Both sand particles and organic substances enhance activity of free-living, nitrogen-fixing bacteria inhabiting soil of permafrost terrain in East Siberia

Yasuyuki Hashidoko*, Tomoya Ohchi, Ryusuke Hatano, and Satoshi Tahara

Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

Corresponding author: yasu-h@abs.agr.hokudai.ac.jp

1. INTRODUCTION

Although boreal forests produce large biomass, available nitrogen in the soil is poor, because of low soil temperature. Thus the mineralizing activities of the soil microorganisms are also very low. This nitrogen imbalance in boreal forest ecosystem has long time been a mystery for soil scientists and forest ecologists (Binkley et al. 2000). In 2002, a research group of Swedish University of Agricultural Science had reported that cyanobacteria, symbiotically attaching on the surface of a ground-covering feather moss, play an important role in nitrogen supply to the boreal forest in Sweden (Deluca et al. 2002). In Siberian boreal forest, Taiga, throughout East Siberian plain on the permafrost area, the nitrogen balance suggests the presence of unknown nitrogen source of the forest ecosystem. The East Siberian forest is heathland forest, mainly composed of Siberian larch (*Larix gmelinii*) and heath (*Vaccinium vitis-idaea*) as a predominant tree species and ground-covering vegetation, respectively. So the forest bed is poor in moss carpets, unlike the Swedish boreal forest. This contradictory fact suggested the presence of other major nitrogen-provider in the heathery boreal forest ecosystem.

When a particular oligotrophic bacterium colonizes on the rhizoplane of a plant inhabiting permafrost ecosystem, certain bacterial community often starts to activate metabolisms of the roots, and also nutrient circulation in the rhizosphere. We named it as rhizo-biocomplex (Hashidoko & Tahara 2005) that can supply deficient nutrients in boreal forest soil in Yakutsk. The rhizo-biocomplex effectively accelerates nitrogen-fixation. Although importance of bio-fertilization for the defrosted sandy soil had long been discussed, potent capabilities of the biocomplex have long been ignored. In this respect, it is necessary to do particular study for rhizo-biocomplex that maintains the large biomass production of the heathery larch forest in Yakutsk. In fact, pure inoculants often show only trace acetylene reduction activity (ARA). N-fixers show low activities in single culture but their mixed culture to form bacterial consortium could express relatively high N-fixing abilities. So, to investigate such possibilities, we first sampled defrosted sandy soil from several depth at a sampling site at Viluy in mid-July, 2003 and 2004, respectively. Based on our speculation that main nitrogen-provider in the heathery boreal forest is free-living, nitrogen-fixing soil bacteria, we did some preliminary studies.

2. MATERIALS AND METHODS

2.1 Acetylene reduction assay

For acetylene reduction assay, we initially used soil washings of 2 mg soil, and incubated it in each screw cap septum vial (Pierce, USA) for 7 days. Medium used was 10 ml of N-free azotobacter medium (Winogradsky's mineral mixture, pH 6.2 or 7.4) solidified with 0.3%

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gellan gum. Then 10% acetylene gas was injected into 20 ml of gas phase, and further incubated for another 1 week at 23℃. Produced ethylene gas was analyzed by gas chromatography (G-5000, Hitachi, Japan) equipped with a glass-capillary column, CP-PoraPLOT U 10 m (0.32 mm i.d. Chrompack, The Netherlands), under the analytical conditions described as follows: injection at 150°C, detection at 250°C, FID for detection, head gas pressure at 50 kPa, column temperature at 50°C. Representative peak retention time for ethylene is 0.90 min and 1.30 min for acetylene. Using absolute standard curve (peak intensity (y)/nl for C_2H_4 (x): for equation of y=62 x), the total volume of the produced gas in the screw cap septum vial was calculated.

2.2 Inoculation of soil and/or soil microfloral composers to N-free soft gel medium

We first tested soil washings and soil suspension as the inoculants. Soil washings corresponding to 2 mg of soil used as the inoculants were inactive, although bacteria actively emerged in the medium. In contrast, when sandy soil was directly inoculated, it showed a clear acetylene reduction activity (ARA). As positive control, we used *Beijerinkia indica* subsp. *indica* IFO3744 (Hashidoko et al. 2002).

2.3 Sub-culturing assay

Subculture test from the active (300 mg of V30 soil-inoculated) or inactive (100 mg of V30 soil-inoculated), in which cultured media were used as active one. From both inactive 100 mg soil-inoculated and active 300 mg soil-inoculated media, 400 μl of the gel were respectively re-inoculated to each of new soft gel medium.

2.4 Sand particles-adding assay

We investigated the effect of soluble materials from the soil, and also plain sand particles using 300 mg of Sea Sand C (Nacalai, Kyoto, Japan) of 40-80 mesh in particle size. The process of the medium preparation is illustrated here. The inoculant used was 100 mg soil of V10 and V30, respectively. The assay was triplicated for supernatant and 0.3 g of Sea Sand C. After 7-days cultivation, sand particles were observed under a light microscope (×400, Olympus, Tokyo, Japan).

2.5 Investigation of soil microflora

Screening of culturable soil microflora from V30 soil was done using modified Winogradsky's medium (with 0.005% yeast extract). Among 21 single colonies obtained, we tentatively identified 5 bacterial species, 3 *Burkholderia* species, and 2 gram-positive rods by means of 16S rRNA gene sequencing and successive homology search. All of them were oligotrophic bacteria that cannot grow in potato-dextrose or nutrient broth medium.

2.6 Nitrogen fixing abilities of the bacteria from soil

Using degenerated primers GEM (GACCTGCAGADNGCCATCATYTCNCC) and KAD (ATAGGATCCTGYGAYCCNAARGCNGA) for *nifH* gene detection (Ohkuma et al. 1996), the target gene-like, amplified DNA fragments of 300 bp were searched. The PCR conditions were as follows: 95°C for 15 min for pre-heating, and then 30 cycles of 94°C for 30 sec, 48°C for 30 sec, and 72°C for 2 min, and then 72°C for 10 min to the end of reaction.

2.7 ARA under the presence of plant secondary metabolites

Plant tissues were collected in Yakutsk in summer, 2004, and they were extracted with 70% EtOH to concentrate *in vacuo*. Initially, we prepared 20,000 mg/l sample solution dissolved in dimethylsulfoxide (DMSO), and 5 μl of the solution was dissolved in 10 ml of the N-free, soft gel medium in a screw cap septum vial to make a final concentration of 100 ppm. The medium

containing 5 µl of DMSO only was set up as the control experiment.

3. RESULTS AND DISCUSSION

3.1 Investigation of physical factors that activate N-fixation

We speculated three possible answers for the mystery of nitrogen source: 1) organic and/or inorganic substances in soil affect N-fixation, 2) microfloral compositions in the medium influence N-fixation, and 3) cell population density of the inoculants affect N-fixation. During preliminarily acetylene reduction assay for soil samples from several depths, particularly soils from 10 cm and 30 cm in Viluy, V10 and V30, respectively, characteristically showed clear ARA when 300 mg of the soil particles were inoculated. In contrast, ARA of other soil samples were rarely influenced by amounts of the inoculated soil particles, unlike V10 and V30. Thus, soil microorganisms from different depth show alternative responses of ARA to sand particles. Although it became clear that a necessary amount of sand particles are important in soil microfloral composers in V10 and V30 for expressing higher ARA, how sand particles stimulate ARA of the microfloral composers are not yet understood (Fig. 1).

Under light microscopic observation, it was visible that sand particles allowed bacterial colonization on the surface. Hence, we concluded that sand particles as matrix for habitat is probably necessary for active nitrogen fixation of the bacterial communities of the soil. Additionally, subculture test from active and inactive cultures of V30 soil to new 300 mg soil particle-containing medium revealed that only the sub-cultured medium from the 300 mg-soil-inoculated pre-culture (ARA-positive) showed relatively lower but stable ARA, while those from 100 mg-soil-inoculated pre-culture (ARA-negative) showed none of ARA. We therefore suggest that microfloral composition that is associated with colonization on sand particles determines capability of N-fixation in the soil microfloral community.

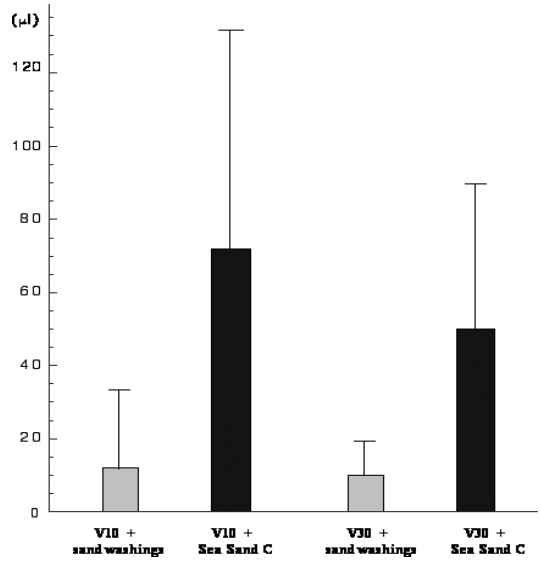

Fig. 1. Effect of sand particles on ARA of heath forest soils in Yakutsk. When commercially available Sea Sand C (1 g) was added to the 100 mg of forest soil, the ARA increased significantly (black columns), while autoclaved soil-washings from 300 mg of the forest soil did not show any positive effect on ARA (grey column). Bars indicate standard deviation. (n=3).

3.2 Isolation of bacteria from defrosted soil as soil microfloral composers

To verify these hypotheses above, we obtained 21 single colonies from V30 soil, and according to 16S rRNA gene sequencing and successive homology search, we tentatively identified five bacterial species, three *Burkholderia* species and two gram-positive rods. All of them were oligotrophic bacteria that cannot grow in potato-dextrose or nutrient broth medium. One of the most dominant bacteria was tentatively identified as *Burkholderia phytofirmans* (no.

2, 3, 4, 7, 8, 9, 10, 12, 13), while others were *Bacillus macroides* (no. 1, 5, 6, 14), *Burkholderia glathei* (16, 17, 18), *Burkholderia sordicola* (no. 15, 19, 20, 21), and *Paenibacillus borealis* (no. 11).

Using degenerated primers GEM and KAD for *nifH* gene detection (Ohkuma et al. 1996), the target gene-like, amplified DNA fragments of 300 bp were obtained from strains no. 2~4, 7~10, 12, 13 and 15~17. In particular, the PCR product clearly appeared in no. 2, identified as *B. phytofirmans*, the predominant bacterium in the soil at this depth (Fig. 2). From the PCR assay and growth performance in nitrogen-free medium, *B. phytofirmans* is likely to be a major N-fixer in the permafrost forest ecosystem. The representative isolates (no. 1, 2, 15, 16) were measured for their nitrogen-fixing abilities in a mono-culture system of each isolate. Isolate no. 16, identified as *B. glathei*, showed a low but significant ARA in 1 g of sand particles-containing medium after 7 day incubation, but the others were all inactive under the same conditions. This result in the mono-culture system suggested that certain microbial consortium and not single species but expressed the nitrogen-fixing activity.

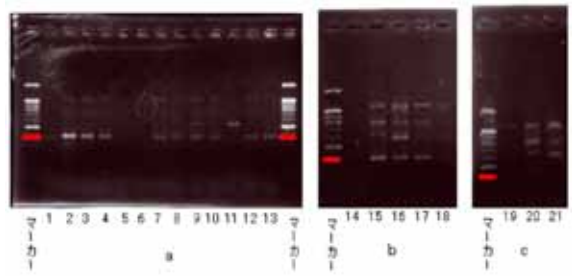

Fig. 2. PCR detection for *nifH* gene from isolated bacteria. Using degenerated primers GEM and KAD for *nifH* gene, the target gene-like, amplified DNA fragments of 300 bp were obtained from strains no. 2~4, 7~10, 12, 13 and 15~17.

3.3 Investigation of chemical factors that activate N-fixation

We also preliminarily investigated a role of heathery plants and other vegetation in nitrogen supply. We observed a ball-like aggregate on the root of *Vaccinium vitis-idaea* which develops its root mat on the topsoil. Some chemical substances including secondary metabolites could

Table 1. Effect of plant extracts on N-fixing activities of microfloral communities from larch forest soils in Yakutsk.

source / soil	Lichen (whole tissue)	Heath (leaves)	Heath (root mat)	Larch (roots)	Control (DMSO)
S30	−	−	++	−	++
V20	++	+++	++++	++	++
V30	−	+	−	−	++

At 100 ppm concentration and incubation for 7 days at 23°C, effects of chemical constituents from plants on N-fixing soil bacteria were evaluated by ARA. ++++: over 40 µl of ethylene production from acetylene gas. +++: over 10 µl of the ethylene production, along with a burst over 40 µl in one sample in triplication. ++: over 10 µl of the ethylene production. +: over background level (5µl). −: no or trace detection of ethylene.

therefore leach into lower, unfrozen sandy soil layers during spring-summer seasons. It is, hence, speculated that *Vaccinium vitis-idaea* widely and densely covering larch forest bed in East Siberia contributes to nitrogen-fixation via chemical components. We tested three plant extracts, larch (root), lichen (whole tissues) and heath (leaves and root mat), including alcoholic extracts from heath root mat.

Unlike the samples V30 and V10 activated by adding sand particles, V20, which did not show any activation by adding sand particles, indicated clear ARA. In particular, extract from heath root mat highly activated nitrogen-fixation of V20 and S30 (30 cm from Spasskaya) soil microflora. In this experiment, the control containing 0.2% DMSO only, also showed significant acetylene reduction activities, and it is expected that the higher ARA of the control is due to sulfur in DMSO. Soil microfloral communities in V20 are unique among all of the soil samples from several depths, because only this sample is highly sensitive to chemicals of heath root mat (Table 1).

V20, hence, may possess one of the most significant microfloral communities in the Siberian Permafrost ecosystem. When exposed to the crude extract of heath root mat, the activation of the nitrogen-fixation in V20-inoculated vials was, in fact, 3-5 fold higher than DMSO only. In our preliminary experiments, silica gel powders do not affect N-fixing activity of the soil bacterial communities, so it is possible to assay directly chemical constituents chromatographed on a silica gel plate in search for unknown chemical principle(s) that activates N-fixation of soil bacteria in V20. If major N-fixers in V20 and ARA activator(s) from heath root were successfully identified, exact evaluation of their nitrogen-fixation activity under exposure of the active substances can be measured again.

4. CONCLUSION

Using the acetylene reduction assay in gellan gum medium, it was found that sand particles are necessary for active N-fixation. The sand particles are probably 3-dimensional matrix for stable colonization of soil microfloral communities. Moreover, mono-culture assay indicated that not single species but the bacterial consortium actively expresses nitrogen-fixing ability in permafrost soil in East Siberia. To understand stability of the heathery larch forest ecosystem on permafrost soil, effect of plant secondary metabolites on N-fixation by soil microfloral communities was tested, and heath root extract was found to be one of the most powerful candidates of N-fixation activator in our test samples. Several experiments to reveal genuine nitrogen source in boreal forest established in permafrost terrain is still on going. Our approach may lead to development of new strategy for conserving the permafrost forest ecosystem.

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