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Nitrogen fixation in *Rhodopseudomonas palustris* co-cultured with *Bacillus subtilis* in the presence of air

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Nitrogen fixation in purple non-sulfur bacteria (PNSB) does not take place even in N-free medium when they are cultured under aerobic conditions. It is assumed that PNSB might possess inadequate capability to protect their cellular components from exposure to air (20.95 vol.% oxygen). In this study, therefore, *Bacillus subtilis* was inoculated together with a purple non-sulfur bacterium *Rhodopseudomonas palustris* in N-free medium in order to examine whether nitrogen fixation in *Rps. palustris* takes place when the co-culture is exposed to 20.95 vol.% oxygen. *Rps. palustris* grew and formed biofilm only when it was inoculated together with *B. subtilis*. When the biofilm formed in the co-culture was inoculated in N-free medium, diazotrophic growth was observed in the sequential subcultures. Expression of *nifH* gene, derepression of nitrogenase activity, an increase of total nitrogen, and a decrease of C/N in the co-culture of *Rps. palustris* and *B. subtilis* demonstrated the occurrence of nitrogen fixation under aerobic conditions. The diazotrophic growth was suppressed at a lower medium-to-air ratio in a sealed culture vessel, and growth of *B. subtilis* preceded growth of *Rps. palustris* in the co-culture. These results suggest that growth of *B. subtilis*, which is usually accompanied with oxygen consumption, might cause a decrease of dissolve oxygen concentration in medium and contribute to the occurrence of nitrogenase activity in *Rps. palustris*.

[**Key words:** Co-culture; Photosynthetic bacterium; Nitrogen fixation; *Bacillus subtilis*; *Rhodopseudomonas palustris*; Nitrogenase]

Ammonia is industrially produced from nitrogen and hydrogen gases with the Haber–Bosch process. It is conducted at typical temperatures of 500°C and pressures around 200 bar, resulting in ammonia concentrations in the exhaust gas of up to 18 vol.% (1). This success led to the large-scale production of chemical nitrogen fertilizers with consumption of large amount of fossil fuel. On the other hand, biological nitrogen fixation, which does not require such high temperature and pressure, produces ammonia in nature. It is estimated that the amount of ammonia produced through biological nitrogen fixation is equivalent to that produced through the chemical nitrogen fixation (2). However, nitrogenase that catalyzes biological nitrogen fixation is highly sensitive to oxygen (3). Because of this feature in nitrogenase, application of biological nitrogen fixation to agricultural production is limited to bacterial species possessing protection mechanisms of nitrogenase from exposure to oxygen. Therefore, approaches to develop methods enabling oxygen-sensitive diazotrophs to produce ammonia in crop fields must be effective in increasing an opportunity that bacterial nitrogen fertilizers are chosen instead of chemical nitrogen fertilizers.

The vast majority of purple non-sulfur bacteria (PNSB) are also diazotrophs, and their nitrogenases are highly sensitive to oxygen (4). Nitrogen fixation in PNSB is dependent on the anoxygenic photosynthesis, which is due to the absence of oxygen evolution from the photosystem II (5). PNSB are versatile in terms of growth conditions, which include photoautotrophic, photoheterotrophic, and chemolithoautotrophic modes (6). Therefore, during the diazotrophic growth under photoautotrophic or photoheterotrophic conditions, their nitrogenases are not subjected to the inhibition by endogenous oxygen from the photosystem II. On the other hand, it is known that nitrogenases in filamentous cyanobacteria are protected from endogenous oxygen evolved during photosynthesis in natural habitats (7). It has been suggested in a purple non-sulfur bacterium *Rhodobacter capsulatus* that FeSII protein might be responsible for protection of its molybdenum nitrogenase from oxygen (8). However, the wild type strain of *Rba. capsulatus* was not able to grow diazotrophically under conditions where oxygen above 15 vol.% was sealed in nitrogen in a culture vessel. Considering that *Azotobacter vinelandii* can grow diazotrophically in the presence of air and has been applied as a free-living N_2 fixer from nitrogen in air (9), the ability to protect nitrogenase from oxygen in *Rba. capsulatus* is not sufficient when PNSB are considered as biocatalysts to supply nitrogen sources to plants.

In this study, diazotrophic growth of a purple non-sulfur bacterium *Rhodopseudomonas palustris* under aerobic conditions was examined in the presence of nitrogen as a sole nitrogen source. In order to explore the possibility that an aerobic heterotroph enables diazotrophic growth of *Rps. palustris* under aerobic conditions, an aerobic bacterium *Bacillus subtilis* was co-cultured with *Rps. palustris*. *B. subtilis* has been used as one of bacterial sensors to measure the biochemical oxygen demand because of its high sensitivity

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towards oxygen and superior oxygen consumption ability (10,11). In addition to these features, it has been reported that a *B. subtilis* strain has beneficial effects as a bacteriological fertilizer on plant and root growth and plant defense against phytopathogens (12). Therefore, effects of co-culture with *B. subtilis* on diazotrophic growth and nitrogen fixation of *Rps. palustris* under aerobic conditions were examined.

MATERIALS AND METHODS

Bacterial strains and culture conditions *Rps. palustris* CGA009 (13) was cultivated at 32°C under photo-heterotrophic conditions using incandescent lump as a light source. *Rps. palustris* was pre-cultivated in modified Okamoto medium (MOM) (14), which contains 0.27 g NH_4Cl, 1.0 g disodium succinate, 1.0 g sodium DL-malate, 1.0 g sodium pyruvate, 1.0 g sodium acetate, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.2 g $CaCl_2 \cdot 2H_2O$, 0.2 g NaCl, 0.02 g $FeSO_4 \cdot 7H_2O$, 41 mg KH_2PO_4, 495 mg K_2HPO_4, 1 ml trace-metal mixture A_5', 500 μg vitamin B1 hydrochloride, 500 μg nicotinic acid, 300 μg *p*-aminobenzoic acid, and 50 μg D-Biotin in 1000 ml deionized water. Trace-metal mixture A_5' contains 2.86 g H_3BO_4, 1.81 g $MnCl_2 \cdot 4H_2O$, 0.22 g $ZnSO_4 \cdot 7H_2O$, 0.08 g $CuSO_4 \cdot 5H_2O$, 0.021 g Na_2MoO_4, 0.01 g $CoCl_2 \cdot 6H_2O$, and 50 g ethylenediaminetetraacetic acid (EDTA) disodium salt in 1000 ml deionized water. The pre-culture was inoculated in MOM not containing NH_4Cl (N-free medium), and diazotrophic cultivation was performed under aerobic conditions in 96-well transparent microplate sealed with parafilm (Bemis Flexible Packaging, Neenah, WI, USA) or 7 ml screw-capped glass test tube with silicone septum. *Rps. palustris* was anaerobically cultured by incubating 96-well transparent microplates in an Anaeropack (Mitsubishi Gas Chemical, Tokyo, Japan). *B. subtilis* ATCC6633 pre-cultured in LB medium was inoculated with or without *Rps. palustris* to N-free medium in the transparent microplate or screw-capped glass test tube. Growth in microplate cultures was evaluated with OD_{600} using a microplate reader (SH-1000, Hitachi, Tokyo, Japan). For microscopic observation using an upright microscope (BX53, Olympus, Tokyo, Japan), *Rps. palustris* and *B. subtilis* were stained with malachite green and safranin solutions for bacterial staining (Kanto chemical, Tokyo, Japan), respectively. In case of larger scale cultivation, culture broth in the screw-capped glass test tube was inoculated to 100 mL N-added or N-free medium in 200 mL rectangular glass bottle. After inoculation, the bottle was sealed with a rubber cap with double seal stopper.

For cultures with different medium-to-air ratios, 6-d co-culture of *Rps. palustris* and *B. subtilis* was inoculated by diluting 6-times with 2 ml, 4 ml, or 6 ml N-free medium in 7 ml screw-capped glass test tube with silicone septum. Cells were collected immediately after inoculation and after 6-d cultivation. For growth monitoring during the co-culture, *Rps. palustris* and *B. subtilis* were inoculated to 4 ml N-free medium in 7-ml screw-capped glass test tubes, and cultured under the conditions as described above. Sampling was performed by scraping the biofilm formed during cultivation from the glass surface and centrifuging the total volumes of cell suspension at 10,000 ×*g* for 10 min. After removing supernatant, cell precipitates were stored at −25°C for DNA extraction.

Preparation of standard curves of cell number versus DNA concentration Cell suspensions with different cell density were prepared using exponentially growing cultures of *Rps. palustris* and *B. subtilis* by diluting those with fresh medium or concentrating those with centrifugation at 10,000 ×*g* for 10 min. Then, 2 ml each of the cell suspension was centrifuged, and precipitated cells were stored at −25°C for DNA extraction. Cell number of the suspensions was counted with microscopic observation in a Thoma's hemacytometer. Standard curves showing cell number in *Rps. palustris* suspension versus concentration of *nifH*, which encodes dinitrogenase reductase, and cell number in *B. subtilis* suspension versus concentration of *ywsC*, which encodes γ-polyglutamic acid synthetase (16), were prepared.

Analysis of *nifH* gene expression After 5-d cultivation with 5 ml medium in 15 ml screw-capped glass test tube with silicone septum, 0.5 mL culture was sampled and mixed with 1.0 ml RNAprotect bacteria reagent (Qiagen KK, Tokyo, Japan). Then, cells were collected by centrifugation at 4°C for 10 min and immediately stored at −80°C. For RNA extraction, the frozen cells were suspended in 100 μL TE buffer (10 mM Tris−HCl, 1.0 mM EDTA disodium salt, pH 8.0) containing 1.0 mg/mL lysozyme. The cell suspension was incubated at room temperature for 5 min. Then, 350 μL buffer RLT (Qiagen KK) containing 2-mercaptoethanol was added to the cell suspension, followed by the standard protocol of RNeasy Mini Kit (Qiagen KK). Expression of *nifH* was quantified by the real-time reverse transcription PCR (RT-PCR) assay using ReverTra Ace qPCR RT kit and KOD SYBR qPCR Mix (both from Toyobo, Osaka, Japan). cDNA corresponding to *nifH* gene was quantified using the *nifH* primer set (5′-TATGGGAAAGGCGGGATCGG-3′ and 5′-TCGAGTCCGCCTTGGGATCG-3′) (15). Expression intensity was normalized by a molar ratio to cDNA corresponding to 16S rRNA gene using the primer set (5′-CCTACGGGAGGCAGCAG-3′ and 5′-GTATTACCGCGGCTGCTG-3′) (15). PCR was performed with the following thermal cycler program: 1 cycle of denaturation at 98°C for 2 min, followed by 40 cycles of denaturation at 98°C for 10 s, annealing at 60°C for 10 s, and elongation at 68°C for 30 s. Threshold PCR cycle number (Ct) was determined with ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA), and cDNA concentration was determined using an equation of $1/(2^{Ct})$.

Measurement of nitrogenase activity After 6-d cultivation in the sealed rectangular glass bottle, 5 ml acetylene was injected with a syringe through a rubber cap. After culturing for 2 h at 30°C with light irradiation, ethylene produced was detected with a gas chromatograph (GC-4000, GL Sciences, Tokyo, Japan) equipped with a flame ionization detector and a capillary column (Rt-alumina bond/KCl, 0.32 mm inner diameter × 30 m length, Restek, Bellefonte, PA, USA). Nitrogen was used as a carrier gas at a flow rate of 3 ml/min, and oven, injector, and detector temperatures were set at 100°C, 200°C, and 200°C. Ethylene concentration was calculated from a standard curve prepared using ethylene gas (99.5%) (GL Sciences).

Measurement of nitrogen and carbon contents Culture broths containing cells and medium before and after 7-d culture in the sealed rectangular glass bottle were frozen at −25°C and lyophilized to remove water. The biofilm formed during the co-culture was scraped off from the glass surface into the culture broth. Total nitrogen concentration in the co-culture was quantified with the Kjeldahl method using a micro Kjeldahl distillation apparatus (Sibata Scientific Technology, Saitama, Japan). Carbon and nitrogen contents in the lyophilizate were measured using an NC analyzer (Sumigraph NC-220F, Sumika Chemical Analysis Service, Osaka, Japan).

Quantification of bacterial DNA and cell density by real-time quantitative PCR DNA in the precipitated cells was extracted using DNeasy blood and tissue kit (Qiagen KK) according to the manufacturer's instructions. Genes encoding 16S rRNA were amplified using the quantitative real-time PCR using KOD SYBR qPCR Mix (Toyobo) and primer sets of 5′-CTGGAAGTCTTGAGTATGGC-3′ and 5′-GCCTCAGCGTCAGTAATGGC-3′ for *Rps. palustris* and 5′-GAACAAAGG GCAGCGAAACC-3′ and 5′-GGGTGTTACAAACTCTCG-3′ for *B. subtilis*. These primer sets designed in this study could amplify 16S rRNA gene of each bacterium exclusively.

nifH in *Rps. palustris* and *ywsC* in *B. subtilis* were amplified using the *nifH* primer set as shown above (15) and a *ywsC* primer set comprising of 5′-GCAAAC-GACGCTTCTAC-3′ and 5′-GGCTTTTACGATCGGTTCTG-3'. Ct was determined with ABI 7500 real-time PCR system (Applied Biosystems), and DNA concentration (arbitrary unit per ml culture) was determined using an equation of $1/(2^{Ct})$. Concentrations of *nifH* and *ywsC* in DNA extract were converted to cell numbers of *Rps. palustris* and *B. subtilis* in the co-culture using the standard curves, respectively.

RESULTS AND DISCUSSION

Diazotrophic growth of *Rps. palustris* under aerobic conditions Diazotrophic growth of *Rps. palustris* was evaluated in N-free medium under aerobic and anaerobic conditions. *Rps. palustris* could grow diazotrophically under anaerobic conditions, whereas it did not grow in N-free medium under aerobic conditions (Fig. 1A). The result implies that *Rps. palustris* could not utilize nitrogen as a substrate for nitrogen fixation under aerobic conditions. Therefore, an aerobic bacterium *B. subtilis* was considered as a biocatalyst to reduce dissolved oxygen concentration in a culture vessel.

Growth of *B. subtilis* was severely restricted during the axenic culture from 3 days after inoculation but OD_{600} increased more significantly than the increase of N-free medium without inoculation of bacterial cells (Fig. 1B). The result indicates that *B. subtilis* could not grow diazotrophically but grew with consumption of oxygen depending on the amount of N source derived from pre-culture. The lack of diazotrophic growth is also supported by the report that *nifH* gene was not detected in the type strain of *B. subtilis* (17).

On the other hand, when both *Rps. palustris* and *B. subtilis* were inoculated in N-free medium, increases of OD_{600} were observed in all the cultures tested (Fig. 2). When the co-culture on 5 days after inoculation, the first subculture on 7 days after inoculation, and the second subculture on 6 days after inoculation were re-inoculated to N-free medium, increases of OD_{600} were repeatedly observed though the increases within 1 day after inoculation were not so marked as that in the first co-culture. The marked increase in the first co-culture would be caused by nitrogen compounds from the pre-culture, which were included in residual medium and bacterial

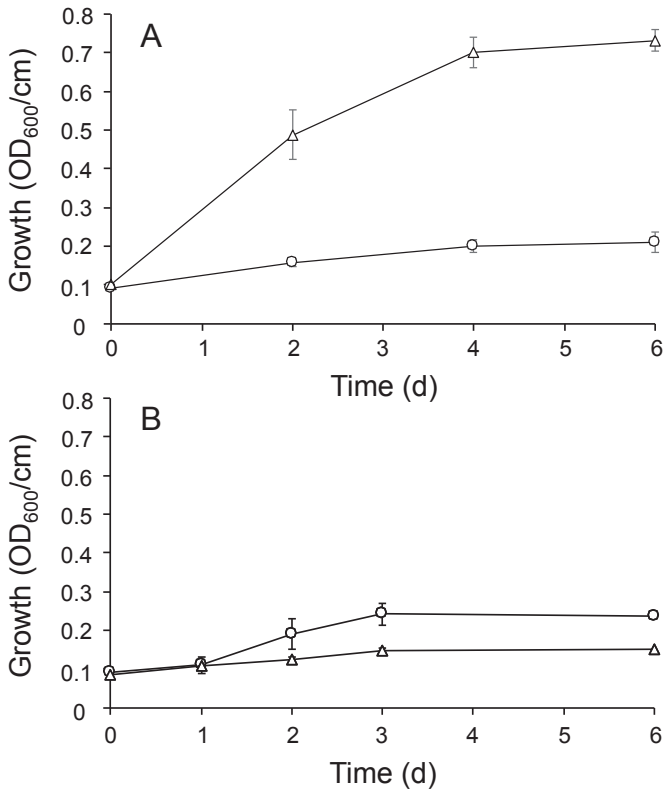

FIG. 1. Growth of *Rps. palustris* and *B. subtilis* in N-free medium. OD_{600} was monitored during cultivation of *Rps. palustris* under aerobic (circles) and anaerobic (triangles) conditions (A) and during cultivation of *B. subtilis* under aerobic conditions (circles) and during medium incubation without bacterial inoculation (triangles) (B).

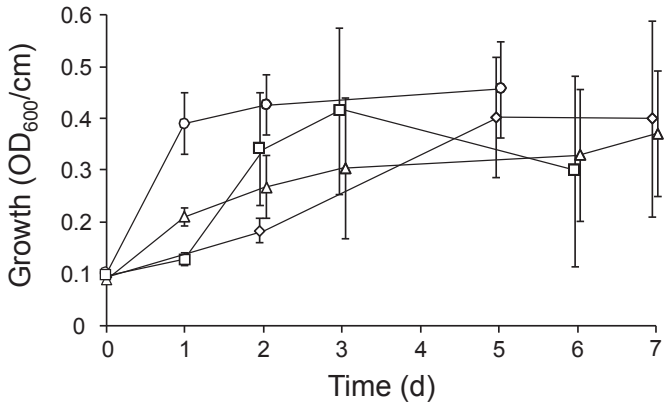

FIG. 2. Growth in the co-culture of *Rps. palustris* and *B. subtilis* in N-free medium under aerobic conditions. The primary culture (circles) was inoculated to the first subculture (triangles), the first subculture was inoculated to the second subculture (squares), and the second subculture was inoculated to the third subculture (diamonds). The growth was monitored by measuring OD_{600}.

cells for inoculation. However, the repeated increases of OD_{600} in the subcultures indicate that diazotrophic growth using nitrogen took place in the co-cultures under aerobic conditions. Because the co-cultures of *Rps. palustris* and *B. subtilis* diazotrophically grew in the first co-culture and repeated subcultures, expression of *Rps. palustris nifH* gene and total N concentration in the co-culture were measured.

The expression of *nifH* gene markedly increased in the co-culture (31.2 ± 5.1 arbitrary units) compared to that in the axenic culture of *Rps. palustris* grown with NH_4Cl (8.9 ± 1.9 arbitrary units). Total nitrogen concentration in the co-cultures was 0.474 ± 0.017 mM, while nitrogen was detected neither in a culture broth where only *Rps. palustris* was cultured in N-free medium nor in N-free medium before co-culture where both *Rps. palustris* and *B. subtilis* cells were suspended. These results suggest that the dinitrogenase reductase gene in *Rps. palustris* was derepressed in N-free medium and N_2 in the culture vessel was converted to inorganic and organic forms of nitrogen based on nitrogen fixation in *Rps. palustris* co-cultured with *B. subtilis*.

Derepression of nitrogenase activity and decrease of C/N in the co-culture Nitrogenase activity and C/N of *Rps. palustris* and *B. subtilis* cultures in N-free medium were measured in their axenic cultures and co-culture. Nitrogenase activity was detected only in the co-culture (Table 1). In addition, C/N of the lyophilizate from co-culture decreased compared to the lyophilizates of N-free media, where either *Rps. palustris* or *B. subtilis* was inoculated (Table 1). The carbon content decreased and the nitrogen content increased markedly in the co-culture, compared to those of the axenic cultures. It is likely that the decrease in C/N was probably caused by the partial conversion of organic carbon sources in medium to carbon dioxide gas and the conversion of N_2 in gas phase to inorganic and organic forms of nitrogen in the co-culture. These results demonstrate that nitrogenase activity and nitrogen fixation were maintained in the co-culture even though the co-culture was exposed to 20.95 vol.% oxygen in the culture vessel.

Population changes of *Rps. palustris* and *B. subtilis* in the co-culture Growth of *Rps. palustris* and *B. subtilis* in the co-culture was investigated by the 16S rRNA gene quantification and microscopic observation. To confirm whether the diazotrophic growth is affected by volumes of medium and air, the medium-to-air ratio in the sealed glass test tube was changed in the co-culture. When the co-culture was inoculated to 6 and 4 ml N-free medium, DNA content for *Rps. palustris* increased on 6 days after inoculation (Fig. 3). On the other hand, DNA content for *Rps. palustris* did not increased in 2 ml N-free medium. DNA content for *B. subtilis* decreased regardless of the medium-to-air ratios. In microscopic observation, cell aggregates were mainly composed of *Rps. palustris* cells stained with malachite green at the inoculation time and after 6 days (Fig. 4A,C). However, after 2 days, an increased number of *B. subtilis* vegetative cells stained with safranin were also observed (Fig. 4B). Many small and transparent cells predicted as *B. subtilis* spores were found in the cell aggregates after 6 days (Fig. 4C). The difficulty of extracting DNA from spores of *Bacillus* sp. has been demonstrated (18). These results together with the literature information indicate that the decreases of DNA content for *B. subtilis* after 6 days were due to spore formation under growth limitation and lower recovery of DNA extracted from the spores. It is likely that shallower medium depth and a larger amount of oxygen source in a sealed test tube might participate in the diazotrophic growth suppression at the lower medium-to-air ratio.

Population changes in *Rps. palustris* and *B. subtilis* cells were followed during the primary co-culture by the *nifH* and *ywsC* quantification, respectively. Though bacterial quantification

TABLE 1. Derepression of nitrogenase activity and decrease of C/N in the co-culture.

	Co-culture	*Rps. palustris*	*B. subtilis*
Nitrogenase activity (μmol C_2H_4/100 ml/h)	8.16 ± 0.33	ND	ND
Total N content (g/100 g)	1.486 ± 0.009	0.080 ± 0.003	0.108 ± 0.009
Total C content (g/100 g)	18.22 ± 0.11	21.1 ± 0.2	19.2 ± 0.3
C/N	12.26 ± 0.14	264 ± 9	180 ± 15

ND, not detected.

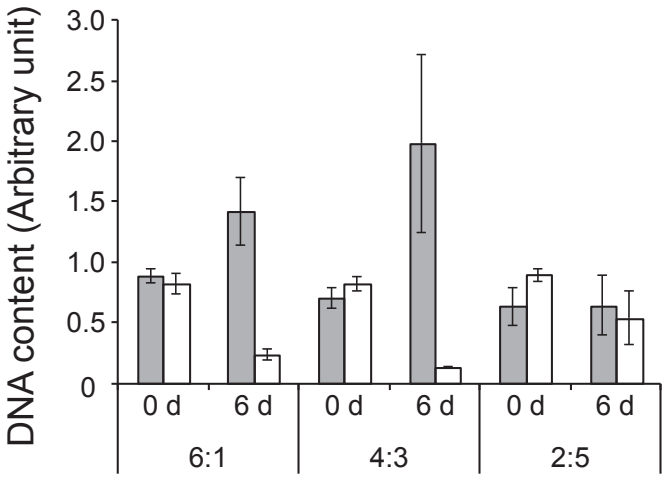

FIG. 3. Effect of medium-to-air ratio in the sealed glass test tube on diazotrophic growth of vegetative cells in the co-culture. DNA concentrations of *Rps. palustris* (shaded bars) and *B. subtilis* (open bars) were determined by the quantification of 16S rRNA gene in the co-cultures.

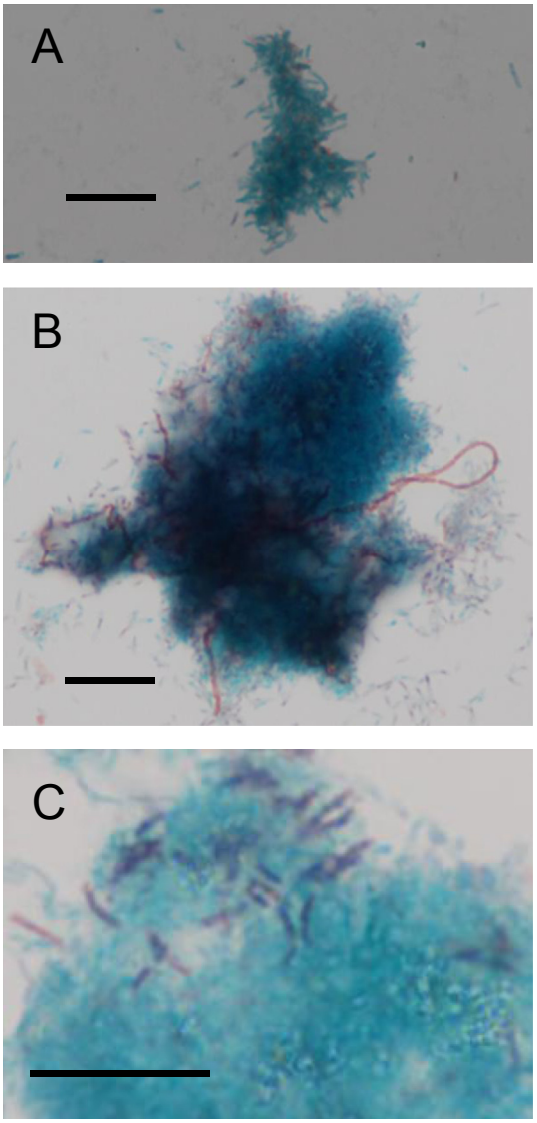

FIG. 4. Microscopic observation of biofilm formation in the second subculture of *Rps. palustris* and *B. subtilis* on day 0 (A), day 2 (B), and day 6 (C) after inoculation. Scale bars indicate 10 μm.

targeted to 16S rRNA genes is generally accepted, DNA quantification with more bacteria-specific genes was chosen, in addition to the 16S rRNA gene quantification, in order to exclude or reduce effects of bacterial contamination in PCR reagents and samples and less-specific cross-reactivity between primers and genes on quantification of targeted genes. *B. subtilis* cells markedly proliferated at the early stage of culture while, at the middle stage of culture, growth of *Rps. palustris* cells became prominent and cell density of *B. subtilis* decreased (Fig. 5). The growth of *B. subtilis* was insufficient compared to that of *Rps. palustris*. It has been reported that *B. subtilis* can grow on most of the TCA cycle intermediates (19) and sodium pyruvate (20) as sole carbon sources. Though carbon sources used in this study (disodium succinate, sodium malate, sodium pyruvate, and sodium acetate) were chosen by the preference of PNSB, disodium succinate, sodium malate, and sodium pyruvate could be utilized by *B. subtilis*. Therefore, insufficient growth of *B. subtilis* might be due to the absence of nitrogen source in medium and limitation of nitrogen flux from *Rps. palustris* to *B. subtilis*. At the late culture stage, cell density of *Rps. palustris* also decreased. These results suggest that the growth of *B. subtilis* at the early stage of co-culture plays an important role in directing *Rps. palustris* to derepress nitrogenase activity unless permeation of oxygen from gas phase into medium is efficient in a culture vessel.

A key factor that participated in the derepression of nitrogenase activity in co-culture Inorganic and organic forms of nitrogen converted from N_2 through nitrogen fixation can be mediators to link the metabolisms between different microorganisms (9,21–23). In this study, growth of both *Rps. palustris* and *B. subtilis* in N-free medium continued in the subcultures (Fig. 4C) and ammonia was not detected in the supernatants of co-cultures (data not shown), suggesting that nitrogen sources formed through nitrogen fixation might be partially supplied to *B. subtilis* within the biofilm. If nitrogen sources derived from nitrogen fixation by *Rps. palustris* might not be supplied, *B. subtilis* cells could not be observed after several sequential subcultures via repeated dilutions with fresh N-free medium. It has been reported that the co-cultures of non-nitrogen fixer that can degrade a polysaccharide and diazotrophic bacterium increase the activities of nitrogen fixation when the polysaccharide is exclusively provided as the sole carbon source (24,25). In this study, however, diazotrophic growth in the axenic culture of *Rps. palustris* was observed with the carbon sources preferred by PNSB only when it was cultured under anaerobic conditions. In addition to the availability of carbon sources by *Rps. palustris*, the restriction of oxygen permeation from gas phase to medium in the culture vessel improved its diazotrophic growth in the co-

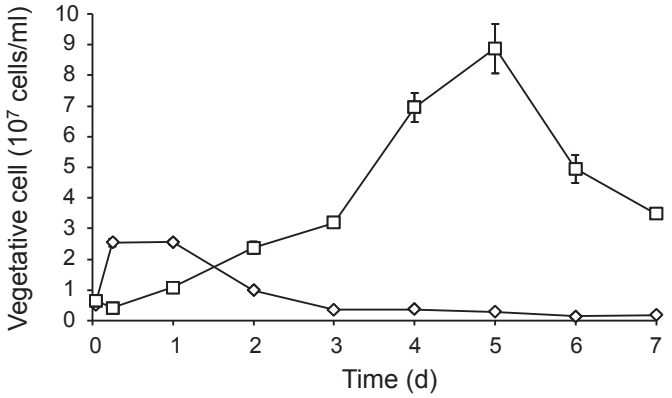

FIG. 5. Population changes of vegetative cells in the co-culture. Cell densities of *Rps. palustris* (squares) and *B. subtilis* (diamonds) were calculated from concentrations of *nifH* and *ywsC* in DNA extract, respectively.

culture. Even though dissolved oxygen concentrations located in the biofilm of co-culture could not be measured, these results indicate that the nitrogen fixation of *Rps. palustris* co-cultured with *B. subtilis* was initiated by oxygen consumption by *B. subtilis*, which is accompanied with a decrease of dissolved oxygen concentration. Therefore, it can be concluded that the N-limited growth of *B. subtilis* at the early stage of co-culture and restriction of oxygen permeation from gas phase to medium might mainly participate in the occurrence of nitrogen fixation ability in *Rps. palustris* in the presence of 20.95 vol.% oxygen. These results show possibilities of biomass production as a source of bacteriological fertilizer based on the diazotrophic growth of *Rps. palustris* through the aid of *B. subtilis* functionality.

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References

1. **Kandemir, T., Schuster, M. E., Senyshyn, A., Behrens, M., and Schlogl, R.:** The Haber-Bosch process revisited: on the real structure and stability of "ammonia iron" under working conditions, Angew. Chem. Int. Ed. Engl., **52**, 12723–12726 (2013).
2. **Vitousek, P. M., Aber, J. D., and Howarth, R. W.:** Human alteration of the global nitrogen cycle: sources and consequences, Ecol. Appl., **7**, 737–750 (1997).
3. **Oelze, J. and Klein, G.:** Control of nitrogen fixation by oxygen in purple non-sulfur bacteria, Arch. Microbiol., **165**, 219–225 (1996).
4. **Masepohl, B. and Hallenbeck, P. C.:** Nitrogen and molybdenum control of nitrogen fixation in the phototrophic bacterium *Rhodobacter capsulatus*, Adv. Exp. Med. Biol., **675**, 49–70 (2010).
5. **Jahn, A., Keuntje, B., Dorffler, M., Klipp, W., and Oelze, J.:** Optimizing photoheterotrophic H_2 production by *Rhodobacter capsulatus* upon interposon mutagenesis in the *hupL* gene, Appl. Microbiol. Biotechnol., **40**, 687–690 (1994).
6. **Nybo, S. E., Khan, N. E., Woolston, B. M., and Curtis, W. R.:** Metabolic engineering in chemolithoautotrophic hosts for the production of fuels and chemicals, Metab. Eng., **30**, 105–120 (2015).
7. **Fay, P.:** Oxygen relations of nitrogen fixation in cyanobacteria, Microbiol. Rev., **56**, 340–373 (1992).
8. **Hoffmann, M. C., Muller, A., Fehringer, M., Pfander, Y., Narberhaus, F., and Masepohl, B.:** Coordinated expression of *fdxD* and molybdenum nitrogenase genes promotes nitrogen fixation by *Rhodobacter capsulatus* in the presence of oxygen, J. Bacteriol., **196**, 633–640 (2014).
9. **Ortiz-Marquez, J. C., Do Nascimento, M., Dublan Mde, L., and Curatti, L.:** Association with an ammonium-excreting bacterium allows diazotrophic culture of oil-rich eukaryotic microalgae, Appl. Environ. Microbiol., **78**, 2345–2352 (2012).
10. **Hu, J., Li, Y., Gao, G., and Xia, S.:** A mediated BOD biosensor based on immobilized *B. subtilis* on three-dimensional porous graphene-polypyrrole composite, Sensors (Basel), **17**, 2594 (2017).
11. **Jia, J., Tang, M., Chen, X., Qi, L., and Dong, S.:** Co-immobilized microbial biosensor for BOD estimation based on sol-gel derived composite material, Biosens. Bioelectron., **18**, 1023–1029 (2003).
12. **Kilian, M., Steiner, U., Krebs, B., Junge, H., Schmiedeknecht, G., and Hain, R.:** FZB24® *Bacillus subtilis*–mode of action of a microbial agent enhancing plant vitality, Pflanzenschutz-Nachrichten Bayer, **53**, 72–93 (2000).
13. **Larimer, F. W., Chain, P., Hauser, L., Lamerdin, J., Malfatti, S., Do, L., Land, M. L., Pelletier, D. A., Beatty, J. T., Lang, A. S., and other 9 authors:** Complete genome sequence of the metabolically versatile photosynthetic bacterium *Rhodopseudomonas palustris*, Nat. Biotechnol., **22**, 55–61 (2004).
14. **Yoshida, K., Inoue, K., Takahashi, Y., Ueda, S., Isoda, K., Yagi, K., and Maeda, I.:** Novel carotenoid-based biosensor for simple visual detection of arsenite: characterization and preliminary evaluation for environmental application, Appl. Environ. Microbiol., **74**, 6730–6738 (2008).
15. **Naito, T., Sachuronggui, Ueki, M., and Maeda, I.:** Light-enhanced bioaccumulation of molybdenum by nitrogen-deprived recombinant anoxygenic photosynthetic bacterium *Rhodopseudomonas palustris*, Biosci. Biotechnol. Biochem., **80**, 407–413 (2016).
16. **Urushibata, Y., Tokuyama, S., and Tahara, Y.:** Characterization of the *Bacillus subtilis ywsC* gene, involved in gamma-polyglutamic acid production, J. Bacteriol., **184**, 337–343 (2002).
17. **Achouak, W., Normand, P., and Heulin, T.:** Comparative phylogeny of *rrs* and *nifH* genes in the *Bacillaceae*, Int. J. Syst. Bacteriol., **49 Pt 3**, 961–967 (1999).
18. **Kuske, C. R., Banton, K. L., Adorada, D. L., Stark, P. C., Hill, K. K., and Jackson, P. J.:** Small-scale DNA sample preparation method for field PCR detection of microbial cells and spores in soil, Appl. Environ. Microbiol., **64**, 2463–2472 (1998).
19. **Asai, K., Baik, S. H., Kasahara, Y., Moriya, S., and Ogasawara, N.:** Regulation of the transport system for C_4-dicarboxylic acids in *Bacillus subtilis*, Microbiology, **146 Pt 2**, 263–271 (2000).
20. **Makkar, R. S. and Cameotra, S. S.:** Biosurfactant production by microorganisms on unconventional carbon sources, J. Surfactants Deterg., **2**, 237–241 (1999).
21. **Gauthier, F., Neufeld, J. D., Driscoll, B. T., and Archibald, F. S.:** Coliform bacteria and nitrogen fixation in pulp and paper mill effluent treatment systems, Appl. Environ. Microbiol., **66**, 5155–5160 (2000).
22. **Gonzalez-Bashan, L. E., Lebsky, V. K., Hernandez, J. P., Bustillos, J. J., and Bashan, Y.:** Changes in the metabolism of the microalga *Chlorella vulgaris* when coimmobilized in alginate with the nitrogen-fixing *Phyllobacterium myrsinacearum*, Can. J. Microbiol., **46**, 653–659 (2000).
23. **Ortiz-Marquez, J. C., Do Nascimento, M., and Curatti, L.:** Metabolic engineering of ammonium release for nitrogen-fixing multispecies microbial cell-factories, Metab. Eng., **23**, 154–164 (2014).
24. **Khammas, K. M. and Kaiser, P.:** Pectin decomposition and associated nitrogen fixation by mixed cultures of *Azospirillum* and *Bacillus* species, Can. J. Microbiol., **38**, 794–797 (1992).
25. **Shrestha, A., Toyota, K., Okazaki, M., Suga, Y., Quevedo, M. A., Loreto, A. B., and Mariscal, A. A.:** Enhancement of nitrogen-fixing activity of *Enterobacteriaceae* strains isolated from sago palm (*Metroxylon sagu*) by microbial interaction with non-nitrogen fixers, Microb. Environ., **22**, 59–70 (2007).