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Physiological Characterization and Molecular Identification of Denitrifying Bacteria Possesing Nitrous Oxide High Reduction Activity Isolated from Rice Soils

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Rice fields are one of the main sources of high nitrous oxide N_2O emission. Soil denitrifiers possessing high N_2O reduction activity are important for controlling N_2O emission. Nitrous oxide reduction is the last step of denitrification process. The aims of this study were to characterize and to identify denitrifying bacteria isolated from rice soils possessing high activity of N_2O reduction. Soil samples were taken from 6 locations of rice fields in Bogor (West Java) and Tangerang (Banten), Indonesia. Physiological characterization was performed using API 20 NE, while molecular identification was conducted based on the 16S rRNA gene sequence. It was found that ten isolates of denitrifying bacteria were able to grow using N_2O as an electron acceptor as indicated by decreasing N_2O concentration in the headspace of the cultures. The bacterial growth indicated by optical density, increased up to 0.12-0.47 after 5 days incubation. Isolate BL2 had the highest activity of N_2O reduction followed by BL1 and BLN1 at up to 5.41, 4.09, and 3.91 μmol mL^{-1} bacterial cultures, respectively. The BL1, BL2, and BLN1 isolates had some different physiological characteristics. Based on their 16S rRNA sequence, BL1 and BLN1 were closely related to *Ochrobactrum anthropi* ATCC 49188 with similarity of 99%.

Key words: denitrifying bacteria, N_2O reduction, rice soils

Nitrous oxide is an environmentally important trace gas because of its contribution to global warming and its role in the destruction of the stratospheric ozone layer. N_2O has 298 times higher global warming potential than that of carbon dioxide and it has 114 years atmospheric lifetime. The atmospheric N_2O abundance has risen from 270 ppb in 1750 to 319 ppb in 2005 (Forster and Ramaswamy 2007). Rice fields are one of the main sources of high N_2O emission. In irrigated rice fields, N_2O is emitted simultaneously since the biochemistry of submerged soils support the production and emission of this gas (Majumdar 2003). The input of large amount of nitrogen fertilizer influences some microbial process especially nitrification and denitrification and results in increased production of N_2O (Hayatsu *et al.* 2008).

The complete denitrification process leads to N_2 formation while the incomplete denitrification produces N_2O. During the last step of complete denitrification, N_2O is reduced to N_2. A truncated version of denitrification is due to lack of activity or the absence of the gene for N_2O reductase. Some denitrifying bacteria are able grow using N_2O as the sole electron acceptor for oxidation of organic compounds (Zumft 1997). The composition of the end gas produced in denitrification varies among complete denitrifiers. Carlson and Ingraham (1983) reported that *Pseudomonas stutzeri* produced only N_2, *P. aeruginosa* and *Paracoccus denitrificans* produced N_2O as well, and under certain condition *P. aeruginosa* produced even more N_2O than N_2.

Differences in denitrifier community composition in soils affects N_2O emission (Holtan-Hartwig *et al.* 2000). Denitrifying bacteria with the ability to reduce exogenous N_2O may have an important role in determining the level of N_2O emission from the environment. The aims of this study were to characterize and to identify bacteria with a high N_2O reduction activity from rice soils. These study represent a preliminary research to obtain potential bacteria isolates to reduce N_2O emission in rice fields.

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MATERIALS AND METHODS

Soil Samples and Media. Soil samples were taken from 6 locations of rice fields in Bogor (West Java) and Tangerang (Banten), Indonesia. Truncated 2.5 mL syringes capped with a rubber seal were used for collecting soil samples. Experiments were conducted using denitrification media containing: 2.72 g $CH_3COONa{\cdot}3\ H_2O$, 0.8 g K_2HPO_4, 0.3 g KH_2PO_4, 0.4 g NH_4Cl, 5.2 g $MgSO_4{\cdot}7\ H_2O$, 1L aquadest (Barford *et al.* 1999) and added with 2.55 g $NaNO_3$ and 2 mL trace element. The trace element composition was 50 g NaEDTA, 1.407 g $ZnCl_2$, 5.5 g $CaCl_2$, 5.06 g $MnCl_2{\cdot}4H_2O$, 4.86 g $FeCl_3{\cdot}6\ H_2O$, 1.1 g $(NH)_4Mo_7O_{24}{\cdot}4\ H_2O$, 1.57 g $CuSO_4{\cdot}5\ H_2O$, 1.61 g $CoCl_2{\cdot}6\ H_2O$, 1L aquadest.

Bacterial Isolation. Thirty mL sterile media in 70 mL serum bottles sealed using rubber seals were used to grow bacteria. Anaerobic conditions were achieved by flushing sterile N_2 gas into the bottles for 10 min. One millilitre soil suspension (five times dilution) was injected aseptically into the bottles. The cultures were incubated at room temperature (28-30^{O}C) for 5-7 days. The bacteria were isolated using streak method on agar media and incubated either anaerobically (in an anaerobic chamber) or aerobically at room temperature (28-30^{O}C) for 7 days. Separately growing colonies were purified by streaking on fresh agar media. The pure isolates were tested for their ability to perform oxidative or fermentative metabolisms using Hugh and Leifson (1953) method. The isolates performing oxidative metabolism were selected for further experiment.

Bacterial Growth and Nitrous Oxide Reduction Test. The selected isolates were grown anaerobically in 7 mL liquid media without No_3^- in test tubes capped with rubber seals. Nitrous oxide was added to the headspace using sterile syringe to obtain a final concentration of 3.54 μmol mL^{-1} headspace volume. The inoculum used (2%, v/v) was a culture in exponential growth ($OD_{\lambda 550\ nm}$ 0.6). Incubation was conducted for 5 on a shaker at room temperature (28-30°C). Bacterial growth was measured turbidimetrically using a Bausch and Lomb Spectronic 20 spectrophotometer at 550 nm and headspace N_2O concentration were analysed using Shimadzu gas chromatography-14B with electron capture detector (ECD). Sterile medium without bacteria added with N_2O was used as a control.

Characterization and Identification. Bacterial isolates were examined using light microscope and their characteristics i.e. colour, elevation, margin, and shape of colony; also shape and motility of cells were recorded. In addition, isolates were characterized on the basis of Gram staining. Physiological characterization was performed using API 20 NE (Biomérieux). Molecular identification was carried out based on 16S rRNA sequence. Deoxyrobonucleic acid (DNA) extraction was done according to Lazo *et al*. (1987).

Amplification of 16S rRNA genes was performed using polymerase chain reaction (Perkin Elmer GeneAmp PCR System). Amplification was carried out using GoTaq Flexi DNA Polymerase (Promega) with the primers 63f (5'-CAG GCC TAA CAC ATG CAA GTC) and 1387r (5'-GGG CGG WTG GTA CAA GGC) (Marchesi *et al.* 1998). The thermal-cycling conditions were initial denaturation step at 95°C for 5 min; followed by 30 cycles of 95°C for 30 s, 55°C for 1 min, 72°C for 1 min; and a final extension step at 72°C for 5 min. The nucleotide sequence of the PCR products were determined using an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems, USA) 4 capillary and Big Dye Terminator cycle sequencing kit v3.1 (Applied Biosystems, USA). Sequence data were compared with sequences in The National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) data bank using Basic Local Alignment Search Tool (BLAST). A phylogenetic tree was constructed using Molecular Evolutionary Genetic Analysis (MEGA) version 4.0 program (Tamura *et al.* 2007).

RESULTS

Bacterial Isolates. Twenty four bacteria were isolated from 6 sampling locations using NO_3^- enrichment media. Soil sample pH was between 5 and 6. Ten isolates were obtained under aerobic condition and the remaining 14 isolates were isolated anaerobically. Fifteen isolates showed oxidative metabolism, while 9 isolates showed fermentative metabolism (Table 1). The oxidative isolates were considered as denitrifying bacteria and selected for further study.

Bacterial Growth and N_2O Reduction. As many as 10 isolates were tested for their ability to use N_2O (Table 2). BL2 isolate showed the highest N_2O reduction activity among tested isolates, although the growth of BL1, Sg1**,** and BLN1 were faster than that of Bl2. BL2 isolate could reduce up to 5.41 μmol mL^{-1} bacterial culture of the N_2O after 5 of incubation at room temperature (28-30°C) followed by BL1 and BLN1 isolates which could reduce up to 4.09 and 3.91 μmol mL^{-1} bacterial cultures of the N_2O respectively. The N_2O reductions of other isolates varied between 1.02-3.55 μmol mL^{-1} bacterial cultures of the N_2O.

Characteristics and Identity of Bacteria. The morphology of three selected colonies possessing high N_2O reduction activity (BL1, BL2, and BLN1) was cream in colour, convex elevation, and entire margin. BL1 and BLN1 colonies were circular, while BL2 was ellipse. Cells were

Table 1 Bacteria isolated from samples of rice soils in Bogor and Tangerang

Sampling locations	Soil pH	Isolates	Growth conditions	Metabolisms
Bogor				
Leuwisadeng	5.5	LSN1	Anaerobe	Oxidative
		LSN2	Anaerobe	Oxidative
Sipak	5.0	SP1	Aerobe	Fermentative
		SP2	Aerobe	Fermentative
		SPN1	Anaerobe	Fermentative
		SPN3	Anaerobe	Fermentative
		SPN4	Anaerobe	Fermentative
Situgede	6.0	SG1	Aerobe	Oxidative
		SG2	Aerobe	Oxidative
		SGN1	Anaerobe	Oxidative
		SGN3	Anaerobe	Oxidative
		SGN4	Anaerobe	Oxidative
		SGN5	Anaerobe	Oxidative
		SGN6	Anaerobe	Oxidative
		SGN7	Anaerobe	Oxidative
Situgede (seedbed)	6.0	SS1	Aerobe	Fermentative
		SS2	Aerobe	Oxidative
		SS3	Aerobe	Fermentative
		SSN1	Anaerobe	Fermentative
Tangerang				
Belendung	5.0	BL1	Aerobe	Oxidative
		BL2	Aerobe	Oxidative
		BLN1	Anaerobe	Oxidative
		BLN2	Anaerobe	Oxidative
Cipete	5.5	CP1	Aerobe	Fermentative

Table 2 Growth and N_2O reduction of denitrifier isolates incubated in 5 days at room temperature (28-30°C)[a]

Isolates	Growth ($OD_{\lambda 550\ nm}$)	N_2O reduced (μmol mL^{-1} culture)
SG1	0.45 ± 0.00	3.55 ± 0.23
SS2	0.37 ± 0.02	1.30 ± 0.00
BL1	0.47 ± 0.00	4.09 ± 0.92
BL2	0.39 ± 0.03	5.41 ± 0.03
LSN1	0.25 ± 0.02	1.33 ± 0.00
LSN2	0.20 ± 0.10	1.02 ± 0.00
SGN4	0.35 ± 0.01	1.02 ± 0.04
SGN6	0.12 ± 0.01	1.11 ± 0.00
SGN7	0.21 ± 0.02	1.51 ± 0.04
BLN1	0.43 ± 0.01	3.91 ± 0.12

[a] Initial concentration of N_2O was 3.54 μmol mL^{-1} headspace volume

rod-shaped, motile, and Gram negative. Based on the API 20 NE assay (Table 3), the three isolates had different physiological characters on hydrolysis of esculine and P-nitrophenyl-β-D-galactopyranoside, and also for the assimilation of potassium gluconate and trisodium citrate. From their physiological characters, BLN1 isolate was identified as *Ochrobactrum anthropi* with 99.9% similarity, while the profiles of Bl1 and BL2 isolates were unknown. However based on 16S rRNA sequences analysis using BLAST (500 bp), BL1, BL2, and BLN1 isolates had similarity of 99, 95, and 99% respectively with *O. anthropi* ATCC 49188 (accession number NC 009668) (Table 4). The phylogenetic tree of the 16S rRNA gene sequence of the isolates shows that BL1, BL2, and BLN1 are members of *Alphaproteobacteria* and the same class with *Ochrobactrum*, *Azospirillum brasilense* and *Azhorhizobium caulinodans* (Fig 1).

DISCUSSION

This study has shown that denitrifying bacteria possessing N_2O reduction activity could be isolated from rice soils. Denitrifiers were isolated under both aerobic and anaerobic isolation conditions. Denitrification is exclusively a facultatively anaerobic or microaerophilic trait. Its expression is triggered in the cell by the low environmental oxygen tension and the availability of a nitrogen oxide. Anaerobically grown cells do not lose their capacity to respire oxygen (Zumft 1997). Some bacteria can carry out the denitrifying process on varying of oxygen levels. However, oxygen has an effect on the denitrification rate and gas end product ratio (Davies *et al*. 1989; Takaya *et al*. 2003). Most denitrifying bacteria are incapable of growing anaerobically by fermentative metabolism (Garcia and Tiedje 1982). Thus, the referred fermentative isolates were not denitrifier.

Table 3 Physiological characteristics of BL1, Bl2, and BLN1 isolates

Physiological tests	BL1	BL2	BLN1
Reduction of nitrate	+	+	+
Indole production	-	-	-
Glucose fermentation	-	-	-
Hydrolysis			
Arginine	+	+	+
Urea	+	+	+
Esculine	+	+	-
Gelatine	-	-	-
p-nitrophenyl-β-D-galactopyranoside	+	-	-
Assimilation			
D-glucose	+	+	+
L-arabinose	+	+	+
D-mannose	+	+	+
D-mannitol	-	-	-
N-acetyl glucosamine	+	+	+
D-maltose	+	+	+
Potassium gluconate	+	-	-
Capric acid	-	-	-
Adipic acid	-	-	-
Malic acid	+	+	+
Trisodium citrate	+	-	+
Phenylacetic acid	-	-	-
Oxidase test	+	+	+

Table 4 Identification results of BL1, Bl2, and BLN1 isolates based on API 20 NE tests and 16S rRNA sequences

Isolates	API 20 NE test	16S rRNA sequences
BL1	Unknown	*O. anthropi* ATCC 49188 (99%)
BL2	Unknown	
BLN1	*Ochrobactrum anthropi* (99.9%)	*O. anthropi* ATCC 49188 (95%)
		O. anthropi ATCC 49188 (99%)

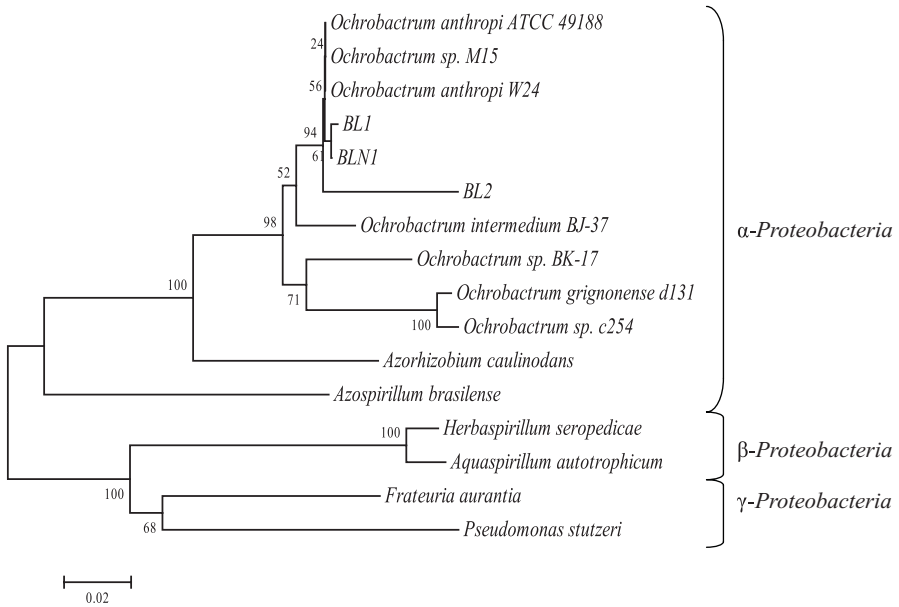

Fig 1 Neighbour-joining dendrogram derived from 16S rRNA gene sequences of the bacteria isolated from rice soils and other bacteria members of the phylum *Proteobacteria*. The scale bar represents 2% sequence divergence. Bootstrap test was conducted by 500 replicates.

All tested isolates could grow using exogenous N_2O so that N_2O concentration in the headspace was decreased while OD of cells was increased. Nitrous oxide reduction was coupled to electron transport phosphorylation and thereby to cell growth. However, the ability to grow on N_2O varied among the isolates tested. Snyder *et al*. (1987) suggested that synthesis and destruction of enzyme may be the mechanisms that control N_2O reductase activity. Not all denitrifiers can reduce exogenous N_2O to N_2 efficiently. Bazylinski *et al*. (1986) reported that *P. aeruginosa* P2 grew abundantly on exogenous N_2O but the exogenous N_2O had little effect on the cell yields of PAO1 and P1 strains. According to Snyder *et al.* (1987), the inability of a number of *P. aeruginosa* strains to grow well on N_2O is therefore a consequence of the nearly complete loss of N_2O reductase activity.

The high similarities (99%) among BL1 and BLN1 with *O. anthropi* ATCC 49188 indicate that these isolates are closely related to the bacterium. Isolate BL2 may be a new species in genus *Ochrobactrum* because it has 95% similarity with *O. anthropi* ATCC 49188. The most widely accepted definition is that strains with over 97% similarity over entire gene are the same species (Stackebrandt and Goebel 1994). Aside of being closely related to *O. anthropi* ATCC 49188, in phylogenetic tree BL1 and BLN1 isolates also closely related to *Ochrobactrum* sp. M15 and *Ochrobactrum anthropi* W24 used as comparators (Fig 1).

From this study, 10 isolates of N_2O reducing denitrifier have been obtained from rice soils. Two of three isolates identified were closely related to *O. anthropi* ATCC 49188. Genus *Ochrobactrum* is member of family *Brucellaceae*, order *Rhizobiales*, class *Alphaproteobacteria* (Garrity and Holt 2001). According to Zumft (1997) denitrification carried out by a diversity of bacteria taxonomically belonging to the group of *Proteobacteria*. In the rice soil with strong denitrification activity, Ishii *et al.* (2009) found that the dominant bacteria were members of *Bukholderiales* and *Rhodocyclales* (*Betaproteobacteria*).

Bacteria with high N_2O reduction activity have the potential application to regulate N_2O emission in rice fields. Three isolates that showed high N_2O reduction activity have been selected in this study. Nevertheless, some experiments still needed to measure the N_2O reduction activity in more detail, at laboratory and also at the field scale.

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