Soil Biology & Biochemistry 97 (2016) 83–91

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

# Inoculation with nitrous oxide ($N_2O$)-reducing denitrifier strains simultaneously mitigates $N_2O$ emission from pasture soil and promotes growth of pasture plants

Nan Gao [a], Weishou Shen [a, *], Hiroko Kakuta [a], Nobuhiro Tanaka [a], Toru Fujiwara [a], Tomoyasu Nishizawa [b], Noriko Takaya [c], Tadashi Nagamine [c], Kazuo Isobe [a], Shigeto Otsuka [a], Keishi Senoo [a]

[a] Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan
[b] Department of Bioresource Science, College of Agriculture, Ibaraki University, Ibaraki 300-0393, Japan
[c] ROM Co., Ltd., Sapporo 060-0062, Japan

ARTICLE INFO

*Article history:*
Received 17 November 2015
Received in revised form
1 March 2016
Accepted 6 March 2016
Available online 17 March 2016

*Keywords:*
Biofertilizer
Climate change
Greenhouse gas
Mitigation technology
Plant growth-promoting rhizobacteria

ABSTRACT

The aim of this study was to screen nitrous oxide ($N_2O$)-reducing denitrifier strains showing both $N_2O$ mitigation and plant growth-promoting (PGP) effects in soil systems, and the effects of selected strains were monitored in soil and plant, analyzed, and comparatively evaluated. Forty denitrifier strains affiliated with *Azospirillum* and *Herbaspirillum*, previously isolated from three different paddy soils, were evaluated. Of these, 11 produced indole-3-acetic acid (>5 μg $mL^{-1}$), 9 promoted the growth of red clover (*Trifolium pratense* L. var. Medium) or timothy (*Phleum pratense* L. var. Horizon) on agar plates, and 7 were inoculated into two different soils for cultivating red clover and timothy in a greenhouse. Compared with non-inoculated control, $N_2O$ flux from red clover soil and from timothy soil were significantly lower 8 and 14 days and 9 days onwards, respectively, after inoculation with these seven strains. Cumulative $N_2O$ emissions from red clover soil were significantly lowered through inoculation with these seven strains. The growth parameters, including plant height, leaf area, fresh weight or dry weight, of the two pasture plants were significantly greater in soils inoculated with most of these seven strains than in non-inoculated soils. The uptake of C and N by the two pasture plants was significantly greater in soils inoculated with most of these seven strains than in non-inoculated soils. In conclusion, inoculating $N_2O$-reducing denitrifiers to pasture soil could mitigate $N_2O$ emissions and simultaneously promote the growth of pasture plants in a greenhouse. These strains will be invaluable microbiological resources for developing novel biofertilizers.



## 1. Introduction

Nitrous oxide ($N_2O$) is a long-lived greenhouse gas and potent ozone-depleting substance in the stratosphere. Agricultural $N_2O$ emissions are rapidly increasing and are estimated to double by 2030 unless mitigation strategies are undertaken (IPCC, 2007). Agricultural soils are the main source of $N_2O$ emissions (Reay et al., 2012) and contribute to 25% total anthropogenic $N_2O$ sources in Japan (National Greenhouse Gas Inventory Report of Japan, 2012). Microbial pathways dominate $N_2O$ formation in agricultural soils (Butterbach-Bahl et al., 2013). $N_2O$ reductase ($N_2OR$) is the only enzyme currently known that utilizes $N_2O$ as a primary substrate (Thomson et al., 2012; Butterbach-Bahl et al., 2013; Hu et al., 2015) and can be applied for $N_2O$ mitigation strategies designed to decrease $N_2O$ emission. Large $N_2O$ emissions from the agricultural soils may result from the failure of $N_2OR$ to operate, thereby terminating the denitrification process at $N_2O$ rather than innocuous dinitrogen ($N_2$) (Thomson et al., 2012).

Mitigation technologies and practices have been developed globally to reduce $N_2O$ emissions from agricultural soils (Itakura et al., 2013; Nishizawa et al., 2014; Bell et al., 2015; Nayak et al., 2015). These studies mainly focus on the influences of N fertilizer types, tillage managements, and N fertilization rates on $N_2O$

* Corresponding author.
*E-mail addresses:* awsshen@mail.ecc.u-tokyo.ac.jp, wsshen79@hotmail.com (W. Shen).

http://dx.doi.org/10.1016/j.soilbio.2016.03.004
0038-0717/

emissions (Akiyama et al., 2015; Bell et al., 2015; Nayak et al., 2015; Yang et al., 2015). Strategies to ameliorate $N_2O$ emissions arising from intensive agricultural practices should be developed to forestall further predicted rises. Thus, both the reduction of N fertilization rate and mitigation of $N_2O$ emissions from applied N fertilizer are necessary. The environmentally friendly approaches inspire a wide range of applications of plant growth-promoting rhizobacteria (PGPR) that could lead to improved nutrient uptake and plant growth (Reddy, 2014; Chauhan et al., 2015; Pii et al., 2015; Sarma et al., 2015). Studies have shown that the growth, yield, and quality of certain plants significantly increased after the application of biofertilizers containing bacterial N fixer, phosphorus (P)- and potassium (K)- solubilizing bacteria, and other functional microbial strains (Sahoo et al., 2013; Bhardwaj et al., 2014; Vassilev et al., 2015). For example, a biofertilizer could increase crop yields by 10%–60% (Urashima et al., 2005; Mia et al., 2010; Habibi et al., 2014). Although many biofertilizers have been extensively investigated, few microbiological mitigation methods have been attempted (Itakura et al., 2013; Nishizawa et al., 2014). To the best of our knowledge, there has been no report about the development of a novel biofertilizer with both $N_2O$ mitigation and plant growth-promoting (PGP) effects—defined as effects that promote plant growth parameters and yields.

Recently, we have isolated hundreds of denitrifying strains from various rice paddy soils using the functional single-cell isolation method (Ashida et al., 2010; Ishii et al., 2011b; Tago et al., 2011; Nishizawa et al., 2013). We have found that many of those are active $N_2O$-reducing denitrifiers in culture conditions (Ishii et al., 2011b; Tago et al., 2011), mainly belonging to *Azospirillum* and *Herbaspirillum* genera. Studies have shown that *Azospirillum* sp. and *Herbaspirillum* sp. were PGPR (Sahoo et al., 2013; Neiverth et al., 2014; Chauhan et al., 2015), which would be invaluable microbiological resources for developing biofertilizer. The current study was, therefore, conducted with the aim to screen $N_2O$-reducing denitrifier strains, showing both $N_2O$ mitigation and PGP effects in soil conditions. Our hypotheses were as follows: (1) inoculation with $N_2O$-reducing denitrifier strains from *Azospirillum* and *Herbaspirillum* genera decreases $N_2O$ flux and emission from N-fertilized soil and (2) inoculation with these $N_2O$-reducing denitrifier strains promotes leguminous plant and non-leguminous plant growth. To test these hypotheses, $N_2O$ and plant samples were collected from greenhouse pot experiments and were analyzed for $N_2O$ flux and emissions, plant growth parameters, and plant nutrients.

## 2. Materials and methods

### 2.1. Candidate strains

In total, 40 candidate strains, previously isolated from three different paddy soils, were denitrifier strains affiliated with *Azospirillum* and *Herbaspirillum* (Table S1). To evaluate the potential to promote plant growth, the production of indole-3-acetic acid (IAA) by each strain was determined as described by Glickmann and Dessaux (1995). In brief, each strain was pre-cultured in R2A liquid culture medium, and 0.5 mL pre-cultured medium was inoculated to 4.5 mL fresh R2A medium with the addition of L-tryptophan (0.1 g $L^{-1}$). After cultivation at 27 °C with shaking at 120 rpm for 48 h, the strain suspension was centrifuged at 7700 rpm for 10 min to collect supernatant; 0.5 mL supernatant was then mixed with 0.5 mL Salkowski reagent and 0.5 mL distilled water and incubated at 37 °C for 1 h. The IAA concentration was measured spectrophotometrically at 530 nm.

To confirm the PGP effect, the candidate strains with IAA production >5 μg $mL^{-1}$ were inoculated to seeds of red clover (*Trifolium pratense* L. var. Medium) and timothy (*Phleum pratense* L. var. Horizon) on agar plates, and the root length and fresh weight were measured. In brief, the seeds were soaked in 1% sodium hypochlorite solution for 15 min, and then washed with sterile distilled water. The surface-sterilized seeds were kept in 5 mL of 0.5 mM $CaCl_2$ solution at 25 °C in dark until further use. Each strain was anaerobically cultivated in R2A liquid culture medium using an Anaero Pack (Mitsubishi Gas Chemical Co., Inc, Tokyo, Japan), centrifuged and re-suspended in 0.5 mM $CaCl_2$ solution to achieve OD660 = 0.8. The surface-sterilized seeds were soaked in a 300-fold diluted strain suspension for 1 min, and 10 inoculated seeds were placed on each 1/10 Murashige and Skoog (MS) medium (0.75% agar, Wako-Junyaku, Osaka, Japan). The seeds were cultivated at 25 °C for 7 days under a 16 h photoperiod with a photosynthetically active radiation of 120 μmol $m^{-2}$ $s^{-1}$, and then subjected to measurements of root length and fresh weight.

The candidate strains capable of promoting plant growth on agar plates and mitigating $N_2O$ emissions in soil microcosms (unpublished) were inoculated into two different soils for cultivating red clover and timothy in a greenhouse (Table 1). To clarify denitrification end products, each strain was inoculated into 5 mL nutrient broth culture medium in a 10-mL glass serum vial, where $^{15}N$-labeled $NaNO_3$ (0.3 mM) and sodium succinate (4 mM) were added. The headspace air was replaced with helium gas three times before incubation. After incubation at 30 °C for 2 weeks, the concentrations of $^{46}N_2O$ and $^{30}N_2$ were measured with a gas chromatography-mass spectrometer (GC–MS, QP2010, Shimadzu, Kyoto, Japan). The proportion of $^{30}N_2$ in the end products of denitrification ($^{46}N_2O + {}^{30}N_2$) was calculated for each strain. Polymerase chain reaction was performed to detect *nosZ* genes of each strain using primers nosZ-F-1181 and nosZ-R-1880 (Rich et al., 2003). PCR amplification was performed using a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) in 50 μl reaction volumes, containing 1 × Ex Taq buffer ($Mg^{2+}$ plus), 0.4 μM of each primer, 0.2 mM of each dNTP, 1.25 U Ex*Taq* DNA polymerase hot start (TaKaRa, Kusatsu, Shiga, Japan), and approximately 25 ng genomic DNA. The thermal profiles were as follows: 98 °C for 10 s, followed by 29 cycles of 98 °C for 10 s, 55 °C for 30 s, 72 °C for 45 s, 72 °C for 7 min, and 4 °C for 10 min.

### 2.2. Greenhouse pot experiments

The greenhouse pot experiments were carried out in the Graduate School of Agricultural and Life Sciences, the University of Tokyo, Tokyo. The growth media were two different pasture soils collected in the spring and summer of 2014 from pastures located in Kamihoromui, Iwamizawa City, Hokkaido (43°10′22″, 141°42′52″). Soil for the cultivation of red clover was collected in the spring, and soil for that of timothy was collected in the summer. These two soils were classified as Eutric Fluvisol, and their physicochemical properties were analyzed by Tokachi Nokyoren (Ogawa, 1997; Hokkaido Central Agricultural Experiment Station, 2012) as shown in Table 2. The soil was air-dried, homogenized through a 2-mm sieve, and stored at room temperature until use. Compound fertilizer (A-COOP 055, $NH_4^+$-N 10%, citric acid-soluble P 25%, soluble K 15%, and citric acid-soluble Mg 5%) was applied to the soil at 1.2 g $kg^{-1}$ air-dry soil. The soil corresponding to each treatment was mixed thoroughly, and placed in plastic pots (15 cm top diameter × 10 cm depth) at 1.0 kg air-dry soil per pot.

Seeds of red clover and timothy were purchased from Snow Brand Seed Co., Ltd., Sapporo, Hokkaido. The seeds were soaked in sterile tap water for 10 min, then washed in 2% sodium hypochlorite solution for 5 min, and followed by 10 serial rinses in sterile tap water. The seeds were broadcast onto the soil surface, and then 30 mL of strain suspension at OD660 = 1.0 ($10^7$–$10^8$ CFU $mL^{-1}$) were inoculated into the soil. To achieve strain suspension at