The isolation and identification of useful bacteria that decrease nitrous oxide emission from agricultural field

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Abstract
Increases in atmospheric concentration of nitrous oxide (N_2O) are partly attributed to soil denitrification. To overcome the problem, we propose utilising useful bacteria *in situ* to suppress N_2O emission from agricultural soil by inhibiting the activities of microorganisms that produce high levels of N_2O . The useful bacteria are the bacteria that convert nitrate ion (NO_3^-) to another nitrogen compound under aerobic conditions, or aerobic denitrifyfing bacteria that reduce NO_3^- to nitrogen under aerobic condition. Therefore, we attempted screening to find bacteria that have either of those properties, and then, four strains (K, N-I, N-II, and N-III) that could remove NO_3^- and NH_4^+ under aerobic conditions were isolated and identified. Of the total nitrogen originally provided, 55% to 75% was taken up into the cells and the residual nitrogen went into the culture supernatant. The strains K, N-I, and N-II were identified as *Enterobacter cloacae*. The strain N-III showed 98.2% gene sequences identical to *Enterobacter cloacae* and 98.1% to *Klebsiella pneumonia*, indicating that the strain N-Ⅲ has the potential to be a new species of bacterium.

Key Words
Aerobic denitrifying bacteria, identification, nitrous oxide.

Introduction
Nitrous oxide (N_2O) has a 200- to 300-fold-stronger greenhouse effect than carbon dioxide (CO_2). It has been reported that N_2O has the potential to destroy the ozone layer (Takaya *et al*. 2003). Recently, the concentration of N_2O in atmosphere is increasing. One of the proposed sources of N_2O is soil denitrification of nitrogenous compounds (Figure 1), resulting from excess agricultural fertilizer. The nitrate ion by fertilization is reduced by denitrifying bacteria to gaseous nitrogen (N_2) under anaerobic condition. However, the agricultural field is aerobic, so the reduction of N_2O to N_2,, that is, the final step of denitrification, is suppressed by oxygen . Therefore, considerable amounts of N_2O are released in agricultural fields. To decrease N_2O emission, we propose dispersing useful bacteria over the agricultural field where the aerobic denitrifers grow and suppress activities of microorganisms that produce high levels of N_2O. The useful bacteria we propose convert NO_3^- to another nitrogen compound or denitrify releasing low levels of N_2O under aerobic condition. Therefore, we attempted the isolation and identification of bacteria that have either of those properties.

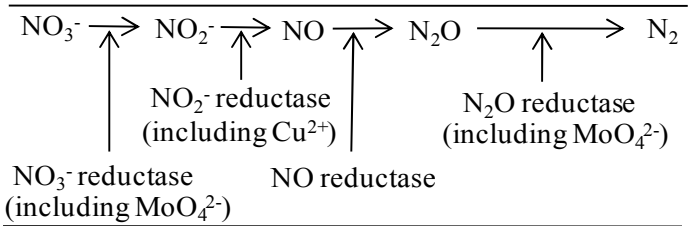

Figure 1. The given course of denitrification steps.

Materials and Methods
Isolation of aerobic denitrifiers
At first, we collected 77 samples of rhizosphere soil from agricultural fields in Kurokawa, Machida, and Nishinomiya (Japan), and then transferred to them saline (0.8%) to adjust the suspensions. Some drops of the suspensions were transferred to 5 mL of screening media in flasks with cotton plugs, respectively and cultured by shaking at 130 rpm at 30 ^oC. The screening medium contained the following compounds: 0.1% NH_4NO_3, 2% Glucose (Glc), and trace metallic salts. Two of the trace metallic salts, molybdate ion (MoO_4^{2-}) and copper ion (Cu^{2+}), are needed for enzymes to catalyze some steps of denitrification (reduction of NO_3^- to NO_2^-, of NO_2^- to NO, and of N_2O to N_2) (Figure 2). Bacterial growth, consumption of NO_3^- , NH_4^+ (N-

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compounds), and Glc and production of NO_2^- were monitored by quantitative analysis. After some independent experiments as described previously, positive strains were isolated and cultured further in 3mL of the same media at 130 rpm at 30 °C, to study the properties of isolated strains.

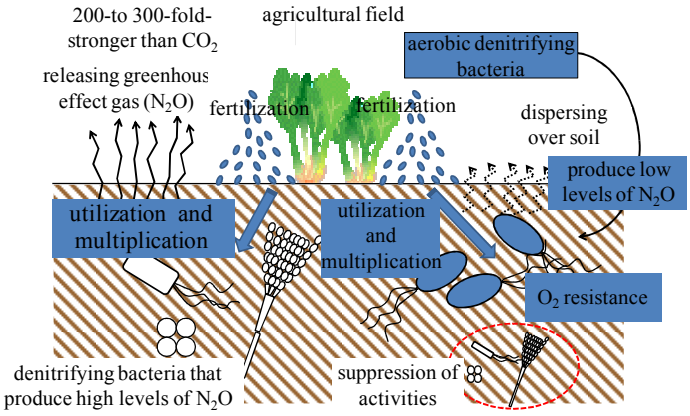

Figure 2. Suppression of releasing N_2O by aerobic denitrifying bacteria.

Analysis of total nitrogen concentration in cultural supernatant and cells
Total nitrogen in the culture supernatant and the cells was determined by Kjeldahl apparatus.

Identification of the strains
The 16S rRNA genes (1500 bp) from the total DNA extracted from the four strains were amplified by PCR. The PCR products were ligated into a pGEM-T easy vector and transformed into *Escherichia coli* cells and then, clones were sequenced. Sequence results were compared with reference sequences using DNA Data Bank of Japan (DDBJ) service.

Results

Isolation of aerobic denitrifiers
It was found that four strains of 77 samples have the potential to be aerobic denitrifers and we defined the four strains as K, N-Ⅰ, N-Ⅱ, and N-Ⅲ. N-Ⅱ was able to remove more NO_3^- and NH_4^+ than other three strains, so the result of N-Ⅱ was shown in Figure 3. The remaining NO_3^-, NH_4^+, and Glc in the medium decrease sharply for the first 20 hours. The production of NO_2^- was too little to detect. Although NO_3^- and NH_4^+ were almost removed, the value of growth was under 6 (optical density at 660 nm [OD_{660}]). This result may indicate that NO_3^- is not being assimilated because this would expect a growth value much higher than 6. To further study the nitrogen prevalence, the four strains were cultured in 50 mL of same media under same conditions. After NO_3^-, NH_4^+ were removed, to analyze total nitrogen, the cultural supernatant and cells were collected.

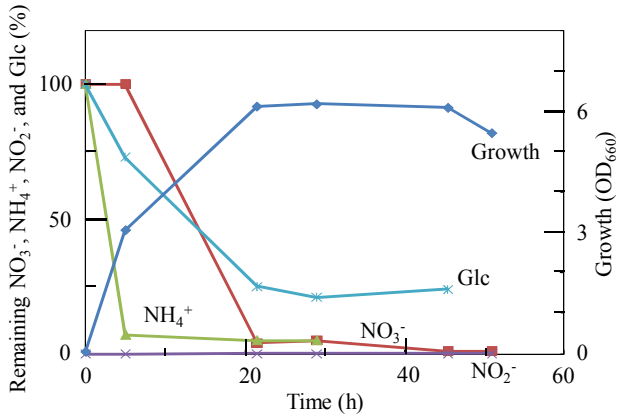

Figure 3. Growth profile of strain N-Ⅱ and of N-compounds in the medium with MoO_4^{2-} and Cu^{2+}consumption

Analysis of total nitrogen in cultural supernatant and cells
Table 1. shows utilization of 0.1% NH_4NO_3 (initially 1.24 mmol) and total nitrogen. *Paracoccus denitrificans* and *Pseudomonus stutzeri* were used as controls in that they are known to denitrify releasing

low level of N_2O under aerobic conditions. The controls removed almost all NH_4^+ but did not remove NO_3^-. With the four isolated strains, 58% to 82% of the total nitrogen originally provided was observed in the cells and 18% to 42% of total residual nitrogen was in the cultural supernatants. Considering remaining NH_4^+ and NO_3^-, it was found that the supernatants contained another nitrogen compound.

Table 1. Total nitrogen in cultural supernatant and cells

strain	Initial (mmol)		Finish (mmol)			remaining rate
	Sup.	Cells	Sup.	(NO_3^-)	(NH_4^+)	Cells	(%)
K	1.24	0	0.60	(0.21)	(0.04)	0.79	112
N-I	1.24	0	0.57	(0.15)	(0.04)	0.75	106
N-II	1.24	0	0.33	(0.00)	(0.03)	1.02	109
N-III	1.24	0	0.59	(0.14)	(0.03)	0.73	106
Paracoccus denitrificans	1.24	0	1.00	(0.58)	(0.04)	0.48	119
Pseudomonus stutzeri	1.24	0	1.08	(0.67)	(0.05)	0.41	121

Identification of the strains

All of the identification results of four strains are shown in Table 2. The strain K showed 99.0% identity to *Enterobacter cloacae* isolate 766 and 98.4% identity to *Enterobacter cloacae* B5. The strain N-Ⅰ showed 99.1% identity to *Enterobacter cloacae* strain FR and 98.6% identity to *Enterobacter cloacae* isolate 766. The strain N-Ⅱ showed 98.6% identity to *Enterobacter cloacae* isolate 766 and 98.0% identity to *Enterobacter cloacae* strain B5. The three strains had significantly-high identity to *Enterobacter cloacae*. The strain N-Ⅲ showed 98.2% identity to *Enterobacter cloacae* isolate 766 and 98.1% identity to *Klebsiella pneumoniae* strain TCCC1, indicating that N-Ⅲ does not have significantly-high identity.

Table 2. The identification of strains K, N-Ⅰ, N-Ⅱ, and N-Ⅲ

Isolate	Homologous strains	Identity (%)
K	*Enterobacter cloacae* isolate 766	99.0
	Enterobacter cloacae strain B5	98.4
N-I	*Enterobacter cloacae* strain FR	99.1
	Enterobacter cloacae isolate 766	98.6
N-II	*Enterobacter cloacae* isolate 766	98.6
	Enterobacter cloacae strain B5	98.0
N-III	*Enterobacter cloacae* isolate 766	98.2
	Klebsiella pneumoniae strain TCCC1	98.1

Conclusions

Isolation of aerobic denitrifiers

Four strains removed NO_3^- and NH_4^+ under aerobic conditions but by analysis of total nitrogen in the supernatant and cells, denitrification did not occur. Considering the remaining NH_4^+ and NO_3^-, it was postulated that the supernatants contained another nitrogen compound.

Identification of the strains

The strains K, N-Ⅰ, and N-Ⅱ were identified as *Enterobacter cloacae*. The nucleotide sequence of 16S rRNA of N-Ⅲcould not be identified with known sequences, so we conclude N-Ⅲ has the potential to be a new species of bacterium.

Acknowledgements
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