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Bio-electrochemical conversion of atmospheric N_2 to ammonium using free-living diazotrophs

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Abstract–The effects of electrochemical reducing power on enrichment, growth, and ammonium production of free-living diazotrophs from rhizosphere soil were evaluated. Soil bacteria were cultivated in a conventional bioreactor (CBR) and an electrochemical bioreactor (EBR), both containing a neutral red-modified graphite felt (NR-GF) cathode and a platinum anode, but with electricity charged to the EBR only. Temperature gradient gel electrophoresis identified 21 species from rhizosphere soil, and 17 and seven species from the CBR and EBR, respectively, after 40 days of incubation. Six species from the CBR and five species from the EBR were diazotrophs. The bacterial community biomass and the ammonium content in the bacterial culture were, respectively, 1.6 and 2 times higher in the EBR than in the CBR. These results indicate that the electrochemical reducing power generated from the NR-GF may be a driving force in the activation of enrichment, growth, and N_2-fixing metabolism of diazotrophs.

Keywords: Bio-electrochemical Reaction, N_2 Fixation, Ammonium Production, Diazotrophs

INTRODUCTION

Most heterotrophic bacteria that inhabit natural soils depend upon nitrogen sources (nitrate or ammonium) originating from free-living or symbiotic diazotrophs, but some can metabolically fix atmospheric nitrogen under nitrogen-limited conditions [1]. The amount of free energy and reducing power required for metabolic reduction of atmospheric nitrogen to hydrazine ($N_2 \rightarrow N_2H_4$) and hydrazine to ammonia ($N_2H_4 \rightarrow 2NH_3$) equates to 16-24 ATP and 3-4 NADH molecules, significantly greater than the amounts consumed for biochemical absorption of the nitrogen sources from the environment. The primary metabolic pathway for NADH regeneration is biochemical sugar oxidation from glycolysis to the tricarboxylic acid cycle, whereas ATP regeneration is dependent on electron transport (electron-driving force) from NADH to gaseous oxygen [2]. Organic carbon sources (sugars and organic acids) are essential for free-living diazotrophs to conserve reducing power and free energy for nitrogen fixation. The carbon sources in natural soil are preserved by the ecological cycle of plant-origin materials (exudates and fallen leaves) but are removed from cropland soil by the harvesting of plant bodies [3,4].

Growth of diazotrophs may be relatively slower than that of non-diazotrophs in the nitrogen source (NO_3^- and NH_4^+) deficient soil, since bacterial growth depends on metabolism for conservation of ATP and NADH [5]. Theoretically, ATP consumption for nitrogen fixation may be 20-30 times higher than for absorption of the nitrogen sources. Presumably, the carbon sources produced by plants growing in cropland soil may induce the activation of metabolic nitrogen fixation by diazotrophs, thereby maintaining the nitrogen sources and allowing non-diazotrophs to grow in proportion to the nitrogen sources generated by diazotrophs [6,7]. Accordingly, the biomass and diversity of diazotrophs may not be higher than that of non-diazotrophs in natural or cropland soil that lack available nitrogen sources. However, diazotrophs may thrive better than non-diazotrophs under environmental conditions with sufficient carbon sources (sugars and organic acids) required for conserving a high balance of ATP and NADH [8]. The balance between consumption and regeneration of ATP and NADH can be influenced and controlled by environmental and nutritional conditions [9].

Electrochemical bioreactors (EBRs) composed of a neutral red-modified graphite felt (NR-GF) cathode and a platinum anode are useful tools to induce biochemical reduction reactions in various bacterial metabolisms. The NR-GF cathode induces bacterial metabolism to regenerate NADH without enzymatic catalysis, by which the metabolic reaction dependent upon NADH may be activated [10,11]. Theoretically, metabolic conservation of a high balance of NADH may be a driving force that induces ATP regeneration through the electron transport system. The high balance of NADH and ATP in the metabolism of diazotrophs may be induced by the electrochemical reducing power, while also being a factor in the activation of nitrogen fixation [12]. Electrochemically induced nitrogen fixation under organic carbon-limited conditions may be a factor causing diazotrophs to flourish and nitrogen sources to increase in bacterial habitats [13].

In this study, the bacterial community isolated from rhizosphere soil in soybean-growing cropland was cultivated in specially designed

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bioreactors: a conventional bioreactor (CBR) and an EBR. In the EBR, electrochemical reduction was applied to evaluate the bio-electrochemical conversion of atmospheric nitrogen to ammonium using free-living diazotroph. In contrast, the CBR was used as a control without such reduction. Variation of the bacterial communities cultivated in the bioreactors for a certain period of time was analyzed and compared with the bacterial community detected from rhizosphere soil using the temperature gradient gel electrophoresis (TGGE) technique.

MATERIALS AND METHODS

1. Collection of Rhizosphere Soil

Five uprooted soybeans, which were grown in a cropland soil for over five weeks, were collected from the middle of a soybean field (0.4 acre). All parts of the rootlets were cut and soaked in 1,000 mL of sterilized saline and shaken mildly for 30 min before the roots were removed. A soil suspension was obtained by filtration with a 200-mesh sieve and used as an inoculum for the CBR and EBR.

2. Inoculation of Rhizosphere Bacterial Community into Bioreactors

A 100 mL sample of the soil suspension was inoculated into 1,900 mL of a defined medium prepared in the CBR and EBR. The defined medium was composed of 20 mM sodium acetate, 20 mM sodium formate, 2 mM potassium phosphate, 5 mM potassium bicarbonate, and 2 mL/L of trace mineral stock solution. The trace mineral stock solution used herein contained 0.01 g/L of $MnSO_4$, 0.02 g/L of $FeSO_4$, 0.01 g/L of $CuSO_4$, 0.01 g/L of $MgSO_4$, 0.01 g/L of $CaCl_2$, 0.002 g/L of $NiCl_2$, 0.002 g/L of $CoCl_2$, 0.002 g/L of $ZnSO_4$, 0.01 g/L of $MoSO_4$, and 10 mM EDTA [14].

3. Immobilization of Neutral Red in the Graphite Felt Anode

The graphite felt (40×60×8 mm; Electrosynthesis GF-S8, USA) was soaked in 1% polyvinyl alcohol (PVA: MW 89,000-98,000) and then completely dried. The PVA fiber was directly combined with graphite fiber to produce a PVA-graphite felt complex. NR was immobilized onto the PVA-graphite felt complex by using thionyl chloride to induce the covalent bond between the amine of NR and the hydroxyl of PVA [15]. The water-soluble PVA was converted to water-insoluble PVNR by substitution of the hydroxyl of PVA with NR. The PVNR-graphite complex is a solid-type electron carrier that mediates electron transfer between bacterial cells and the graphite electrode.

4. Bioreactors

Both bioreactors were structurally identical and composed of the NR-GF cathode (height 140 mm, width 1,000 mm, thickness 10 mm) and a platinum wire anode (length 300 mm, thickness 0.5 mm). The NR-GF was rolled into a cylindrical shape (outer diameter 115 mm, inner diameter 55 mm) and equipped into round-type reactors (diameter 120 mm, height 250 mm). The cathode and anode in the EBR were electrochemically reduced and oxidized, respectively, by charging with DC 3 V electricity. Electricity was not charged to the electrodes in the CBR. The defined medium and 200 mM potassium phosphate buffer (pH 7.5) were the catholyte and anolyte, respectively. Bacteria were cultivated in the cathode compartment (total volume 2,500 mL, working volume 2,000 mL) at 20-25 °C. A glass filter (diameter 90 mm, pore 1-1.6 μm; Duran Group, Germany) was modified with cellulose acetate film (diameter 90 mm, thickness 35 μm; Electron Microscopy Science, USA) and placed between the cathode and the anode compartments to separate them electrically [16].

5. Cultivation of Bacterial Community from Rhizosphere Soil

The bacterial community contained in soil suspension was initially cultivated in the defined medium prepared in three CBRs and three EBRs for ten days. After that, 400 mL of fresh medium per day was continuously fed to the bioreactors. Filtered air was continuously sparged into the bacterial culture at a rate of 20 mL/min, to induce a microaerobic environment. The bacterial culture was discharged with exhaust to protect the outflux from contamination.

6. 16S rDNA Amplification

To analyze the bacterial diversity distribution in the rhizosphere soil, total genomic DNA was directly extracted from the precipitate of a soil suspension, using a bead beater (model FastPrep-24; MP Biomedical, Solon, OH, USA) and a DNA extraction kit (Power Soil DNA isolation kit; MoBio Laboratories, Carlsbad, CA, USA). To analyze the time-course variation of bacterial communities in the bioreactors, total genomic DNA was extracted from bacterial cells collected from the outflux of the CBR and EBR, using a genomic DNA extraction kit (Accuprep; Bioneer, Daejeon, Korea). The 16S ribosomal DNA was amplified via direct polymerase chain reaction (PCR) using the chromosomal DNA template and the following 16S rDNA specific universal primers: forward 5′-GAGTTGGATCCTGGCTCAG-3′ and reverse 5′-AAGGAGGGGATCCAGCC-3′. The PCR mixture (50 mL) consisted of 2.5 U Taq polymerase, 250 mM of each dNTP, 10 mM Tris-HCl (pH 9.0), 40 mM KCl, 100 ng of template, 50 pM primer, and 1.5 mM $MgCl_2$. Amplification was conducted for 30 cycles of 1 min at 95 °C, 1 min of annealing at 55 °C, and 2 min of extension at 72 °C, using a PCR machine (T Gradient model; Biometra, Göttingen, Germany).

7. Temperature Gradient Gel Electrophoresis

The 16S rDNAs amplified with genomic DNAs extracted directly from the rhizosphere soil suspension or the bacterial culture discharged from the CBR and EBR was employed as template for the preparation of TGGE samples (16S rDNA variable region). A variable region of 16S rDNA was amplified using the forward primer 341f 5′-CCTACGGGAGGCAGCAG-3′ and reverse primer 518r 5′-ATTACCG-CGGCTGCTGG-3′. A GC clamp (5′-CGCCCGCCGGCGGGCGGGCGGGGCGGGGGCACGGGGGG-3′) was attached to the 5′ end of the 341f primer [17]. The PCR for the TGGE samples was performed by the same procedure used for the 16S rDNA amplification, except the annealing temperature that was 53 °C. The TGGE system (Dcode; Bio-Rad, Berkeley, CA, USA) was operated in accordance with the manufacturer's specifications. Aliquots (45 mL) of the PCR products were electrophoresed on gels containing 8% acrylamide, 8 M urea, and 20% formamide in a 1.5-times-concentrated TAE (Tris, Acetate, and EDTA) buffer system. Electrophoresis was carried out at a constant voltage of 100 V for 12.5 h, followed by 40 V for 0.5 h, with a temperature gradient of 39-52 °C. Prior to electrophoresis, the gel was equilibrated to the temperature gradient for 30-45 min.

8. Amplification and Identification of DNA Bands on the TGGE Gel

DNA was extracted from the DNA bands on the TGGE gel and

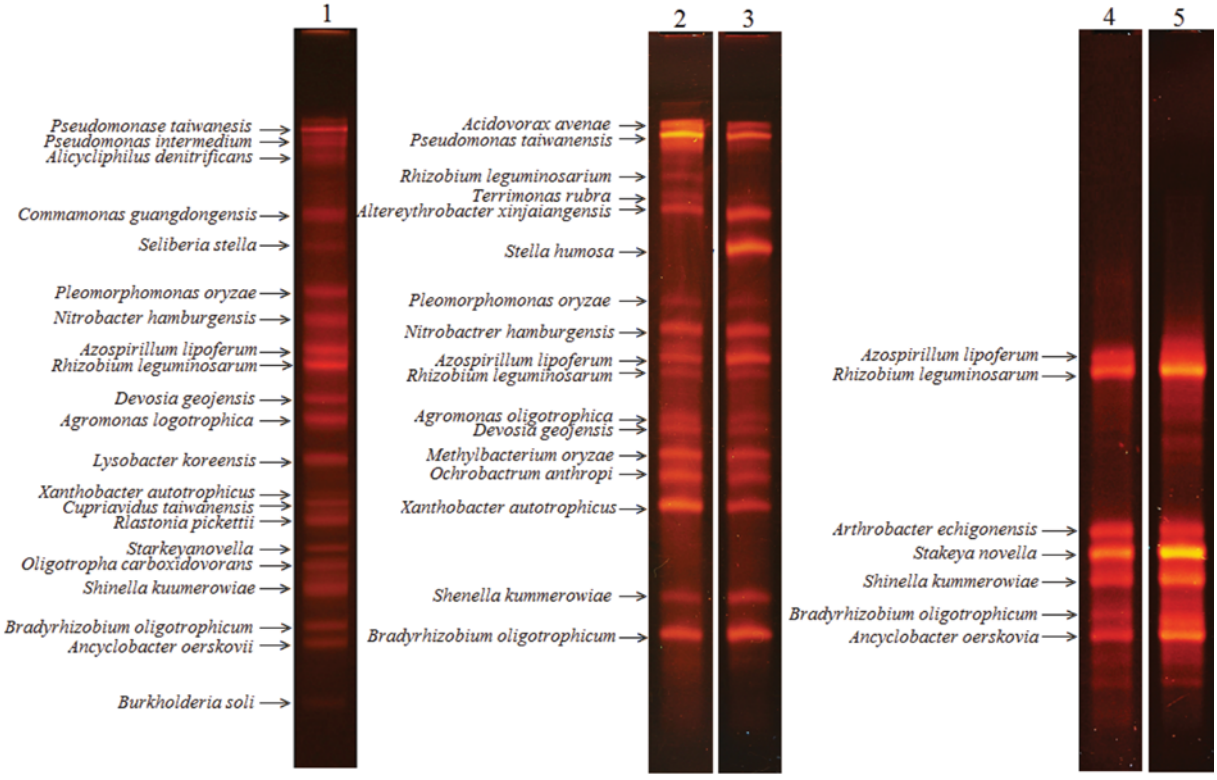

Fig. 1. Temperature gradient gel electrophoresis profile of 16S rDNAs amplified with genomic DNAs extracted from rhizosphere soil (lane 1), and from bacterial communities grown in a conventional bioreactor for 20 days (lane 2) and 40 days (lane 3) and in an electrochemical bioreactor for 20 days (lane 4) and 40 days (lane 5).

purified with a DNA gel purification kit (Accuprep; Bioneer). The purified DNA was then amplified with the same primers and procedures used for TGGE sample preparation, except that the GC clamp was attached to the forward primer. The amplified DNA was sequenced to identify the bacteria on the basis of 16S rDNA sequence homology, using the GenBank database.

9. Analysis

Bacterial culture (10 mL) was collected from the outflux of three bioreactors and centrifuged at 4 °C and 5,000 ×*g* for 30 min. The supernatant was used as a sample for ammonium analysis. Ammonium was determined with an ion chromatograph (Dionex DX-500, USA) equipped with a cation column (IonPac, Dionex CS12A), with 20 mM methanesulfonic acid as the mobile phase. The column temperature and flow rate were adjusted to 35 °C and 1.0 mL/min, respectively. The biomass was determined on the basis of dry cell weight at intervals of three days, for which 200 mL each of bacterial cultures was collected from the outflux of three bioreactors and refrigerated at 4 °C during harvesting for one day. The harvested bacterial culture was centrifuged at 4 °C and 5,000 ×*g* for 30 min and the precipitate was lyophilized. The cell mass was based on the weight of the lyophilized cells and calculated to be a 1,000 mL-based value.

RESULTS AND DISCUSSION

1. Bacterial Diversity in Rhizosphere Soil and Bioreactors

The diversity of heterotrophic bacteria inhabiting rhizosphere soil and those cultivated in the two bioreactors was analyzed by TGGE. As shown in Fig. 1, 21, 17 and 7 heterotrophic bacterial species were identified in the rhizosphere soil, CBR and EBR, respectively. Nine and four species detected from the CBR and EBR, respectively, were identical to heterotrophic bacteria detected from the rhizosphere soil, as shown in Tables 1, 2, and 3. Conversely, all of the heterotrophic bacteria detected from the CBR and EBR on day 20 of incubation were conserved until the 40th day of incubation, according to the DNA bands separated by TGGE. These results suggest that most, though not all, of the heterotrophic bacteria inhabiting the rhizosphere soil may have adapted to the nutritional and environmental conditions of the bioreactors and grew stably for at least 40 days. Seven of the species detected from the rhizosphere soil, six from the CBR, and five from the EBR were free-living diazotrophs (nitrogen-fixing bacteria), as shown in Tables 1, 2, and 3 (bold-lettered species). Two species (*Starkeya novella* and *Ancylobacter oerskovia*) in the EBR grew autotrophically in the electrochemically reduced and carbon source-limited conditions because the bacteria were capable of facultative chemolithotrophic, heterotrophic, and methylotrophic growth. This may indicate that the CBR is relatively more suitable than the EBR for growth of heterotrophic bacteria originating from the rhizosphere soil, although the EBR induced diazotrophs to actively fix atmospheric nitrogen and selectively grow.

Carbon and nitrogen sources are essential nutrients that influence maintenance of the bacterial diversity and growth of the heterotrophic bacteria inhabiting rhizosphere soil and bioreactors [18].

Table 1. Bacteria identified on the basis of sequence homology of DNA separated by TGGE (Fig. 2, lane 1) that was performed with 16S rDNA extracted from the rhizosphere soil

Species (GenBank accession no.)	Homology (%)	General characteristics
Pseudolabrys taiwanensis (NR043515)	99	Gram(-), short rod-shaped microorganism isolated from soil
***Pseudoacidvorax intermedium* (NR044241)**	**100**	**Gram(-), short rod-shaped, nitrogen-fixing bacterium isolated from an agricultural area**
Alicycliphilus denitrificans (NR074585)	99	Gram(-), motile, non-spore-forming facultative anaerobe isolated from soil and water
Comamonas guangdongensis (NR108203)	98	Gram(-), nonmotile, facultatively anaerobic bacterium isolated from subterranean forest sediments
Seliberia stellata (NR104886)	98	Gram(-), strictly aerobic bacterium that exists in soil and fresh water
***Pleomorphomonas oryzae* (NR041002)**	**99**	**Nitrogen-fixing bacterium isolated from paddy soil of *Oryzae sativa***
Nitrobacter hamburgensis (NR074313)	99	Gram(-), nitrite-oxidizing bacterium isolated from soil of an old botanic garden in Hamburg
***Azospirillum lipoferum* (NR102897)**	**98**	**Gram(-), nitrogen-fixing bacterium associated with roots of monocots; exerts beneficial effects on plant growth**
***Rhizobium leguminosarum* (NR103919)**	**99**	**Gram(-) bacterium with typical nitrogen-fixing ability by formation of root nodules**
Devosia geojensis (NR044291)	98	Gram(-), obligately aerobic, rod-shaped bacterium that inhabits farm fields
***Agromonas oligotrophica* (NR025876)**	**98**	**Gram(-), nitrogen-fixing oligotrophic bacterium isolated from paddy field soil**
Lysobacter koreensis (NR041014)	99	Gram(-), non-spore-forming bacterium isolated from soil of a ginseng field in Korea
Xanthobacter autotrophicus (NR074255)	98	Gram(-), aerobic, rod-shaped bacterium that inhabits soil containing decaying organic material, and water
***Cupriavidus taiwanensis* (NR07423)**	**98**	**Gram(-), nitrogen-fixing bacterium isolated from soil**
Ralstonia pickettii (NR043152)	98	Gram(-), rod-shaped bacterium inhabiting moist environments such as soils, rivers, and lakes
Starkeya novella (NR074219)	98	Bacterium capable of faculatative chemolithotrophic, heterotrophic, and methylotrophic growth in soil
Oligotropha carboxidovorans (NR074142)	98	Gram(-) soil bacterium solely represented by genus *Oligotropha*
Shinella kummerowiae (NR044066)	98	Bacterial strain first isolated from root nodules of grass (*Kummerowia stipulacea*)
***Bradyrhizobium oligotrophicum* (NR102489)**	**98**	**Nitrogen-fixing, oligotrophic bacterium isolated from paddy field soil; able to grow in extra-low-nutrient environment**
Ancylobacter oerskovii (NR042655)	99	Gram(-), pleomorphic, non-spore-forming bacterium isolated from soil with oxalate
Burkholderia soli (NR043872)	98	Gram(-), strictly aerobic, nonmotile bacterium inhabiting soil in Korean ginseng fields

Species in bold lettering are diazotrophs. TGGE, temperature gradient gel electrophoresis

The environmental and nutritional conditions of the rhizosphere soil, CBR, and EBR for bacterial community growth are impossible to analyze comparatively by chemical techniques. However, the diversity of the bacterial community and the ratio of diazotrophs

Table 2. Bacteria identified on the basis of sequence homology of DNA separated by TGGE (Fig. 2, lanes 2 and 3) that was performed with 16S rDNA extracted from bacterial community grown in the conventional bioreactor

Species (GenBank accession no.)	Homology (%)	General characteristics
Acidovorax avenae (NR044241)	98	Gram(-), biotrophic bacterium; causes seedling blight and bacterial fruit blotch of cucurbits
Pseudolabrys taiwanensis (NR043515)	99	Gram(-), short, rod-shaped microorganism isolated from soil
***Rhizobium leguminosarum* (NR103919)**	**99**	**Gram(-) bacterium with typical nitrogen-fixing ability by formation of root nodules**
Terrimonas rubra (NR109417)	98	Gram(-) bacterium isolated from farmland soil sample in China
Altererythrobacter xinjiangensis (NR108901)	98	Gram(-), nonmotile, strictly anaerobic bacterium isolated from sand soil
***Pleomorphomonas oryzae* (NR041002)**	**99**	**Nitrogen-fixing bacterium isolated from paddy soil of *Oryzae sativa***
Nitrobacter hamburgensis (NR102489)	98	Gram(-) bacterium that oxidizes nitrite to nitrate; inhabits soil, sand stone, and sewage
***Azospirillum lipoferum* (NR102897)**	**98**	**Gram(-), nitrogen-fixing bacterium associated with roots of monocots; exerts beneficial effects on plant growth**
***Rhizobium leguminosarum* (NR103919)**	**99**	**Gram(-) bacterium with typical nitrogen-fixing ability by formation of root nodules**
***Agromonas oligotrophica* (NR025876)**	**98**	**Gram(-), nitrogen-fixing, oligotrophic bacterium isolated from paddy field soil**
Devosia geojensis (NR044291)	98	Gram(-), obligately aerobic, rod-shaped bacterium that inhabits farm fields
Methylobacterium oryzae (NR043104)	98	Methyl compound-catabolizing bacterium isolated from paddy field soil
Ochrobactrum anthropi (NR074243)	98	Obligately aerobic, strictly respiratory bacterium that grows in soil, water, plants, nematodes, and insects
Xanthobacter autotrophicus (NR074255)	98	Gram(-), aerobic, rod-shaped bacterium that inhabits soil containing decaying organic material, and water
Shinella kummerowiae (NR044066)	98	Bacterial strain first isolated from root nodules of grass (*Kummerowia stipulacea*)
***Bradyrhizobium oligotrophicum* (NR102489)**	**98**	**Nitrogen-fixing oligotrophic bacterium isolated from paddy field soil; able to grow in extra-low-nutrient environment**
Stella humosa (NR025582)	98	Gram(-), nonmotile, asporogenic bacterium that inhabits freshwater, soil, and sewage in widely separated geographical areas

Species in bold lettering are diazotrophs. TGGE, temperature gradient gel electrophoresis

to non-diazotrophs may be indicators of the environmental or nutritional conditions [19]. The nutritional conditions of the CBR may not be significantly different from those of the rhizosphere soil when estimated on the basis of bacterial species diversity and diazotrophs/non-diazotrophs ratio (Tables 1 and 2) [20]. The nutritional conditions of the CBR and EBR were identical, as simultaneously prepared medium was used in both bioreactors. Accordingly, any detectable difference in bacterial species diversity and diazotrophs/non-diazotrophs ratio between the CBR and EBR would be reflective of differences in environmental condition between these two bioreactors (Tables 2 and 3) [21]. Whereas the environmental conditions of the CBR may be suitable for maintaining the diversity and growth of both diazotrophs and non-diazotrophs, those of the EBR may be selective for diazotroph growth. The selective growth of diazotrophs in the EBR may be caused by the activation of nitrogen fixation induced by electrochemical reduction [22]. The ecological relationship between diazotrophs and non-diazotrophs growing in the rhizosphere soil and CBR may be syntrophic for the distribution of nitrogen source and growth factors, where the balanced diazotrophs/non-diazotrophs ratio in the rhizosphere soil may be conserved in the CBR [23]. Conversely, this bacterial syntrophism may not be conserved in the EBR, because the nitrogen fixation of

Table 3. Bacterial species identified on the basis of sequence homology of DNA separated by TGGE (Fig. 2, lanes 4 and 5) that was performed with 16S rDNA extracted from the bacterial community grown in an electrochemical bioreactor

Species (GenBank accession no.)	Homology (%)	General characteristics
Azospirillum lipoferum **(NR102897)**	**98**	**Gram(-), nitrogen-fixing bacterium associated with roots of monocots; exerts beneficial effects on plant growth**
Rhizobium leguminosarum **(NR103919)**	**99**	**Gram(-) bacterium with typical nitrogen-fixing ability by formation of root nodules**
Arthrobacter echigonensis **(NR041402)**	**99**	**Gram(+), nitrogen-fixing, obligate aerobe that is generally found in soil; useful for bioremediation**
Starkeya novella (NR074219)	98	Bacterium capable of faculatative chemolithotrophic, heterotrophic, and methylotrophic growth in soil
Shinella kummerowiae **(NR044066)**	**98**	**Bacterial strain first isolated from root nodules of grass (*Kummerowia stipulacea*)**
Bradyrhizobium oligotrophicum **(NR102489)**	**98**	**Nitrogen-fixing, oligotrophic bacterium isolated from paddy field soil; able to grow in extra-low-nutrient environment**
Ancylobacter oerskovia (NR042655)	99	Gram(-), pleomorphic, non-spore-forming bacterium isolated from soil with oxalate

Species in bold lettering are diazotrophs. TGGE, temperature gradient gel electrophoresis

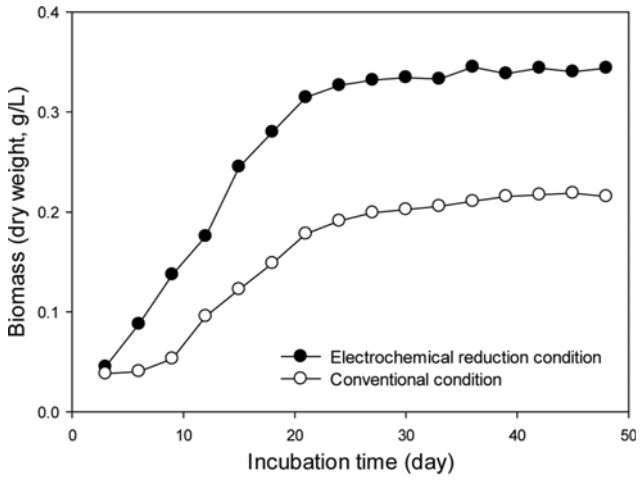

Fig. 2. Biomass variation of the bacterial communities grown in a conventional bioreactor and in an electrochemical bioreactor.

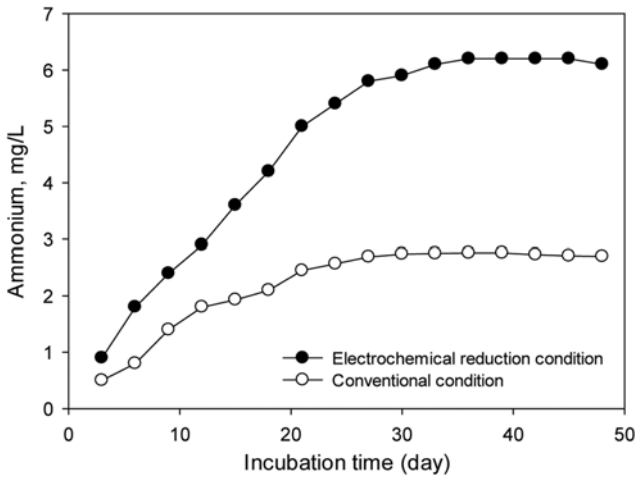

Fig. 3. Variation of the ammonium content in cultures of bacterial communities grown in a conventional bioreactor and in an electrochemical bioreactor.

diazotrophs induced by electrochemical reduction may cause them to exclusively consume carbon, thereby resulting in the non-diazotrophs losing their competitiveness for the carbon source.

The diversity and biomass of diazotrophs and non-diazotrophs are indicators for the evaluation of enrichment. Although diversity can be estimated by TGGE, the biomass of the bacteria growing in CBRs and EBRs cannot be analyzed separately. Accordingly, the estimation of diazotroph enrichment must be based on the relationship between biomass and ammonium production of the bacterial community cultivated in the CBR and EBR (Figs. 2 and 3) [24].

2. Relationship between Biomass and Ammonium Content in the CBR and EBR

Bacterial cultures collected from the outflux of the CBR and EBR contain bacterial cells and metabolites. The bacterial cells were precipitated and lyophilized for measurement of dry cell mass, whereas the ammonium content of the metabolites was selectively analyzed to evaluate the nitrogen-fixing activity of the diazotrophs in the bioreactors. As shown in Figs. 2 and 3, the biomass and ammonium content increased gradually over time from day 1 to day 20 in both the CBR and EBR, and then leveled off. The biomass and ammonium contained in the bacterial culture discharged from the EBR was approximately 1.6 and 6 times higher, respectively, than those from the CBR. The maximal ammonium/biomass ratio was 4.5 (0.9/0.2) in the CBR and 17.7 (6.2/0.35) in the EBR, which correlated to the ratios of diazotrophs to total heterotrophic bacteria grown in both bioreactors. The ratios of diazotrophs to total heterotrophic bacteria in the CBR and EBR were 0.35 (6/17) and 0.71 (5/7), respectively. Nitrogen fixation of the diazotrophs may be activated by the electrochemical reducing power generated by NR-GF cath-

ode, resulting in the relatively higher biomass and ammonium production of bacterial community inhabiting the EBR than the CBR [25]. The ammonium detected from both the CBR and EBR must be produced by diazotrophs, while the ammonium content must be proportional to the biomass of diazotrophs [26]. The selective enrichment and growth of diazotrophs in the EBR were determined on the basis of the ratio of diazotrophs to non-diazotrophs (Tables 2 and 3), the difference of bacterial community biomass (Fig. 2), and the difference of ammonium content produced (Fig. 3) in the bioreactors. Selective enrichment of diazotrophs in the EBR may be established by the selective consumption of carbon source coupled to nitrogen assimilation [27]. The unique non-diazotrophs detected from the EBR were bacteria capable of chemolithotrophic growth in soil, growing with the ATP and NADH regenerated by the electrochemical reducing power and the ammonium produced by diazotrophs [28,29].

The reduction of bacterial diversity (Table 3) and increase of biomass (Fig. 2) in the EBR may cause an increase of ammonium content in the bacterial culture. The electrochemical reducing power may induce physiological changes in bacteria to maximally regenerate ATP and NADH. The carbon source limitation may cause diazotrophs to grow suboptimally, causing more ammonium to be synthesized than is physiologically required, and hence some of the excess ammonium may be excreted to the bacterial culture in the EBR (Fig. 3). The accumulation of free-living diazotrophs and ammonium in the bioreactor may be a useful biological process for continuously producing nitrogen fertilizers, using a low-cost energy source (low-voltage electricity), materials (acetate and formate), and system (EBR) [30]. Chemical processes for ammonium synthesis from atmospheric nitrogen are more rapid than biological processes, but energy consumption for a chemical process may be significantly higher than that for a biological process; additionally, larger volumes of carbon dioxide may be generated from a chemical process than from a biological process.

CONCLUSION

The electrochemical reducing power generated from NR-GF may be a driving force for both non-diazotrophs and diazotrophs to regenerate ATP and NADH. The nitrogen-fixing metabolism of diazotrophs may be selectively activated in a high balance of ATP and NADH. The heterotrophic metabolism of non-diazotrophs cannot be activated, because both carbon and nitrogen sources are absolutely required for biosynthesis coupled to consumption of ATP and NADH. The bio-electrochemical conversion of atmospheric N_2 to ammonium by the free-living diazotroph may be a useful biotechnology for green agriculture and reduction of CO_2 emission.

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