmental pH in the peat soil and water, often lower than pH 4.0, is one of the key factors shaping the specified microbial community (Hashidoko et al., 2008; Palmer et al., 2010; Palmer and Horn, 2012; Takeda et al., 2012). Although the pH of the stagnant water in ecologically developing *Sphagnum* peat bogs under acidic
environments range from 3.5 to 4.5 (Gorham and Janssens, 1992; Opelt et al., 2007a), the bare surface-created boreal peat bogs are highly disturbed by cryoturbation to be hot spots for active N\textsubscript{2}O emissions may provide the ideal conditions (pH 4.0 or more higher) for N\textsubscript{2}O emission in the subarctic ecosystem covered with sphagnum moss bogs (Repo et al., 2009).

Relatively high temperatures (up to 30 °C) unexpectedly resulted in an increase in N\textsubscript{2}O emissions by Burkholderia sp. SF-E2, suggesting the presence of high temperature-responsive denitrifiers in boreal peat ecosystems that may lead to an acceleration of N\textsubscript{2}O emissions in subarctic zone in response to global warming (Fig. 1.8B). This trend is similar to a previous study reported by Hernandez and Mitsch (2007). The N\textsubscript{2}O emitter, characterized as a non-cryophilic bacterium, might experience accelerated emissions with incremental increases in the annual air temperature in the subarctic tundra, as suggested by the finding that their enzyme activities essential for N\textsubscript{2}O emission are often stimulated by heat stress. Conversely, Burkholderia sp. SF-A1 and Burkholderia phenazinium SF-C1, in this study, did not show any linear relationship between N\textsubscript{2}O emissions and temperatures from 4 to 30 °C (Fig. 1.5B). The optimal temperatures for emissions in Burkholderia sp. SF-A1 and B. phenazinium SF-C1 were observed in the range of 15–25 °C, close to the average air temperature in the sampling site (15 to 17 °C) in summer.

It is known that supplemental sucrose can accelerate N\textsubscript{2}O emissions from Janthinobacterium sp., isolated from a tropical peatland in Central Kalimantan, Indonesia (Hashidoko et al., 2010b), and Pseudomonas denitrifiers from Andisol corn
farms in Hokkaido, Japan, (Li et al., 2014) to produce 1,000- and 9,000-fold higher levels of N\textsubscript{2}O respectively. However, the effects of 5 g L\textsuperscript{-1} sucrose supplementation on N\textsubscript{2}O emission by Burkholderia N\textsubscript{2}O emitters, SF-A1, SF-C1, and SF-E2, were statistically significant, but probably ecologically insignificant: only 3–fold higher levels in these N\textsubscript{2}O-emitting bacteria of Burkholderia (Fig. 1.9A). Their low responses to the supplemented sucrose suggest that three isolates of Burkholderia are oligotrophs rather than saprophytic heterotrophs, highly adaptable to conditions with a poor availability of nutrients, including carbon sources.

Regarding the lack of significant difference between exposure and non-exposure of the N\textsubscript{2}O emitters to 10% C\textsubscript{2}H\textsubscript{2} (Fig. 1.9B), the enzyme (N\textsubscript{2}O reductase) catalyzing the reduction of N\textsubscript{2}O into N\textsubscript{2} gas in the final step of the denitrification process seems to be mal-functional or missing. In the PCR assay, two key genes of denitrification, respiratory nitrate reductase large subunit (\textit{narG}) gene and N\textsubscript{2}O reductase catalytic subunit (\textit{nosZ}) gene, were probed. All three N\textsubscript{2}O emitters of Burkholderia were positive for the \textit{narG} but negative for the \textit{nosZ} gene (Fig. 1.11). The results of this assay suggest that these Burkholderia N\textsubscript{2}O emitters are incomplete denitrifiers that are most likely missing the \textit{nosZ} gene. The atypical \textit{nosZ} gene (Sanford et al., 2012) was detected in these N\textsubscript{2}O emitters by PCR assay. To confirm the absence of the \textit{nosZ} gene more directly, pyrosequencing analysis for genomic DNA should be conducted.
Conclusion

In conclusion, *Burkholderia* denitrifiers, especially isolate SF-E2 which was adapted to the acidic, nutrient-poor boreal peat environment and showing an optimal N\textsubscript{2}O emission potential at relatively higher temperature and lower pH, would be a key player in N\textsubscript{2}O emission from disrupted boreal peat ecosystems. Some reports of hyper-active N\textsubscript{2}O emission from degraded *Sphagnum* bogs due to climate change and human impact support the speculation that such denitrifiers play a dominant role in N\textsubscript{2}O emission from the boreal hotspot regions. Further studies on molecular evidence for active N\textsubscript{2}O gas emissions would be linked to development of new technology to repress N\textsubscript{2}O production.
CHAPTER 2. Comparison of N₂O-emitting bacteria in epiphytic and/or endophytic bacterial community between Sphagnum fuscum and Sphagnum capillifolium

Materials and Methods

2.1 Sphagnum moss sampling in 2014 in Finland

*Sphagnum fuscum* and *Sphagnum capillifolium* were collected from the palsa peat bogs that are showing drastic subsidence at a site near Kilpisjärvi in Finland (69°03’N 20°32’E) in August, 2014. The *Sphagnum* samples were stored in zippered plastic bags at 4 °C for further study.

2.2 Screening of N₂O emitters in two Sphagnum mosses and comparisons of their N₂O emission potentials

In order to compare potential of microbial communities, screening of microorganisms from these two *Sphagnum* mosses was performed. As preliminary isolation of the *Sphagnum* moss-associated microorganisms, *Sphagnum* moss leaf was suspended in 10 mL sterile water and vortexed 30 sec. From the suspension solution, 100 µL was added to the N₂O assay medium (10 mL MWG medium with 22.6 mL headspace in each vial) (Hashidoko et al., 2008) without carbon source or 0.1 g moss leaves were directly added to the assay medium. After incubation at 15 °C for 7 days, emitted N₂O assay was measured by using an ECD-gas-chromatograph (Shimadzu GC-14B, 125 Kyoto, Japan) connected to a Porapak N column (1-m long,
Waters, Milford, MS, USA). To observe the microbial communities between these two Sphagnum mosses, PCR-DGGE was performed. Firstly, DNA was extracted from the culture medium after the N₂O assay by using Isoplat II DNA Extraction kit (Nippon Gene, Toyama, Japan), and then PCR products for DGGE were obtained by using the common pair primer of GC-341F (CGC CCG CCG CGC CCC GCG GGG GTC CCG CCG CCC CCG CCC GCC T AC GGG AGG CAG CAG) and 907R (CCG TCA ATT CCT TTR AG T TT) (Ferris et al., 1996).

In order to search N₂O emitters from Sphagnum mosses, 100 µL of the culture medium (after incubation for 7 days) was 10⁴-10⁶ times diluted with sterile water and the resulting solution each 100 µL was used for inoculation to MWG plates. After incubation of 5 days at 20 °C, apparent single colonies were selected to streak cultivation on MWG plates with many repeats until purified. Each pure strain (unique shape size, color, or the outlooks) thus obtained was inoculated to the medium for culture-based N₂O emission assay.

2.3 Characteristics of active N₂O emitters

To examine the optimal nitrogen substrates for N₂O emission, available substrates for N₂O emitters were offered by 1 mM NH₄NO₃, KNO₃, and NH₄Cl. To study the optimal pH for N₂O emitters, pH was adjusted at 4.6, 5.0, 5.7, 6.8, and 7.3 at 15 °C. To find the appropriate temperature, the gradient temperatures were set from 4, 10, 15, 20, 25, and 30 ºC at pH 5.0.
2.4 Supplemental sucrose and (E)-caffeic acid

To observe their response to supplemental sucrose as a carbon source, 0.05%, and 0.5% sucrose were used for comparison with control (without addition of sucrose). To investigate effect of polyphenol on N₂O emission, (E)-caffeic acid at 0, 0.005, 0.01, 0.05, 0.1, 0.5, 1.0 g L⁻¹ were added, and measured its effect on acceleration of N₂O emission. Each treatment was in triplicate, and incubation condition was at 15 °C for 5 days with inoculants for N₂O assay.

2.5 Acetylene blocking assay

Acetylene blocking assay (also called acetylene inhibition assay) is widely used to measure the denitrification rates (Sørensen, 1978). To measure enzyme activity of N₂O reductase upon exposure to acetylene (C₂H₂) at pH 5.0 and 7.0, 10% C₂H₂ gas was injected into the headspace of the assay vial inoculated with N₂O emitters. At the same time, the treatments without injection of C₂H₂ gas were set as control to compare the activity of N₂O reductase.

2.6 Detection of functional genes

Using PCR assay, functional genes of narG, nirS, and nosZ were detected. The target gene was amplified using the pairs of primers, narGF (TCG GGC AAG GGC CAT GAG TAC) and narGR (TTT CGT ACC AGG TGG CGG TCG), nirSCd3Af (AAC GYS AAG GARY ACS GG) (Nie et al., 2015) and nirSR3cd (GAS TTC GGR TGS GTC T) (Throbäck et al., 2004), nirK-1F (GGM ATG GTK CCS TGG CA) and
nirK-5R (GCC TCG ATC AGR TTR TGG) (Braker et al., 1998), nosZ-1111F (STA CAA CWC GGA RAA SG), nosZ-661F (CGG CTG GGG GCT GAC CAA), nosZ-1527R (CTG RCT GTC GAD GAA CAG), and nosZ-1773R (ATR TCG ATC ARC TGB TCG TT) (Scala and Kerkhof, 1998). The reaction conditions of PCR amplifications in details were presented in Table 2.1.

Table 2.1 PCR primers and amplification conditions for narG, nirS and nosZ genes

<table>
<thead>
<tr>
<th>gene</th>
<th>Primer set</th>
<th>Sequence (5’–3’)</th>
<th>Thermal profile</th>
<th>Reference</th>
</tr>
</thead>
</table>
| narG | 2168F, 2391R | 5’-TCGGGCAAGGGCCACGAATAC-3’
5’-TTCTCGTACCACGTCGCG GTC-3’ | 95 °C 10 min; 30 cycles of 95 °C 1 min, 52 °C 1 min, 72 °C 1 min; 72 °C 10 min | This study |
| nirS | cd3AF, R3cd | 5’-GTSAACGTAACGARACSGG-3’
5’-GASTTCGRTGSGTCTTGA-3’ | 95 °C 10 min; 30 cycles of 95 °C 1 min, 52 °C 1 min, 72 °C 1 min; 72 °C 10 min | Throβäck et al., 2004 |
| nosZ | 661F, 1527R | 5’-CGGCTGGGGGCT GACCAA-3’
5’-CTGRCTGTCGADGAACAG-3’ | 95 °C 10 min; 30 cycles of 95 °C 1 min, 55 °C 1 min, 72 °C 1 min; 72 °C 10 min | Scala and Kerkhof, 1998 |

Results

2.1 N₂O emission potential of microbial communities in two Sphagnum mosses

After incubation for 7 days, N₂O emission from microbial communities in Sphagnum capillifolium is 4.8- and 13.7-fold higher than those of Sphagnum fuscum respectively (Fig. 2.1A and 2.1B). This suggested that N₂O emission from the bacterial communities of S. capillifolium was significantly higher than those of S. fuscum.
**Fig. 2.1 Comparison of N$_2$O emission from microbial communities in two Sphagnum mosses**

100 µL leave washing and 100 mg moss leaves as the inoculates were added to the N$_2$O assay medium respectively. The medium with 0.05% sucrose was incubated at 15 °C for 7 days. The pH was adjusted to 5.0 (n=3) * P < 0.05

16S rRNA gene-targeted PCR-DGGE profile of the microbial cultures from two Sphagnum mosses showed that the major bacterial communities culturable in these Sphagnum mosses are quite similar each other. However, S. capillifolium harbored a bacterium of family Enterobacteriaceae, while S. fuscum contained the genus Dyella of class Gammaproteobacteria (Fig. 2.2).
Fig 2.2 Profile of 16S rRNA gene-targeted PCR-DGGE profile of bacterial community from the leaves of *Sphagnum fuscum* and *Sphagnum capillifolium*

PCR-DGGE profiles for bacterial communities in phyllosphere of *S. fuscum* and *S. capillifolium* were examined. DNA was extracted from the culture-based medium (Fig. 2.1) after incubation for 7 days.

### 2.2 N₂O emitters

Two *Burkholderia* spp. were isolated from *S. fuscum*, while three gammaproteobacteria, *Pseudomonas*, *Serratia*, and an unidentified *Enterobacteriaceae* bacterium were isolated from *S. capillifolium* together with a *Burkholderia* sp. (Table 2.2). Among them, *Serratia* sp. SC-K1, an *Enterobacteriaceae* bacterium SC-L1, and *Pseudomonas* sp. SC-H2 showed the most active N₂O emission. Among them, N₂O emission of *Pseudomonas* sp. SC-H2 was
most active, and *Enterobacteriaceae* bacterium SC-L1 was second active (Fig. 2.3).

![N2O emission graph](image)

**Fig. 2.3 Comparison of the N2O-emitting ability between these N2O emitters**

All the inoculants were incubated at 15 °C for 5 days.

<table>
<thead>
<tr>
<th>Sphagnum species</th>
<th>Isolate</th>
<th>Most aligned DNA (accession no.)</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. fuscum</em></td>
<td>SF-B1</td>
<td><em>Burkholderia</em> sp. MS14 (CP009744)</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td>SF-D2</td>
<td><em>Burkholderia cepacia</em> SE-1 (KF681774)</td>
<td>99%</td>
</tr>
<tr>
<td><em>S. capillifolium</em></td>
<td>SC-K1</td>
<td><em>Serratia</em> sp. HUMV-21 (CP011303)</td>
<td>97%</td>
</tr>
<tr>
<td></td>
<td>SC-L1</td>
<td>an <em>Enterobacteriaceae</em> bacterium YG5-6 (KC560019)</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td>SC-H2</td>
<td><em>Pseudomonas</em> sp. 05CF15-5C (LC007966)</td>
<td>99%</td>
</tr>
</tbody>
</table>

**Note:** A1, C1, and E2 were isolated and identified as the same with Chapter 1.

### 2.3 Substrate, temperature and pH

According to N2O emission assay upon exposure to alternative nitrogen source, KNO₃ is the most efficient substrate for N₂O production, followed by NH₄NO₃, while
almost no N₂O emission was observed with NH₄Cl for the substrate. The active N₂O emission from NO₃⁻ probably indicates that all of the three N₂O emitters isolated from *S. capillifolium* are nitrate reducers (Fig. 2.4).

**Fig. 2.4 Response of N₂O-emitting bacterium to different nitrogen source**

Mineral nitrogen (as 1 mM of NH₄Cl, NH₄NO₃ or KNO₃) were added to the N₂O assay medium to test the optimum nitrogen source of N₂O for an *Enterobacteriaceae* bacterium SC-L1, *Serratia* sp. SC-K1, and *Pseudomonas* sp. SC-H2.

**Fig. 2.5 Response of N₂O emitters to incubation temperatures**

An *Enterobacteriaceae* bacterium SC-L1, *Serratia* sp. SC-K1, and *Pseudomonas* sp. SC-H2 were cultured at different temperatures (4, 10, 15, 20, 25, and 30 °C) to examine optimal temperature for N₂O emission. The medium contained 0.05% sucrose and was adjusted to pH 5.0 incubated for 5 days in triplicate.

For the three of the strains used, N₂O emission increased along with the increasing temperature from 4 to 30 °C (Fig. 2.5). This experiment confirmed that the optimal temperature for these three strains is over 30 °C.
Fig. 2.6 Response of N\textsubscript{2}O emitters to different pH
The soft gel medium for the culture-based N\textsubscript{2}O assay was adjusted to alternative pH values (pH 4.6, 5.0, 5.7, 6.8, or 7.3) and incubated at 15 °C with 0.05% sucrose for 5 days, (n=3).

N\textsubscript{2}O emission moderately increased with the increasing pH from 4.6 to 7.3 in the \textit{Enterobacteriaceae} bacterium SC-L1 and \textit{Serratia} sp. SC-K1. Both showed a temporary increase at pH 5.0 but no drastic increase in N\textsubscript{2}O emission through the pH range tested. This indicated that these N\textsubscript{2}O emitters are adapted to acidic environments allowing nitrate respiration at weakly acidic region. Conversely, \textit{Pseudomonas} sp. SC-H2 showed the most drastic response at pH 6.0 and 7.0 for N\textsubscript{2}O emission. The N\textsubscript{2}O emitter produced N\textsubscript{2}O with a drastic increase at pH 6.8 and 7.3, although at pH 5.7 or much more acidic region, level of N\textsubscript{2}O production was almost zero (Fig. 2.6).

2.4 Supplemental (E)-caffeic acid and sucrose

Without carbon source, \textit{Serratia} sp. SC-K1 emitted higher level of N\textsubscript{2}O than \textit{Enterobacteriaceae} bacterium SC-L1 and \textit{Pseudomonas} sp. SC-H2. These two isolated showed much more active N\textsubscript{2}O emission when added 0.05% sucrose. Particularly, response of \textit{Pseudomonas} sp. SC-H2 toward 0.05% sucrose is drastic, and approximately 2 x 10\textsuperscript{3} times higher than that without sucrose (Fig. 2.7). Higher N\textsubscript{2}O emission capability under a condition poor in carbon source implied that \textit{Serratia} sp. SC-K1 is an oligotrophic bacterium able to utilize gellan gum as the most acceptable carbon source. In contrast, \textit{Enterobacteriaceae} bacterium SC-L1 and
*Pseudomonas* sp. SC-H2 are rather saprophytic bacterium.

Fig. 2.7 Response of N$_2$O emitters to supplemental sucrose

Sucrose (0, 0.05%, 0.5%) was added to the medium to examine for culture-based N$_2$O emission assay using *Enterobacteriaceae* bacterium SC-L1, *Serratia* sp. SC-K1, and *Pseudomonas* sp. SC-H2. Incubation was done at 15 °C for 5 days. The culture medium was adjusted to pH 7.0 before inoculation. bar, standard deviation, (n=3). Note the scale of y-axis is 10-timers high in *Pseudomonas* sp. SC-H2.

2.5 Acetylene blocking assay

At pH 5.0, any statistical significance was not observed in between two groups with 10% C$_2$H$_2$ and without C$_2$H$_2$ as control at pH 5.0, but *Pseudomonas* sp. SC-H2 cultured at pH 7.0 showed statistically significant increase of N$_2$O production. Exposure to C$_2$H$_2$ led to drastic increase of the N$_2$O emission as 4-fold of the control. Without 10% acetylene, the level of produced N$_2$O at pH 7.0 was extensively higher than that at pH 5.0 (Fig. 2.8). Peat ecosystem highly disturbed often increases pH values of the soil and logged water. Despite the denitrification process for N$_2$O mission was greatly accelerated, N$_2$O reductase for the final step of denitrification to
reduce N₂O to N₂ was also excessively functioning.

Fig. 2.8 Response of N₂O emitters to acetylene gas at pH 5.0 and 7.0

Culture medium is MWG supplemented with 0.05% sucrose incubation for 5 days at 15 °C. bar, SD (n=3).

2.6 Denitrification-associated genes

By using PCR assay, narG gene was detected in all of the N₂O emitters, but only *Pseudomonas* sp. SC-H2 contained nirS and nosZ genes (Fig. 2.9). Together with the results of C₂H₂ blocking assay, it is speculated that *Pseudomonas* sp. SC-H2 is a complete denitrifier. For *Enterobacteriaceae* bacterium SC-L1 and *Serratia* sp. SC-K1, nirK gene was not detected (data not shown).
Fig. 2.9 PCR assay for the detection of \textit{narG}, \textit{nirS} and \textit{nosZ} genes from the N\textsubscript{2}O emitters

Agarose gel electrophoretic profile of \textit{narG}, \textit{nirS} and \textit{nosZ} gene amplicons for the PCR assays. As a molecular size marker, 2-kbp DNA ladder (Takara) was used.

Discussion

Increased atmospheric N deposition can reduce the growth of some \textit{Sphagnum} moss species, such as \textit{Sphagnum magellanicum} (Aerts et al., 2001; Limpens and Berendse, 2003), while, increased production of \textit{S. fuscum} increased under the elevated N deposition was reported by Vitt et al. (2003). \textit{S. capillifolium} is also can tolerant to high N supply (Bonnett et al., 2010). However, current study offered the evidence that both of them have a strong N\textsubscript{2}O emission potential due to \textit{Sphagnum} moss-associating bacterial communities, especially those of \textit{S. capillifolium}. Bragazza et al. (2006) presented that carbon source decreased with the increasing atmospheric N deposition in peat bogs, leading to reduction of a C/N ratio and
subsequent increment of denitrification potential in the peat bogs. Resulting C/N ratio less than 20 triggered stimulation of N$_2$O emission (Huang et al., 2004; Klemedtsson et al., 2005; Maljanen et al., 2012).

From the analysis of bacterial communities, N$_2$O emission potential of *S. capillifolium* is greater than that of *S. fuscum* (Fig. 2.1). The major microbial communities in these two *Sphagnum* mosses are agree able well each other, and also showed some similarities with those of pristine boreal and tropical forest peats, composed of *Burkholderia*, *Mucilaginibacter*, *Rhodanobacter*, and *Janthinobacterium* (Hashidoko et al., 2008; Sun et al., 2014). *Burkholderia* spp. of class *Betaproteobacteria* are often observed as N$_2$O emitters (Hashidoko et al., 2010b). When environments changed, some new bacterial communities can arise and emerge in *Sphagnum* mosses leaves, such as saprophytic bacteria of class *Gammaproteobacteria*. In some early studies, denitrifying *Pseudomonas* species (*P. denitrificans*, *P. perfectomarinus*, *P. fluorescens*, *P. stutzeri*, *P. aeruginosa*, *P. nautica*) capable of nitrate respiration were found in several reports (Delwiche, 1959; Payne et al., 1971; Balderston et al., 1976; Sørensen et al., 1980; Dooley et al., 1987; Viebrock and Zumft, 1988; SooHoo and Hollocher, 1991; Prudêncio et al., 2000). Anderson and Levine (1986) referred to evidences that a *Serratia* sp. produced N$_2$O.

The three N$_2$O emitters showed a higher N$_2$O production along with the increment temperature, particularly at 30 °C (Fig. 2.5), suggesting that N$_2$O emission from boreal peatlands increased with the global warming (Pfenning and McMahon, 1997). For all of the three active N$_2$O emitters isolated from *S. capillifolium*, N$_2$O
production is relatively high at pH 7.3 (Fig. 2.6). A similar trend has been reported in some previous studies, in which the maximal denitrification rates by pure denitrifiers were found at a neutral pH (Thomas et al., 1994). At pH 7.0, N₂O reduction to N₂ by *Pseudomonas* sp. SC-H2 maintained after 5-days-incubation (Fig. 2.8). This high-response of SC-H2 to relatively high pH is explainable by a report of Palmer and Horn (2015) that N₂O emission hotspots is inclined to neutral peatlands. C₂H₂ blocking assay at pH 5.0 and 7.0, for these three active N₂O emitters, N₂O reduction to N₂ is almost negligible at pH 5.0. This also consistent with a previous study, in which it was reported no function of N₂O reductase at pH region less than 6.1 (Liu et al., 2014).

It was also suggested that the process of N₂O reduction to N₂ was inhibited in boreal *Sphagnum*-peat ecosystem under the acidic environment. As pH of pristine *Sphagnum*-dominant bogs is medium-strongly acidic, N₂O reductase of denitrifiers cannot work under such acidic conditions, and it is suggested that the process of N₂O reduction is not the primary factor to decrease the N₂O gas emission in the pristine *Sphagnum* bogs system. The palsa bogs harboring the ice core at deep layer in the subarctic regions will become neutral catchment region due to surrounding mineral nutrients inputs when it once collapsed and rewetted (Seppälä, 2011). An unexpected big threat of the N₂O emission would be observed in such neutral spots.

For the *Enterobacteriaceae* bacterium SC-L1 and *Serratia* sp. SC-K1, neither of *nirS* and *nirK* genes were detected, and it was suggested both are non-denitrifiers. *Enterobacter* is often reported as N₂O emitter of dissimilatory nitrate reduction to
ammonium (DNRA) type (Smith and Zimmerman, 1981). DNRA might be an player for N₂O emission in S. capillifolium bogs.

**Conclusion**

Two *Sphagnum* mosses, *S. fuscum* and *S. capillifolium*, harbor similar bacterial communities, but the denitrifiers are different. Furthermore, N₂O emission from the bacterial communities of *S. capillifolium* was higher than those of *S. fuscum* collected at the same site. In addition, N₂O-emitting isolates from *S. capillifolium* had higher activity to produce N₂O than those from *S. fuscum*. One of hyper-active N₂O emitters from *S. capillifolium* was a nosZ gene-harboring *Pseudomonas* sp. SC-H2, and N₂O reduction to N₂ catalyzed by N₂O reductase added by nosZ was observed at pH 7.0. However, its level of N₂O production was outstandingly high.
CHAPTER 3. Influence of different concentration of polyphenols on \( \text{N}_2\text{O} \) emission by \( \text{N}_2\text{O} \) emitters.

Materials and methods

3.1 Response of the \( \text{N}_2\text{O} \) emitters to \textit{Sphagnum} powders, and plant polyphenols

To know whether microenvironment on the maintaining or decomposing \textit{Sphagnum} bog affect \( \text{N}_2\text{O} \) production by \textit{Burkholderia} \( \text{N}_2\text{O} \) emitters, effects of plant materials of \textit{S. fuscum} on \( \text{N}_2\text{O} \) emission by \( \text{N}_2\text{O} \)-emitting bacteria was also examined.

![Fig. 3.1 Process for preparation of moss juice and residues](image)
A 10-g portion of air-dried *Sphagnum fuscum* obtained at a mire near Joensuu (66°19'N; 29°29'E) in Finland in August, 2009 was crushed in 50 mL of Milli-Q water, using a pestle and mortar to obtain a moss paste juice. The resulting moss paste juice was centrifuged (14,000 rpm, for 15 min), filtered with a filter paper followed by a 0.45 µm membrane, while the insoluble powdery residues were autoclaved (at 121 °C, for 20 min) for the supplemental matters. Concentration of the water-soluble and the insoluble *S. fuscum* tissue powders were adjusted to amounts equivalent to 0.2, 0.5, 1, 2, 5, 10, and 20 g and 0.1, 0.3, 0.5, and 1.0 g dry weight of *S. fuscum* tissues per liter, respectively (Fig. 3.1). The media inoculated with *Burkholderia* sp. SF-E2 in five replicates were incubated under the culture conditions as described above.

### 3.2 Extraction of polyphenols form *Sphagnum fuscum*

A potion of 192 g *S. fuscum* was soaked in methanol at 4 °C for 10 days. Total 546 mg dry materials were concentrated from the recovered methanol using evaporator. The resulting dry materials were re-dissolved in 30 mL EtOAc and 30 mL 5% NaHCO₃ solution for liquid-liquid partition. After extraction with EtOAc, resulting water layer was acidified with 2 M HCl to pH 3.0 or less, and the aqueous layer was further extracted with 30 mL EtOAc. The resulting acidic fraction of ethyl acetate layer was also dried over anhydrous Na₂SO₄ and concentrated (Scheme 4).
Using CHCl₃:MeOH:H₂O:HCOOH=65:25:4:0.5 as a developing solvent, silica gel TLC was carried out for separation of the target compounds from the acidic fraction. Eventually, 12.6 mg polyphenols were obtained by the preparation TLC (Fig. 3.2). For the N₂O emission assay, this polyphenol was finally adjusted to the concentration of 0.005, 0.05, 0.125, 0.25, 1.25, and 6.25 µg mL⁻¹. In addition, FD-MS was used to identify molecular size of the polyphenols.
3.3 Influence of gallic acid and (E)-caffeic acid on N₂O emitters

The effects of supplementation with gallic acid and (E)-caffeic acid at different concentration on N₂O productivities of the three Burkholderia isolates were examined, using the culture-based N₂O emission assay system. Gallic acid concentration was set at 0 (control), 1, 5, 10, 50, and 100 mg L⁻¹, while supplementation with (E)-caffeic acid was at 0, 0.5, 1, 5, 10, 50, 100, 500, and 1,000 mg L⁻¹ because of high responses at higher range of (E)-caffeic concentration. The polyphenol-supplementation assays were performed in triplicate. Incubation conditions were the same as those described above.
Results

3.1 Increased N₂O emission along with increased concentration of Sphagnum residues

Supplementation with water-insolubles of S. fuscum tissue resulted in a moderate increase of N₂O production (Fig. 3.3), but water-soluble part was not effective even at concentration of equivalent to 10 g d.w. of moss tissues per liter.

![Graph showing N₂O emission](image)

**Fig. 3.3 Effects of tissue powders of Sphagnum fuscum on N₂O emission by Burkholderia sp. SF-E2**

*S. fuscum* (10 g dry weight) was ground with a mortar and pestle in 5 mL Milli-Q water, and the insoluble residues filtered with a filter paper was added to the assay medium at different amount (0.1, 0.3, 0.5, 1.0 g d.w. of *S. fuscum* tissues L⁻¹) were separately added to the N₂O assay medium to examine the effect of tissue materials of *S. fuscum* on N₂O emission by *Burkholderia* sp. SF-A1 and SF-E2 and *B. phenazinium* SF-C1. Incubation was at 15 °C for 7 days.

3.2 N₂O emission upon exposure to Sphagnum moss polyphenols extracts

According to the analysis of FD-MS, the m/z value of the major substances
suggested that the major derivatives are low molecular compounds of polyphenolic derivatives (Fig. 3.4). To identify the structure of these compounds accelerated N₂O emission would be meaningful and important in the further study.

![FD-MS spectrum of crude polyphenol extracts from *Sphagnum fuscum*](image)

**Fig 3.4** FD-MS spectrum of crude polyphenol extracts from *Sphagnum fuscum*

According to FD-MS spectrum of the crude polyphenols mixture from *S. fuscum*, the polyphenols showing m/z 386.3 and 391.2 would be major substances with plant polyphenols.

Polyphenol extracts from *S. fuscum* significantly promoted N₂O emission by *Burkholderia* sp. SF-E2 even at a lower concentration of 0.005 µg mL⁻¹. Meanwhile, N₂O production increased along with the increasing concentration of polyphenol extracts from 0.005 to 6.25 µg mL⁻¹ (Fig. 3.5).
Fig 3.5 Effect of crude polyphenols extracts from S. fuscum on N₂O emission by Burkholderia sp. SF-E2

3.3 Influence of gallic acid and (E)-caffeic acid on N₂O emission by Pseudomonas

Low concentration of (E)-caffeic acid (0.005-0.1 mg mL⁻¹) accelerated N₂O emission by Pseudomonas denitrifiers. According to their responses to (E)-caffeic acid, these Pseudomonas denitrifiers are divided into two types. 1) N₂O production decreased to 39.4, 8.3, and 5.5 µg vial⁻¹ d⁻¹ when the concentration of (E)-caffeic acid is 0.05 g L⁻¹ by Pseudomonas sp. 10CFM 15-6A, 10CFM 15-4D, and 05CF 15-5C respectively, and 2) N₂O emission is lower than control with lower concentration of (E)-caffeic acid (0.005 and 0.01 g L⁻¹) observed in Pseudomonas sp. 10CFM 5-4A and 05CF 15-6B. However, they tolerant to 0.1 g L⁻¹ (E)-caffeic acid and emit higher N₂O than control, with statistic significance (P < 0.05) (Fig 3.6). All are susceptible to 0.5 g L⁻¹ of (E)-caffeic acid, showing almost zero-level of N₂O production. Their bacterial cell growth was also repressed by the supplemental (E)-caffeic acid.
Addition of gallic acid and (E)-caffeic acid in the culture medium led to acceleration of N₂O emission. Gallic acid at concentration of 10 mg L⁻¹ showed the greatest activation of N₂O emission by Burkholderia sp. SF-E2 and B. phenazinium SF-C1 (Fig. 3.7), while supplementation with (E)-caffeic acid led to the highest activation of N₂O emission by Burkholderia sp. SF-E2 at concentration of 100 mg L⁻¹ (Fig. 3.8). Gallic acid led to 4-fold higher N₂O emissions than the control, while 67-fold increase of N₂O emission was observed in the culture medium supplemented with 100 mg L⁻¹ (E)-caffeic acid.
Fig 3.6 Effect of (E)-caffeic acid on N$_2$O emission by *Pseudomonas* denitrifiers from Andisol

Response of *Pseudomonas denitrifiers* upon exposure to different concentration of (E)-caffeic acid was observed. All the culture medium without sucrose was adjusted at pH 5.0 before the incubation. All are in triplicate. *$P < 0.05$, **$P < 0.01$.}
Fig. 3.7 Effects of plant polyphenols also detectable from *Sphagnum fuscum* on N$_2$O emission by *Burkholderia*

Gallic acid and (E)-caffeic acid, both of which are contained in *Sphagnum* mosses as plant polyphenols, were exposed to three isolates and examined for N$_2$O emission.

Fig. 3.8 Effect of high concentrated (E)-caffeic acid on N$_2$O emission of *Burkholderia* sp. SF-E2

Response of *Burkholderia* sp. SF-E2 upon exposure to higher concentration of (E)-caffeic acid was further examined. All the culture medium without sucrose was adjusted at pH 5.0 before the incubation. All are in triplicate.
For *Enterobacteriaceae* bacterium SC-L1 and *Serratia* sp. SC-K1, relatively lower concentration of (E)-caffeic acid (≤ 0.1 g L⁻¹), obviously accelerated N₂O emission, and their optimum concentration is 0.1 g L⁻¹. Among them, *Serratia* sp. SC-K1 showed a sensitive response to 0.1 g L⁻¹ (E)-caffeic acid with 13-fold higher N₂O production. Conversely, N₂O emission of *Pseudomonas* sp. SC-H2 decreased significantly (*P* < 0.01) at 0.01 g L⁻¹ (E)-caffeic acid or more (Fig. 3.9).

Fig. 3.9 Effects of caffeic acid on N₂O emission by N₂O emitters isolated from leaves of *Sphagnum capillifolium*

(E)-caffeic acid was exposed to three isolates (*Enterobacteriaceae* bacterium SC-L1, *Serratia* sp. SC-K1, and *Pseudomonas* sp. SC-H2) and each was examined for N₂O emission. All are in triplicate.
In addition, N\textsubscript{2}O production in the medium for N\textsubscript{2}O emission assay inoculated with \textit{S. capillifolium} under the presence of 0.1 g L\textsuperscript{-1} (\textit{E})-caffeic acid was 5.7, 7.9 and 19.2 fold higher than those of \textit{S. fuscum} during the 4-, 8-, and 15- day incubation times respectively (Fig. 3.8). It indicated that (\textit{E})-caffeic acid can also accelerate the N\textsubscript{2}O emission of microbial communities from these \textit{Sphagnum} species.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3.8.png}
\caption{Effects of (\textit{E})-caffeic acid on N\textsubscript{2}O emission from the culture inoculated with leaf tissues of \textit{S. fuscum} or \textit{S. capillifolium}}
\end{figure}

\textit{S. fuscum} and \textit{S. capillifolium} as inoculants were exposed to 0.1 g L\textsuperscript{-1} (\textit{E})-caffeic acid and examined its effect on N\textsubscript{2}O emission at 4-, 8- and 15- day incubation. All are in triplicate. Statistic significance and \textit{P} value were calculated with student-\textit{t} test. *** \textit{P} < 0.001
Discussion

Betaproteobacteria of genus *Burkholderia* are believed to be capable of utilizing secondary metabolites or pectin-rich cell wall fragments and polyphenols from degrading tissues of *Sphagnum* mosses for their carbon sources or metabolic stimulants (Fig. 3.3). In nutrient-poor bogs, *S. fuscum* is the predominant plant species (Wieder et al., 2010), rich in polyphenols (Turetsky et al., 2008) such as gallic acid, sphagnum acid (Rudolph and Samland, 1985), chlorogenic acid, *(E)-caffeic* acid (Montenegro et al., 2009), catechin, and other flavonoids (Opelt et al., 2007b). In this study, an appropriate concentration of some representative polyphenols accelerated N₂O emission by the N₂O emitters from *S. fuscum*, although the degraded peat soils in relatively dry bogs showed only minimal levels of N₂O emission. The microenvironment in the fresh phyllosphere of *S. fuscum* is probably an important factor for emerging N₂O-emitting denitrifiers.

Polyphenols as the secondary metabolites of *Sphagnum* mosses protect the living and dead cell of the mosses from microbial degradation, however, Bragazza and Freeman (2007) reported that high N inputs reduce the content of polyphenols. Montenegro et al. (2009) pointed out that *(E)-caffeic* acid is an impotant polyphenol of *Sphagnum* mosses, and this study showed that lower concentration of *(E)-caffeic* acid (≤ 0.1 g L⁻¹) can promote N₂O emission by N₂O emitters, especially that of *Serratia* sp. SC-K1 (Fig. 3.9).
Conclusion

Some isolates (Burkholderia and an Enterobacteriaceae bacterium) are adapted to relatively high concentration of (E)-caffeic acid (0.1 g L⁻¹), and they also produce relatively high N₂O. However, Pseudomonas isolates of two distinct types are repressed their N₂O emission upon exposure to different concentration range of (E)-caffeic acid (0.005 to 1 g L⁻¹). All Pseudomonas denitrifiers showed very low N₂O production supplemented with 0.5 g L⁻¹ of (E)-caffeic acid.
CHAPTER 4. Relationship between functional and 16S rRNA gene sequences based on some N₂O-emitting isolates

Materials and Methods

4.1 Primer design

Firstly, to search for protein sequence data of functional enzymes (e.g. Nar) of different species of N₂O emitters available from NCBI database (http://www.ncbi.nlm.nih.gov/), and then alignment of these sequences online by using http://www.genome.jp/tools/clustalw/ to find the conserved region of these sequences. Then, the forward primers and reverse primers were designed according to continuous amino acids (6-8 AA) of the conserved region.

Amino acid sequences of nitrate reductase α-subunit (NarG) that were translated from the DNA sequence of the eubacterial narG gene were randomly collected from phyla Proteobacteria including class Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria, Firmicutes, Actinobacteria, and some other minor ones (more than 20 bacteria). Two conserved regions of the eubacterial NarG, HYVGQEK (located at the 579–585 position in the amino acid sequence, encoding the nitrate reductase α-subunit of Pseudomonas aeruginosa PA7; accession no. ABR84794) (31) and DMHPFIH (located at the 804–810 position for the same protein), were selected from the sequence data for designing the degenerate primer pair (5′-CAY TAY GTS GGS CAR G-3′/5′-TGD ATR AAN GGR TGC A-3′). The PCR conditions were as follows: preheating at 95 °C for 10 min, 35 cycles of
denaturation at 95 °C for 1 min, annealing at 52 °C for 1 min, and extension at 72 °C for 1 min 30 s. In addition, highly conserved regions of narG genes for pseudomonads and others were used to design specific forward (5’-TCG GGC AAG GGC CAT GAG TAC-3’) and reverse (5’-TTT CGT ACC AGG TGG CGG TCG-3’) primers for narG gene amplification.

Table 4.1 PCR primers for narG gene

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4.2 Construction of phylogenetic tree

A region of 1456-bp sequences of their 16S rRNA genes from positions 74 to 1529 for *Escherichia coli* (accession no. J01859.1) were analyzed by the maximum composite likelihood estimation, along with 9 reference species selected from several groups of pseudomonads registered in NCBI DNA database. This neighbor-joining tree method was conducted for identification of the pseudomonads at the species level, using the computing tool for phylogenetic analysis in Molecular Evolutionary Genetics Analysis (MEGA v. 6.06) software (Tamura et al., 2013). Under an estimation of distances between all pairs of the sequences with 1,000 bootstrap replicates, clustering of the complete denitrifiers and incomplete denitrifiers was performed and used for further determination of their emergence in the corn farm Andisol.

Results

4.1 Phylogenetic tree of 16S rRNA genes for *Pseudomonas* denitrifiers isolated from Shizunai Andisol corn farm

The 16S rRNA gene sequences obtained from the incomplete denitrifiers, *Pseudomonas* sp. 10CMF5-1B and 10CMF15-2A, from post-harvest soil were completely agreeable with each other. The 16S rRNA sequence from another incomplete denitrifier, *Pseudomonas* sp. 10CMF5-2D from the same post-harvest soil, was identical to the corresponding gene sequence from two other incomplete denitrifier *Pseudomonas* sp., 10CFM15-4D and 10CFM15-6A obtained from
pre-tilled soil samples. These two groups of pseudomonads formed a cluster (clade A in Fig. 4.1) with two more complete denitrifiers, *Pseudomonas* sp. 10CMF5-4A and 05CF15-6B, which obtained from the different pre-tilled soils were also agreeable with each other. Other complete denitrifiers such as *Pseudomonas* spp. 10CMF5-2B and 05CF15-5C, formed another cluster (clade B in Fig. 4.1) which could be clearly distinguished from the clade A pseudomonads. An incomplete denitrifier, *Pseudomonas* sp. 05CFM15-6D, obtained from pre-tilled soil, was also grouped into clade B.

The phylogenetic relationships among the N₂O-emitting *Pseudomonas* sp. obtained from chemically fertilized corn farmland soil were computed, along with 9 reference species selected from several groups of pseudomonads. This neighbor-joining tree was deduced based on the obtained 16S rRNA gene sequences of the 1456-bp region, using the maximum composite likelihood method for estimation of distances between all pairs of the sequences simultaneously with 1,000 bootstrap replicates by Kimura 2-parameter model. The scale bar represents 0.01 substitutions per nucleotide site. The same region of the sequence from *E. coli* (accession no. J01859.1) was used as the outgroup. Those sequences that behaved similarly to incomplete denitrifiers are marked with an asterisk. Red and blue colors indicate incomplete and complete denitrifiers respectively.
4.2 PCR assay of narG, nirS, and nosZ genes

PCR-amplicons of partial narG gene were obtained using a primer set narG-Ps-2168F (5′-TCG GGC AAG GGC CAT GAG TAC-3′)/narG-Ps-2379R (5′-TTT CGT ACC AGG TGG CGG TCG TCG-3′) (Fig. 4.2).

![Figure 4.2 PCR assay for the detection of narG gene from the N₂O-emitting Pseudomonas bacteria.](image1)

Left panel is an agarose gel plate for all the denitrifiers from the post-harvest soils, and right panel is those from the pre-tilled soils.

Sequences of partial nirS gene were also obtained as PCR-amplicons using a primer set nirSCd3aF (5′-AAC GYS AAG GAR ACS GG-3′)/nirSR3cd (5′-GAS TTC GGR TGS GTC T-3′) (Fig. 4.3).
Fig. 4.3 PCR assay for the detection of nirS gene from the N₂O-emitting Pseudomonas bacteria.

Left panel is an agarose gel plate for all the denitrifiers from the post-harvest soils, and right panel is those from the pre-tilled soils.

PCR-amplicons of partial nosZ gene were also detectable from 4 isolates out of 10 Pseudomonas isolates, using a primer set nosZ-1111F (5’-STA CAA CWC GGA RAA SG-3’)/nosZ-1773R (5’-ATR TCG ATC ARC TGB TCG TT-3’). Another nosZ gene-targeted primer set nosZ-661F (5’-CGG CTG GGG GCT GAC CAA-3’)/nosZ-1527R (5’-CTG RCT GTC GAD GAA CAG-3’) were also tested for 4 pseudomonads from the post-harvest soils and 6 isolates from the pre-tilled soils (Fig. 4.4).
Fig. 4.4 PCR assay for the detection of nosZ gene from the N₂O-emitting *Pseudomonas* isolated.

*Pseudomonas* spp. 10CFM5-2B, 05CF15-5C, 05CF15-6B, and 10CFM5-4A, all positive of this PCR assay among those from post-harvest soils (left panel) and also from pre-tilled soils (right panel), were shown by blue color. Data for translated amino acid sequence are all assignable as nitrous oxide reductase (N₂OR) large subunit.

Sequence data of the PCR-amplicons obtained from 4 pseudomonads using *nosZ*-661F/*nosZ*-1527R are shown in Table 4.2.
Table 4.2 Sequence data of nosZ gene and translated amino acid sequence from 4 pseudomonads

PCR-amplicon sequences of partial nosZ gene using a primer set nosZ-1111F (5’-STA CAA CWC GGA RAA SG-3’)/nosZ-1773R (5’-ATR TCG ATC ARC TGB TCG TT-3’) for the left panel and another set nosZ-661F (5’-CGG CTG GGG GCT GAC CAA-3’)/nosZ-1527R (5’-CTG RCT GTC GAD GAA CAG-3’) for the center and right panels shown above. The sequence data shown below are those obtained for the PCR-amplicons using nosZ-661F/nosZ-1527R.
4.3 Phylogeny tree of narG, nirS and nosZ genes

Unlike the nosZ gene described below, the narG gene was detected in all Pseudomonas sp. capable of emitting N₂O. Phylogenetic analysis using 204-bp sequences of narG genes in the region from positions 2191 to 2394 of P. aeruginosa PA96 narG (accession no. CP007224.1) along with 5 reference species of pseudomonads. Dendrogram thus obtained showed that the phylogenetic relationships among the 10 N₂O-emitting Pseudomonas bacterial isolates are similar to those obtained by the 16S rRNA gene analysis in their clustering patterns (Fig. 4.5).

![Phylogenetic tree of narG, nirS and nosZ genes](image)

**Fig. 4.5** Phylogenetic analyses of functional genes (narG, nirS, and nosZ gene) of the N₂O-emitting Pseudomonas sp. isolated from corn farm Andisol samples

In comparison with dendrogram their accession No. of nirS gene (strain): LC047828 (10CFM5-1B), LC047829 (10CFM5-2D), LC047830 (10CFM15-2A), LC047831 (05CF15-5C), LC047832 (05CF15-6B), LC047833 (05CFM15-6D), LC047834 (10CFM5-4A), LC047835 (10CFM15-4D), and LC047836 (10CFM15-6A).
Among the 10 *Pseudomonas* isolates, 9 strains of them harbored *nirS* gene except for the strain *Pseudomonas* sp. 10CFM5-2B. Phylogenetic analysis of 366-bp of partial *nirS* gene sequence (366-bp) among these 9 *nirS*-type of *Pseudomonas* denitrifiers also showed a congruent pattern of the *nirS* gene phylogeny with their 16S rRNA gene phylogenies.

Conversely, PCR assay for the detection of *nosZ* gene in those N₂O-emitting pseudomonads, amplicons of 700-900 bp were obtained from 10CFM5-2B, CF15-5C, CF15-6B, and CFM5-4A. All the sequenced PCR products were confirmed as *nosZ* fragments by a homology search on the NCBI database. All of the pseudomonads harboring *nosZ* gene also showed positive response to acetylene blocking assay, proving that they are N₂O reductase-positive complete denitrifiers. Their N₂O reductase activities seemed to be relatively low, though phylogenetic tree for partial sequence of *nosZ* is incongruous with the phylogenetic pattern of their 16S rRNA gene sequences.

**Discussion**

In the phylogenetic analysis of genus *Pseudomonas* bacteria using 1455-bp sequences of the 16S rRNA gene in the region positioned 74–1541 for *E. coli* (accession no. J01859.1), almost all of the pseudomonads grouped together as incomplete denitrifiers were involved in clade A (Fig. 4.1). In contrast, three isolates out of 4 complete denitrifiers are in clade B, which consisted of many major and widely known pseudomonads, such as a group of *Pseudomonas fluorescens*, a group
of *P. chlororaphis* group, and a group of *P. syringae* group, according to the 16S rRNA gene-based classification of genus *Pseudomonas* (Anzai et al., 2000). The position of clade C that is composed of *P. putida* group and ungrouped *P. jessenii* and *P. asplenii* showed that *Pseudomonas* spp. in cluster A are clearly distinguishable from known species of *Pseudomonas* bacteria.

The isolates in clade A showed the highest sequence homology to *Pseudomonas* sp. PAMC 26831 without giving species name (accession no. KF011705) that had been isolated from subarctic Alaskan grassland soil (Park and Kim, 2015), and uncultured *Pseudomonas* sp. RF3-C12 (accession no. JN379403) reported as a catechin degrader in the rhizosphere of *Rhododendron formosanum* (Wang et al., 2013); it is unknown whether these are N$_2$O-emitting denitrifiers. In contrast, three isolates in clade B were closely related to the *P. fluorescens* group (Fig. 4.1). Although two isolates of 10CFM5-2B and 05CFM15-6D were unknown *Pseudomonas* species, another isolate (05CF15-5C) was identical to *Pseudomonas veronii*, isolable from natural water (Elomari et al., 1996).

The phylogenetic tree of the N$_2$O-emitting *Pseudomonas* bacteria, constructed using sequence variation of their partial *narG* gene, is consistent with the dendrogram constructed from the 16S rRNA gene sequences of the pseudomonads. The clustering based on their *narG* sequences also resulted in two groups (clade A and B), and the members of clade A were separable into two sub-group, A1 and A2, similarly to the 16S rRNA gene-based dendrogram (Fig. 4.1). Thus, it is reasonable to speculate that isolates in sub-group A1 are a group that could easily have lost the downstream gene (*nos*) in denitrification-associated gene clusters.

The *nirS* phylogeny showed a high level of similarity to phylogenetic tree of 16S rRNA genes as supported by Jones et al. (2008). However, similarity between
phylogenetic tree of nosZ and 16S rRNA gene among nosZ-harboring Pseudomonas was remarkably low.

**Conclusion**

Both of the phylogenetic trees of nirS and narG genes showed a close relationship with 16S rRNA gene phylogeny. In contrast, almost no similarity was observed in between nosZ and 16S rRNA gene phylogenetic trees of these denitrifiers.
TOTAL DISCUSSION AND CONCLUSION

Discussion

In this study, the major culturable N₂O-emitting bacterium (Burkholderia, Enterobacteriaceae bacterium, and Pseudomonas) from boreal peat mosses S. fuscum and S. capillifolium were isolated and investigated. Among these N₂O emitters, Pseudomonas sp. SC-H2 showed the most active N₂O emission in the 0.05% sucrose-containing medium, followed by an Enterobacteriaceae bacterium SC-L1, Serratia sp. SC-K1 and Burkholderia spp. SF-A1, SF-C1, and SF-E2 from S. fuscum showed relatively low ability to emit N₂O although these Burkholderia isolates are highly adaptable to the acidic environments in the boreal peat ecosystems (Fig. 2.3).

Influence of gallic acid or (E)-caffeic acid on N₂O emission from the culture of pure isolates was performed, and appropriate concentration of the polyphenols in the Sphagnum mosses promoted N₂O emission of the N₂O-emitting bacteria. In addition, the environmental factors, such as pH, temperature, nitrogen sources, polyphenols (gallic acid or (E)-caffeic acid), and nosZ gene affected or regulated N₂O emission. To explore why some Pseudomonas isolates from Andisol harbor or lack nosZ gene, phylogenetic analyses of narG, nirS, and nosZ genes compared to that of 16S rRNA gene sequences.

pH

Soil pH is a key factor for N₂O emission (Van den Heuvel et al., 2011). This study showed Burkholderia (Proteobacteria) adapted to the acidic pH (pH 5.0) (Fig. 1.8A), however, N₂O emission by bacteria of Enterobacteriaceae and Pseudomonas
of class Gammaproteobacteria in boreal peat bogs are much more active in neutral pH regions rather than acidic regions (Fig. 2.6B). Low soil pH generally decreases rate of denitrification process, but it leads to increase of N₂O emission as reported by Van den Heuvel et al. (2011). There is no difference of N₂O emission in between with C₂H₂ and control (without C₂H₂ gas) (Fig. 1.9B and Fig. 2.8), and no functionality of N₂OR under the lower pH (pH ≤ 6.1) was widely accepted active N₂O emission at acidic region despite of harboring nosZ gene. It probably due to a posttranscriptional interference on the expression of nosZ gene (Liu et al., 2014).

**Temperature**

All the N₂O emitters isolated from boreal peat bogs showed higher N₂O emission at relatively high temperature over 25 °C or 30 °C (Fig. 1.8B and Fig. 2.5). This may indicate that global warming promotes N₂O emission from boreal Sphagnum bogs. Previous study also offered the evidence that N₂O emission decreased along with decreased temperature (Pfenning and McMahon, 1997). This response to high temperature is probably caused by increasing cell growth of denitrifiers along with increased temperature from 15 °C to 25 °C as demonstrated in Fig. 1.10.

**Nitrogen source**

This study also showed that pure cultures of the isolated N₂O emitters most adapted to NO₃⁻ rather than NH₄⁺ as inorganic nitrogen substrate for N₂O production, indicating denitrification or DNRA as the major pathways for N₂O emission in boreal
peat ecosystem. Denitrification is regarded as the major pathway for \( \text{N}_2\text{O} \) emission in soils (Mosier, 1998). Dong et al. (2009) reported that potential of DNRA increased as the concentration of \( \text{NO}_3^- \) declined. So this may illustrate that the concentration of \( \text{NO}_3^- \) is another important key on the \( \text{N}_2\text{O} \) emission.

**Polyphenols and plant insolubes**

This study strongly suggested that polyphenols as the important secondary metabolites of *Sphagnum* mosses also play a key role in the boreal peat bogs. Lower concentration of \( (E) \)-caffeic acid \(< \) 0.1 g L\(^{-1}\) and gallic acid \(< \) 10 mg L\(^{-1}\) accelerated \( \text{N}_2\text{O} \) emission, while *Burkholderia* sp. SF-E2 and *Serratia* sp. SC-K1 showed clear acceleration of its \( \text{N}_2\text{O} \) emission upon exposure to relatively high concentration of \( (E) \)-caffeic acid \((0.1 \text{ g L}^{-1})\) (Fig. 3.8 and Fig. 3.9B). And different strains even in the same species showed different responses to different concentration of \( (E) \)-caffeic acid. In addition, \( \text{N}_2\text{O} \) production increased under the treatment with plant tissue powders, and this indicated the *Sphagnum* moss tissue stimulate cell growth of these \( \text{N}_2\text{O} \) emitters.

**nosZ gene**

In this study, some \( \text{N}_2\text{O} \)-emitting bacteria are found to be harboring *nosZ* gene while some others are missing *nosZ* gene (Fig. 1.11 and Fig. 2.9). To date, the reason for malfunctional or missing *nosZ* gene in some hyper-active \( \text{N}_2\text{O} \) emitters is still mystery. Shiina et al. (2014) reported that soil types and \( \text{N}_2\text{O} \) reductase genotype \((\text{nosZ})\) of indigenous soybean nodulation bacterium *Bradyrhizobium japonicum* show
a tight relationship between them. This group also discovered the present nosZ-missing B. japonicum as dominant in Andosols. In addition, atypical nosZ gene has been found in nondenitrifiers (the DNRA soil bacterium Anaeromyxobacter dehalogenans) (Sanford et al., 2012).

**Phylogenetic analysis**

Phylogenetic trees of functional genes (narG and nirS) displayed a high similarity with those of 16S rRNA gene among the Pseudomonas denitrifiers. While, phylogeny tree of nosZ gene showed incongruence with 16S rRNA gene. It is maybe a reason why some strains harboring nosZ gene and some strains missing nosZ gene excessively produce N$_2$O.

**Methods for mitigation of N$_2$O emission**

Under acidic conditions, the most reliable reasons for active N$_2$O emission are malfunctionalities of N$_2$O reductase (N$_2$OR), coded by nosZ gene in acidic-adapted N$_2$O-emitting microorganisms. In contrast, neutral or alkaline pH ( $\geq 6.1$) is conducive to N$_2$O reductase assembly for the model N$_2$O emitter, Paracoccus denitrificans (Liu et al., 2014). It is also consistent with the isolates, an Enterobacteriaceae bacterium SC-L1 and Pseudomonas sp. SC-H2 as shown in the pH-dependent N$_2$O emission assay (Fig. 2.8).

In fact, complete denitrifiers that exactly harbored nosZ gene can mitigate N$_2$O emission due to reduction of N$_2$O into N$_2$ gas catalyzed by N$_2$OR. However,
incomplete denitrifiers that are missing nosZ gene or lacking N₂OR functionalities are often found in acidic soils and become a big threat for N₂O fluxes. In addition, atypical nosZ genes is more dominant and abundant in some soils than their typical counterparts of denitrifiers (Orellana et al., 2014). This report indicated that the atypical NosZ proteins seem to play an important role in mitigation of N₂O emission in soil, and indeed, Sanford et al. (2012) reported that atypical NosZ protein of a DNRA soil bacterium, *Anaeromyxobacter dehalogenas*, is an effective N₂O reductase.

*Enterobacteriaceae* bacteria commonly possess the pathway of DNRA (Philippot and Germon, 2005). In this study, an *Enterobacteriaceae* bacterium SC-L1 also showed the functional N₂O reduction capacity at pH 7.0 (Fig. 2.8) although it did not harbor any typical nosZ gene. Hence, current hypothesis is that the atypical nosZ gene is functioning in the strain of the *Enterobacteriaceae* bacterium SC-L1. It should be continued related study to detect the atypical nosZ gene and to measure N₂O reductase activity in future line of the research.

**Conclusion**

From the perspective of microbial communities and pure-culture of denitrifiers, the author observed N₂O emission potentials of some *Sphagnum* moss-associated eubacteria from the boreal peat bogs. Some *Burkholderia* spp., SF-A1, SF-C1, and SF-E2, a *Pseudomonas* sp. SC-H2, and *Enterobacteriaceae* bacteria SC-L1 and *Serratia* sp. SC-K1 characterized as N₂O emitters were isolated from the leaves of *Sphagnum* mosses and their physiological traits were also examined. N₂O emission increased by *Enterobacteriaceae* bacterium and *Pseudomonas* isolates at relatively
high temperature and relatively high pH conditions. In addition, appropriate concentration of (E)-caffeic acid and gallic acid significantly promoted N₂O emission by the N₂O emitters. Phylogenetic analyses of nirS and narG genes showed a close relationship between these denitrification-associated genes and 16S rRNA gene in their phylogeny. In contrast, similar pattern in phylogeny of nosZ with phylogeny of 16S rRNA gene was not found among these denitrifiers.
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Permafrost Conference, pp. 36-42.


APPENDIX

Primer design

Amino acid sequences of bacterial NarG protein translated from nucleotide sequences and nucleotide sequences themselves were collected from 50 strains registered in NCBI database to search conserved regions of *narG* gene.
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<td>Nitrobacter_vulgaris</td>
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**.****.* * :** *:**:::** * :** : . * .**** ** :
43_Bacillus_licheniformis_ATCC
44_Bacillus_subtilis_DS518263
42_Geobacillus_thermodenitrifi
46_Rhodococcus_equi_103S
50_Mycobacterium_tuberculosis_
45_Staphylococcus_aureus_subsp
47_Staphylococcus_aureus_subsp
50_Mycobacterium_tuberculosis_
45_Streptomyces_kanamyceticus_
48_Propionibacterium_acnes_SK1
41_Aromatoleum_aromaticum_EbN1
41_Thiobacillus_denitrificans
37_Burkholderia_thailandensis
40_Burkholderia_mallei_NCTC102
5_112-CB-20S
2_Burkholderia_sp._CCGE1003
3_Burkholderia_phymatum_STM815
4_Methylbium_petroleiphilum_P
6_Acidovorax_ebreus_TPSY
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27_Escherichia_cholerae_S88
31_E.coli_S88
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38_Salmonella_enterica_subsp._
25_Enterobacter_cloacae_SCF1
21_Pectobacterium_wasabiae_WPP
23_Erwinia_carotovora_subsp._a
26_Dickeya_dadantii_Ech586
32_Dickeya_dadantii_Ech703
20_Marinobacter_aquaeolei_VT8
49_Nitrobrocher vulgaris
| 43 | Bacillus licheniformis_ATCC | EENG------TFTAGRFLHAKDIGRAT---EHDEWKPAVWDETDGFAIP |
| 44 | Bacillus subtilis_DQ518263 | KENG------VTAGRFLHAKDIGRKT---KHDQKPAVWDEQTTSSFAIP |
| 42 | Geobacillus thermodenitrifi | KHDG------RYAAGRFLASLGMLQ---QYAEKWTIWDQSEQQFAIP |
| 47 | Staphylococcus equi_103S | EDEN------GYKAGRFLDGLQQ---EDAGWKPAVWDEQTSSFAIP |
| 50 | Mycobacterium tuberculosis_ | EDNG------RYAAGRFLRASDLGQ---EDEWKPAVWDETDGSAVP |
| 45 | Streptomyces kanamyeticus_ | ERDG------AYVPGRFLRASDLGQ---EGEMWKPAVWDEQSDGAV |
| 48 | Propionibacterium acnes_SK | RFGDPQADGAAYVGKFLTDKLVAQ---PDHPFRPLMVEADSQKDP |
| 41 | Armatoleum aromaticum EbN1 | PFGDPQADGAAYVPGRYVRASDFEGHLGQ---EDEWKPAVWDEQSDGVP |
| 42 | Thiobacillus denitrificans_ | PFGDPQADGAAYVPGRYVRASDFEGHGLQ---EDEWKPAVWDEQSDGVP |
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| 49 | Burkholderia mallei_NCTC102 | THTLPDGRT-TFVPGRYVRASDFEGHLGQ---EDEWKPAVWDEQSDGVP |
| 1 | N2O Sarawak | RQTLPDGRT-TLVPGRYVRASDFEGHLGQ---EDEWKPAVWDEQSDGVP |
| 112-CB-20S | RQTLPDGRT-TFVPGRYVRASDFEGHLGQ---EDEWKPAVWDEQSDGVP |
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| 3 | Burkholderia phymatum_STM815 | THTLPDGRE-TLVPDRYLRASDFNGNLGQ---EDEWKPAVWDEQSDGVP |
| 4 | Hydrogenophaga_sp_PBC | THTLPDGRE-TLVPDRYLRASDFNGNLGQ---EDEWKPAVWDEQSDGVP |
| 5 | Acidovorax ebreus_TPSY | THTLPDGRE-TLVPDRYLRASDFNGNLGQ---EDEWKPAVWDEQSDGVP |
| 7 | Alicyclobifilus_sp. | THTLPDGRE-TLVPDRYLRASDFNGNLGQ---EDEWKPAVWDEQSDGVP |
| 9 | Alicyclobifilus denitrificans | THTLPDGRE-TLVPDRYLRASDFNGNLGQ---EDEWKPAVWDEQSDGVP |
| 11 | Delftia acidovorans_SPH-1 | THTLPDGRE-TLVPDRYLRASDFNGNLGQ---EDEWKPAVWDEQSDGVP |
| 12 | Thiomonas intermedia_K12 | THTLPDGRE-TLVPDRYLRASDFNGNLGQ---EDEWKPAVWDEQSDGVP |
| 30 | Burkholderia vietnamiensis_ | THTLPDGRE-TLVPDRYLRASDFNGNLGQ---EDEWKPAVWDEQSDGVP |
| 34 | Burkholderia pseudomallei_1 | THTLPDGRE-TLVPDRYLRASDFNGNLGQ---EDEWKPAVWDEQSDGVP |
| 24 | Burkholderia ambifaria_MC40 | THTLPDGRE-TLVPDRYLRASDFNGNLGQ---EDEWKPAVWDEQSDGVP |
| 28 | Burkholderia multivorans_AT | THTLPDGRE-TLVPDRYLRASDFNGNLGQ---EDEWKPAVWDEQSDGVP |
| 35 | Burkholderia gladioli_BSR3 | THTLPDGRE-TLVPDRYLRASDFNGNLGQ---EDEWKPAVWDEQSDGVP |
| 13 | Ralstonia pickettii_12J | THTLPDGRE-TLVPDRYLRASDFNGNLGQ---EDEWKPAVWDEQSDGVP |
| 14 | Brucella melitensis_M5-90 | THTLPDGRE-TLVPDRYLRASDFNGNLGQ---EDEWKPAVWDEQSDGVP |
| 22 | Brucella abortus_S19 | THTLPDGRE-TLVPDRYLRASDFNGNLGQ---EDEWKPAVWDEQSDGVP |
| 18 | Ochrobactrum anthropi_ATCC4 | THTLPDGRE-TLVPDRYLRASDFNGNLGQ---EDEWKPAVWDEQSDGVP |
| 17 | Oligotropha carboxidovorans | THTLPDGRE-TLVPDRYLRASDFNGNLGQ---EDEWKPAVWDEQSDGVP |
| 16 | Azorhizobium caulinarodans_OR | THTLPDGRE-TLVPDRYLRASDFNGNLGQ---EDEWKPAVWDEQSDGVP |
| 32 | Dickeya dadantii_Ech703 | THTLPDGRE-TLVPDRYLRASDFNGNLGQ---EDEWKPAVWDEQSDGVP |
| 20 | Marinobacter aqaeolei_VT8 | THTLPDGRE-TLVPDRYLRASDFNGNLGQ---EDEWKPAVWDEQSDGVP |
| 49 | Nitroacter vulgaris | THTLPDGRE-TLVPDRYLRASDFNGNLGQ---EDEWKPAVWDEQSDGVP |
43_Bacillus_licheniformis_ATCC YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
44_Bacillus_subtilis_DQ518263 YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALS
24_Geobacillus_thermodenitrificans YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
46_Rhodoferax_ferrireducens_T1 YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
50_Mycobacterium_tuberculosis YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
45_Streptomyces_kanamyricus_ YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
48_Propionibacterium_acnes_SK1 YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
41_Thiobacillus_denitrificans YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
5_112-CB-20S YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
2_Burkholderia_sp._CCGE1003 YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
4_Burkholderia_phymatum_STM815 YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
11_Delftia_acidoavorans_SPH-1 YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
8_Polaromonas_naphthalenivorans YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
10_Rhodoferax_ferrireducens_T1 YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
12_Thiomonas_intermedia_K12 YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
30_Burkholderia_vietnamiensis_ YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
34_Burkholderia_pseudomallei_1 YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
29_Hyphomicrobium_denitrificans YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
13_Ralstonia_pickettii_12J YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
14_Brucella_melitensis_M5-90 YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
22_Brucella Abortus_S19 YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
18_Ochrobactrum_undulinum ATCC YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
25_Enterobacter_cloacae_SCF1 YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
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21_Pectobacterium_wasabiae_WPP YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
33_Citrobacter_koseri_ATCCBA- YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
38_Salmonella_enterica_subsp._ YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
20_Marinobacter_aquaeolei_VT8 YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
49_Nitrobeta_carniformis YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
19_Methylobacterium_radiotolerans YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
16_Azorhizobium_caulinodans_ YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
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5_Pseudomonas_fluorescens_AF1 YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
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27_Escherichia coli S88 YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
31_E coli S88 YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
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27_Escherichia coli S88 YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA
31_E coli S88 YSDIVLPAATYWEDLSSTDMHPFIHPNPAISAWEKSDWDFFKALA

43_Bacillus_licheniformis_ATCC      TGNQ-RDLNVVIRKLK--EVDWLED----------------------
44_Bacillus_subtilis_DQ518263       TGNQ-RDLNVVIRKLK--EVDWLED----------------------
42_Geobacillus_thermodenitrifi      TGNQ-RDEQVMIRKLEKREVNDL-------------------------
47_Staphylococcus_equi_103S         TGNQ-RDEVTVIRRRSQ-EVQY------------------------
50_Mycobacterium_tuberculosis_      TGNQ-RDEVTVIRRRSQ-EVRY-------------------------
45_Streptomyces_kanamyceticus      TGNQ-RDEVTVIRRRSQ-EVQY------------------------
48_Propionibacterium_acnes_SK1      TGNQ-RDEVTVIRRRSQ-EVQY------------------------
41_Aromatoleum_aromaticum_EbN1     TGNQ-RDEQVMIRKLEKREVDWLES----------------------
112-CB-20S                        TGNQ-RDEQVMIRKLEKREVDWLES----------------------
2_Burkholderia_sp._CCGE1003         TGNQ-RDEQVMIRKLEKREVDWLES----------------------
3_Burkholderia_phymatum_STM815     TGNQ-RDEQVMIRKLEKREVDWLES----------------------
4_Hydrogenophaga_sp_PBC            TGNQ-RDEQVMIRKLEKREVDWLES----------------------
5_Methylum_petroleiphilum_P        TGNQ-RDEQVMIRKLEKREVDWLES----------------------
6_Acidovorax_ibreus_TPSY           TGNQ-RDEQVMIRKLEKREVDWLES----------------------
7_Alicyciphilus_sp.                TGNQ-RDEQVMIRKLEKREVDWLES----------------------
11_Delftia_acidovorans_SPH-1       TGNQ-RDEQVMIRKLEKREVDWLES----------------------
8_Polaromonas_naphthalenivoran     TGNQ-RDEQVMIRKLEKREVDWLES----------------------
10_Rhodoferax_ferrireducens_T1     TGNQ-RDEQVMIRKLEKREVDWLES----------------------
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19_Methylobacterium_radiotoler     TGNQ-RDEQVMIRKLEKREVDWLES----------------------
16_Azorhizobium_caulinodans_OR    TGNQ-RDEQVMIRKLEKREVDWLES----------------------
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5_Pseudomonas_aeruginosa_PA74#    TGNQ-RDEQVMIRKLEKREVDWLES----------------------
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*  ** :