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Effect of Electrochemical Redox Reaction on Biochemical Ammonium Oxidation and Chemical Nitrite Oxidation

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A modified graphite felt electrode with neutral red (NR-electrode) was shown to catalyze the chemical oxidation of nitrite to nitrate under aerobic conditions. The electrochemically oxidized NR-electrode (EO-NR-electrode) and reduced NR-electrode (ER-NR-electrode) catalyzed the oxidation of 1,094±39 mg/l and 382±45 mg/l of nitrite, respectively, for 24 h. The electrically uncharged NR-electrode (EU-NR-electrode) catalyzed the oxidation of 345±47 mg/l of nitrite for 24 h. The aerobic bacterial community immobilized in the EO-NR-electrode did not oxidize ammonium to nitrite; however, the aerobic bacterial community immobilized in the ER-NR-electrode bioelectrochemically oxidized 1,412±39 mg/l of ammonium for 48 h. Meanwhile, the aerobic bacterial community immobilized on the EU-NR-electrode biochemically oxidized 449±22 mg/l of ammonium for 48 h. In the continuous culture system, the aerobic bacterial community immobilized on the ER-NR-electrode bioelectrochemically oxidized a minimal 1,337±38 mg/l to a maximal 1,480±38 mg/l of ammonium to nitrate, and the community immobilized on the EU-NR-electrode biochemically oxidized a minimal 327±23 mg/l to a maximal 412±26 mg/l of ammonium to nitrate every two days. The bacterial communities cultivated in the ER-NR-electrode and EU-NR-electrode in the continuous culture system were analyzed by TGGE on the 20th and 50th days of incubation. Some ammonium-oxidizing bacteria were enriched on the ER-NR-electrode, but not on the EU-NR-electrode.

Keywords: Ammonium oxidation, nitrite oxidation, chemolithotroph, electrochemical reduction, neutral red, TGGE

The ammonium oxidizer *Nitrosomonas* sp. and the nitrite oxidizer *Nitrobacter* sp. generate biochemical energy (ATP) and reducing power (NADH) for biosynthesis, which is dependent on the redox equivalent of ammonium and nitrite [1–3, 29]. The biochemical oxidation of NH_4^+, NH_2OH, or NH_2NH_2 in the respiration of *Nitrosomonas* sp. generated proton translocation when being coupled with the reduction of oxygen to H_2O [4, 5, 8, 13]. The electrochemical gradient of protons gives rise to Δp, which is responsible for the coupling of metabolic energy to the transport of a number of nutrients and to ATP synthesis [11, 14, 16, 37]. The membrane-bound nitrite-cytochrome *c* oxidoreductase, soluble cytochrome *c*-550, and membrane-bound cyotochrome *c* oxidase serve as the electron transport chain from NO_2^- to O_2 in combination with ATP and NADH regeneration in the nitrite-oxidizing bacteria [30, 39, 40]. Cytochrome *c*-550 was identified as an alternative electron mediator between nitrate-cytochrome *c* oxidoreductase and cytochrome *c* oxidase [18], which is linked to the proton-pumping activity component of ATP generation [25].

Ammonium-oxidizing and nitrite-oxidizing bacteria are syntrophically related to the production and consumption of nitrite, functioning respectively as a metabolite and a substrate. The nitrite-oxidizing bacteria are faced with fluctuating nitrite availability with a temporary excess supply, followed by various periods of nutrient deficiency, owing to the dependence of those bacteria on ammonium-oxidizing bacteria to obtain nitrites in an ammonium-contaminated habitat [17, 33].

The conversion of ammonia to nitrite by *Nitrosomonas* sp in matrix systems has previously been metabolically characterized on the basis of proton translocation and membrane potential generation [13, 34]; however, the metabolic conversion of nitrite to nitrate by *Nitrobacter agilis* has not been verifiably linked, thus far, to proton translocation [31]. Ammonium oxidizers and nitrite oxidizers must be balanced for the syntrophic oxidation of ammonium to nitrate; however, ammonium oxidizers have been shown to persist for a longer time than nitrite oxidizers in wastewater treatment reactors, owing to fluctuations in the chemical parameters and the underlying bacterial community in these environments [9]. Nitrite was previously shown to

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be electrochemically oxidized to nitrate *via* an electrocatalytic reaction [7, 27] and aeration under acidic conditions [31]. This process may induce ecological fluctuations in the population of nitrite oxidizers in ammonium-containing wastewater.

In this study, an aerobic bacterial community growing in a wastewater treatment reactor was immobilized on the NR-electrode in an effort to characterize the mechanism by which ammonium and nitrate are electrochemically and bioelectrochemically oxidized. The electrochemical oxidation of ammonium and nitrite was compared with the bioelectrochemical oxidation efficiency. The TGGE technique was employed to analyze the aerobic bacterial community variation in proportion to the incubation time.

Materials and Methods

Organisms and Medium

An aerobic bacterial community was obtained from a wastewater treatment reactor in the Jungrang wastewater treatment complex (Seoul, Korea), and then cultivated in an inorganic ammonium medium (IAM) composed of 100 mM NH_4Cl, 10 mM K_2HPO_4, 100 mM $NaHCO_3$ and 2 ml/l of trace mineral stock solution. The trace mineral stock solution contained 0.01 g/l of $MnSO_4$, 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 $SeSO_4$, 0.002 g/l of WSO_4, 0.002 g/l of $ZnSO_4$, 0.002 g/l of $Al_2(SO_4)_3$, 0.0001 g/l of $TiCl_3$, 0.002 g/l of $MoSO_4$, and 10 mM EDTA.

A 2,000-ml volume of wastewater treatment reactant was settled to induce the sedimentation of solid particles for 20 min, after which the supernatant was centrifuged for 40 min at 5,000 ×*g* and 20°C. The centrifugal precipitant was then resuspended in IAM and cultivated for 60 days at 25°C in order to enrich the proportion of chemolithotrophic nitrifying bacteria. The enriched chemolithotrophic nitrifying bacterial community was cultivated continuously in the IAM or immobilized on the NR-electrode, in accordance with the purpose of this research. The enrichment was assessed *via* the analysis of ammonium, nitrite, and nitrate concentrations *via* an ion chromatographic technique.

Bioreactor

A specially designed electrochemical bioreactor (ECB) was organized to achieve the experimental objective, as is shown in Fig. 1A. The working compartment was separated using the cellulose acetate and ceramic membrane from the counter-electrode, as is shown in Fig. 1B

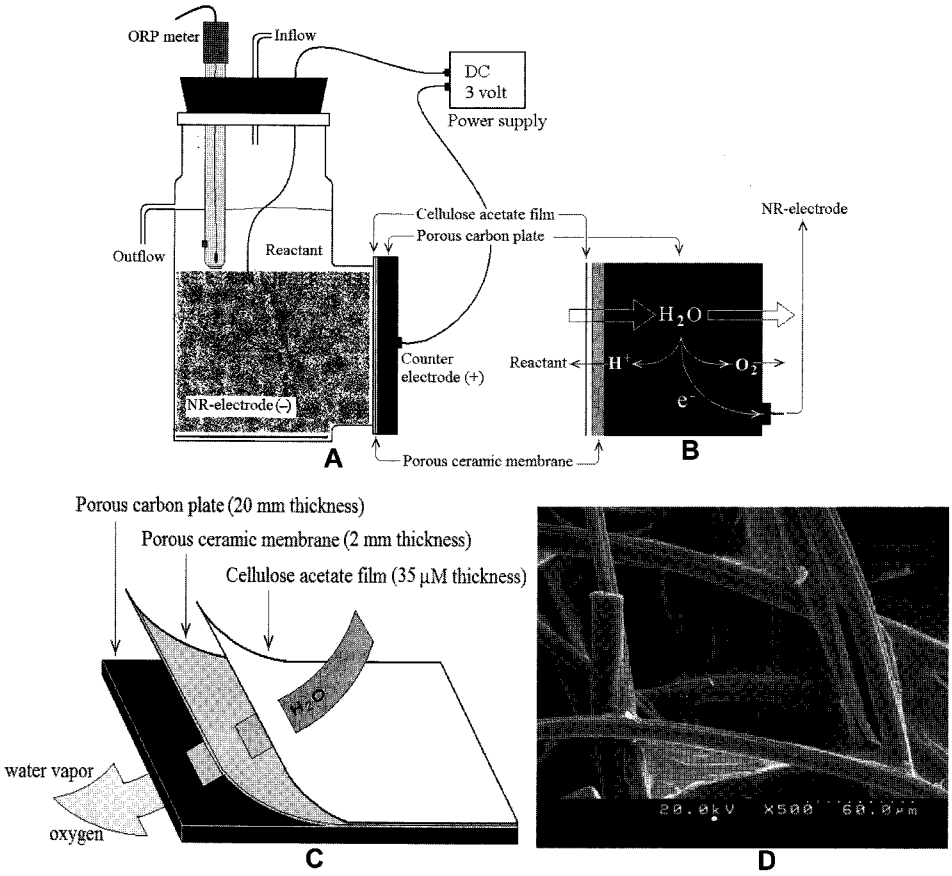

Fig. 1. Schematic structure of an electrochemical bioreactor (**A**), the operating mechanism (**B**), and counter-electrode (**C**), and SEM picture of the NR-graphite felt electrode (**D**).
The film-like structure between graphite fibers in the SEM picture is the modified PVA with NR, which was not soluble in water and was stable during autoclaving.

and 1C. The triple bioreactors, of which the function and structure were completely identical, were utilized for the batch and continuous culture tests. The IAM was supplied continuously to the bioreactors (300 ml) at the speed of 150 ml/day in the continuous culture test. Ammonium, nitrite, and nitrate contained in the outflow were determined *via* ion chromatography. All reactors were commonly aerated at a rate of 100 ml/min/l.

Electrodes

A modified graphite felt electrode (0.47 m^2/g; Electrosynthesis, U.S.A.) with neutral red (NR-electrode) was used as a working electrode to activate electron transfer from the electrode to bacterial cells, or in the reverse direction [29]; the felt was rolled to maximize its surface area (thickness 6 mm×length 180 mm×height 100). A modified porous carbon plate with cellulose acetate film was utilized as a counter-electrode, as is shown in Fig. 1B and 1C. The working and counter-electrode were charged with 3 volts of DC electricity using a solar cell panel, as is shown in Fig. 1A. The working electrodes were prepared in the following three variations: an electrochemically uncharged NR-electrode (EU-NR-electrode) that was not electrically charged; an electrochemically reduced NR-electrode (ER-NR-electrode) that was charged with cathodic electricity (−); and an electrochemically oxidized NR-electrode (EO-NR-electrode) that was charged with anodic electricity (+).

Immobilization of NR in Graphite Felt

Neutral red was immobilized onto the graphite felt by a covalent bond between neutral red and polyvinyl alcohol (mean molecular weight, 80,000; Sigma) *via* the following procedure. The graphite felt (10 mm×100 mm×40) was soaked into 1% (w/v) of polyvinyl alcohol solution (100 ml) at 60°C under vacuum for 3 h, and then dried for 12 h at 100°C. The completely dried graphite felt was then soaked for 6 h in pure chloroform containing 10% thionylchloride and 0.1% neutral red to induce a covalent bond between the -OH of polyvinyl alcohol and the $-NH_2$ of neutral red, after which the graphite felt was washed in methanol to remove the unbound neutral red. In this reaction, the water-soluble polyvinyl alcohol was converted to water-insoluble polyvinyl neutral red and the graphite felt electrode was exchanged with a more rigid one, such as a plastic sponge. The polyvinyl neutral red–graphite felt complex (NR-graphite felt) was quite stable, and remained unchanged after more than 10 autoclave sessions, as is shown in Fig. 1D.

Immobilization of Bacterial Community in Graphite Felt

One thousand ml of a bacterial community precultivated for 30 days in IAM at 25°C was harvested *via* 40 min of centrifugation at 4°C and 5,000 ×*g*. The harvested cells (10 ml) were mixed, without washing, with 10 ml of 25 mM phosphate buffer (pH 7.0) containing 4% alginate. A mixture of 20 ml of bacterial cells and alginate (final 2%) was absorbed into the NR-graphite felt (20 mm×40 mm×100) using a micropipette. The NR-graphite felt containing the bacterial cells and alginate mixture was then soaked in 100 mM $CaCl_2$ solution to induce calcium alginate coagulation for 30 min in a refrigerator and washed with 25 mM phosphate buffer, which was instantly used as the biocatalyst for the batch, fed-batch, and continuous cultures. This biocatalyst is quite helpful in that it allows the lag phase to be skipped over, owing to the ammonium oxidation efficiency. The final cell mass immobilized on the NR-graphite felt (20 mm×40 mm×100) was adjusted to a dry weight of 2.5 g/l.

16S rDNA Amplification

The bacterial cells were collected from the continuous culture system at the 2nd, 4th, 6th, 8th, and 10th weeks of incubation during cultivation for more than 70 days. Chromosomal DNA was extracted directly from the bacteria grown in the bioreactors equipped with the ER-NR and EU-NR electrodes. 16S ribosomal DNA was amplified *via* direct PCR using the chromosomal DNA template and the following 16S-rDNA-specific universal primers: forward 5'-GAGTTGGATCCTGGCTCAG-3' and reverse 5'-AAGGAGGGGA TCCAGCC-3'. The PCR reaction mixture (50 μl) consisted of 2.5 U of Tag polymerase, 250 μM 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; Biometera, German).

Thermal Gradient Gel Electrophoresis

Samples were isolated from three identical electrochemical bioreactors operated under conventional and electrochemical reduction conditions. The 16S rDNA amplified from chromosomal DNA was utilized as a template for TGGE sample preparation. A variable region of 16S rDNA was amplified with forward primer (eubacteria, V3 region) 341f 5'-CCTACGGGAGGCAGCAG-3' and reverse primer (universal, V3 region) 518r 5'-ATTACCGCGGCTGCTGG-3'. The GC clamp (5-CGCCCG CCGCGCGCGGCGGGC GGGGCGGGGGCACGG GGGGCCTACGGGAGGCAGCAG-3') was attached to the 5'-end of the GC341f primer [26]. The procedures for PCR and DNA sequencing were identical to those utilized for 16S rDNA amplification, except for an annealing temperature of 53°C. The TGGE system (Bio-Rad, Dcode, Universal Mutation Detection System, U.S.A.) was operated in accordance with the manufacturer's instructions. Aliquots (45 μl) of the PCR products were triply electrophoresed in gels containing 8% acrylamide, 8 M urea, and 20% formamide with a 1.5× TAE buffer system at a constant voltage of 100 V for 12.5 h and then at 40 V for 0.5 h, applying a thermal gradient of 39 to 52°C. Prior to electrophoresis, the gel was equilibrated to the temperature gradient for 30 to 45 min.

Amplification of TGGE Band

DNA was extracted from the TGGE band and purified with a DNA gel purification kit (Accuprep; Bioneer, Korea). The purified DNA was amplified with the same primers and procedures utilized for TGGE sample preparation, in which the GC clamp was not attached to the forward primer.

Analysis

Nitrate and nitrite were analyzed with an ion chromatograph (Dionex DX-500, U.S.A.) equipped with an anion column (IonPac, Dionex AS14A). Five mM sodium hydroxide was used as the mobile phase. Ammonium was analyzed with an ion chromatograph (Dionex DX-500, U.S.A.) equipped with a cation column (IonPac, Dionex CS12A). A 100% 20 mM methanesulfonic acid solution was used as the mobile phase. The column temperature and flow rate were adjusted to 35°C and 1.0 ml/min.

Cyclic Voltammetry

Cyclic voltammetry was conducted using a cyclovoltammetric potentiostat (BAS model 50W, U.S.A.) employing an Ag/AgCl reference electrode, a glassy carbon working electrode (3 mm diameter), and a platinum

wire counter-electrode (0.5 mm diameter and 40 mm length) with a reaction mixture containing 50 mM Tris buffer (pH 7.5), 100 μM NR, and the aerobic bacterial community (biomass, 10 mg/l). The aerobic bacterial community was excluded from the reaction mixture for analysis of the electrochemical reaction of the electrode with ammonium or nitrite. The scan range was adjusted from −800 mV to +200 mV vs. Ag/AgCl, and the scan ratio was adjusted to 25 mV/s. The aerobic bacterial community was excluded from the reaction mixture for the control test. In order to analyze the redox reaction between bacterial cells and substrate (NH_4^+ and NO_2^-), ammonium or nitrite was added to the reaction mixture at the moment of switching from the 6th to the 7th cycle during 12 scan cycles, and this procedure was repeated continuously.

Results

Chemical Nitrification

The chemical oxidation of ammonium to nitrite and nitrite to nitrate was evaluated under aerobic conditions. As is shown in Fig. 2, the EO-NR-electrode, ER-NR-electrode, and EU-NR-electrode did not oxidize ammonium to nitrite, but actively oxidized nitrite to nitrate as follows: NO_2^-+ NR-electrode→NO_3^-+NR-electrode. In this reaction, the NR-electrode may function as a catalyst but not as a reactant, based on the result that nitrite was not oxidized under conventional conditions without the NR-electrode (data not shown). The initial and final concentrations of ammonium (mean 1,395±35 mg/l) and nitrite (mean 1.3±0.07 mg/l) were unchanged during 24 h of reaction (Fig. 2A). The nitrite oxidation efficiencies evidenced by the EO-NR-electrode, ER-NR-electrode, and EU-NR-electrode were, respectively: 1,094±39 mg/l, 382±45 mg/l, and 345±47 mg/l, for 24 h (Fig. 2B). The NR-electrode is supposed to function as a catalyst for the chemical oxidation of nitrite to nitrate under aerobic conditions, and the electrochemical oxidation energy may induce the NR-electrode to chemically oxidize nitrite. The electrochemical reduction energy neither activated nor inhibited the chemical oxidation of nitrite.

Biological Nitrification

The immobilized bacterial community in the ER-NR-electrode and the EU-NR-electrode catalyzed ammonium oxidation to nitrate, but the EO-NR-electrode did not, as is shown in Fig. 3. Here, 1,412±39 and 449±22 mg/l of nitrate were produced from ammonium by the bacterial community

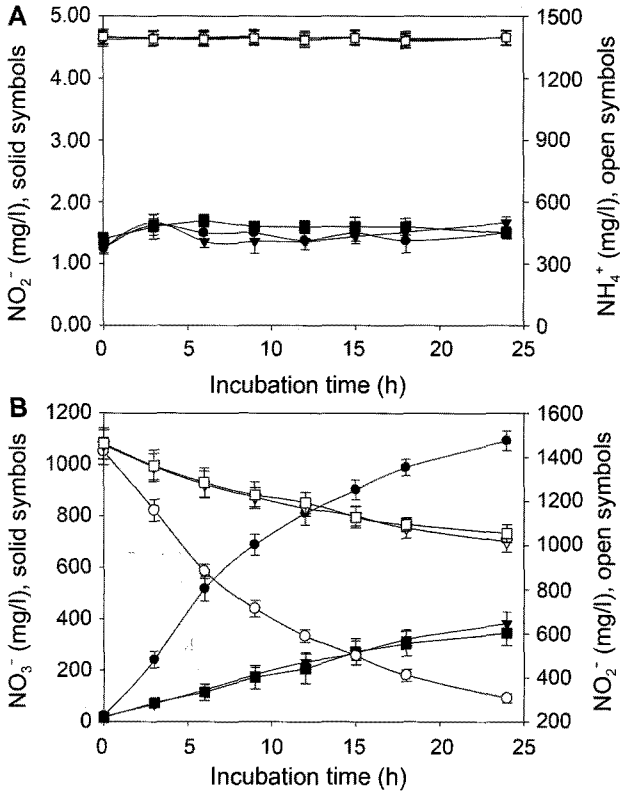

Fig. 2. Chemical oxidation of ammonium to nitrite (**A**) and nitrite to nitrate (**B**) *via* catalysis of the EO-NR-electrode (●, ○), ER-NR-electrode (■, □), and EU-NR-electrode (▼, ▽) under aerobic conditions.

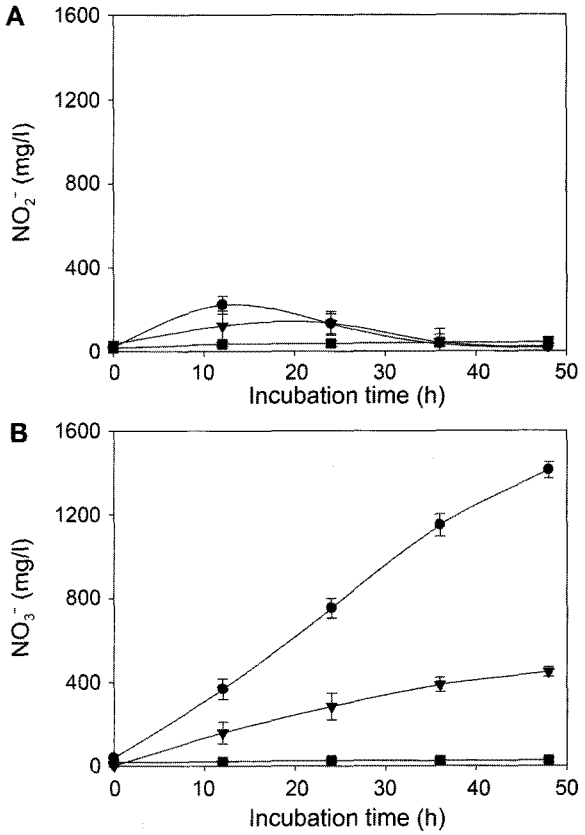

Fig. 3. Nitrite (**A**) and nitrate (**B**) produced from ammonium by the aerobic bacterial community immobilized on the ER-NR-electrode (●), EO-NR-electrode (■), and EU-NR-electrode (▼).

immobilized on the ER-NR electrode and the EU-NR-electrode, respectively, for 48 h; however, nitrate was not produced by the bacterial community immobilized on the EO-NR-electrode. A maximal 222±42 mg/l and 135±56 mg/l of nitrite were detected in the bioreactor equipped with the ER-NR-electrode and the EU-NR-electrode, respectively, during 48 h of incubation. These phenomena may be attributable to the biochemical ammonium oxidation activation and chemical nitrite oxidation catalysis functions of the ER-NR-electrode. Nitrite generated *via* the biochemical ammonium oxidation may be oxidized chemically to nitrate by the ER-NR-electrode, thus preventing nitrite from accumulating in the bioreactor. Meanwhile, the biochemical oxidation efficiency of ammonium by the aerobic bacterial community immobilized on the EU-NR-electrode may be lower than the nitrite oxidation efficiency of the NR-electrode, in which the oxidation of ammonium to nitrate was limited to 449±22 mg/l. No biochemical nitrite generation from ammonium resulted in any chemical nitrate generation in the bioreactor containing the EO-NR-electrode.

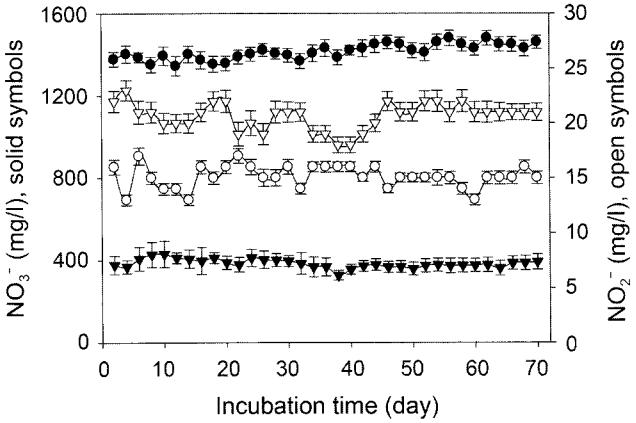

Fig. 4. Nitrate generated from ammonium by an aerobic bacterial community immobilized on the ER-NR-electrode (●) and the EU-NR-electrode (▼) in a continuous culture system over 70 days.
Significantly low levels of nitrite were detected in bioreactors equipped with the ER-NR-electrode (○) and the EU-NR-electrode (▽).

Long-Term Stability for Nitrification

The bioreactors utilized for the biological nitrification test (Fig. 3) were operated for more than 70 days in order to assess the biochemical stability of the aerobic bacterial community immobilized on the ER-NR and EU-NR electrodes as a catalyst. An outflow of 150 ml/day was continuously discharged in proportion to the inflow of fresh medium. The mean values of nitrate and nitrite contained in the outflow for 70 days were maintained at a minimal 1,337±38 mg/l to a maximal 1,480±38 mg/l and a minimal 13±0.5 mg/l to a maximal 17±0.7 mg/l in the ER-NR-electrode, and a minimal 327±23 mg/l to maximal 412±26 mg/l and minimal 18±0.7 to a maximal 23±0.9 mg/l in the EU-NR-electrode, respectively, as is shown in Fig. 4. Nitrite may be oxidized to nitrate *via* either chemical or biochemical means. It is difficult to determine which oxidation reaction is superior for nitrite oxidation; however, chemical oxidation is supposed to be superior to biochemical oxidation for nitrite oxidation. The bacterial cells contained in the outflow were analyzed by TGGE.

TGGE Pattern

The bacterial communities contained in the outflow from the bioreactors with the ER-NR and EU-NR electrodes were analyzed *via* the TGGE technique. Each triple identical

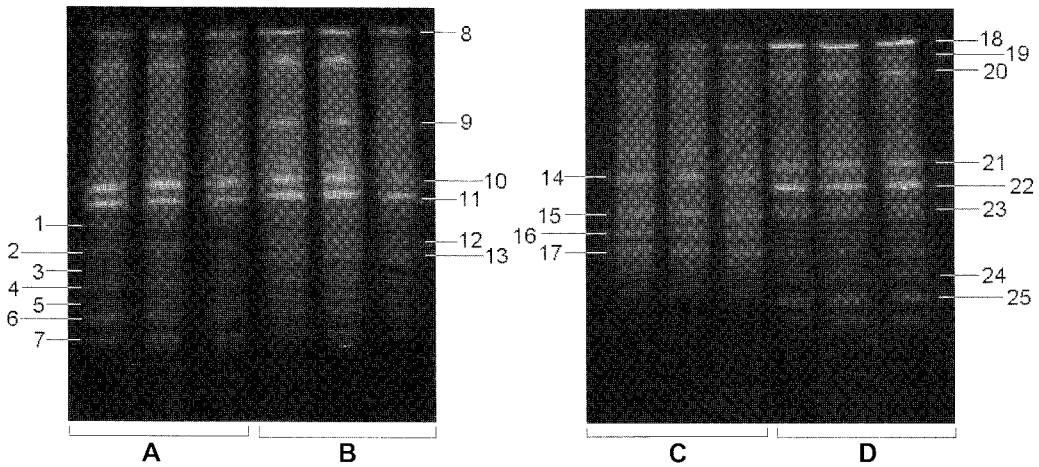

Fig. 5. TGGE patterns of 16S rDNA isolated from a bacterial community grown under conventional conditions (**A**) and electrochemical reduction conditions (**B**) on day 20 of incubation, and the conventional bioreactor (**C**) and electrochemical bioreactor (**D**) on day 50 of incubation.
Three ammonium-oxidizing bacteria were identified, but nitrite-oxidizing bacteria were not, *via* sequence homology of DNA extracted from the TGGE gel. Bands 8, 18, and 19 were identified with *Nitrosomonas* sp. (AB000702), *Nitrosomonas* sp. (AF363293), and an uncultured ammonia-oxidizing bacterium (AB239747.1), respectively. Other DNAs extracted from the TGGE gel were identified with uncultured bacteria.

TGGE patterns were obtained from triple identical electrophoresis as shown in Fig. 5A, 5B, 5C, and 5D. As demonstrated in Fig. 5, the TGGE pattern for DNA extracted from the bacterial community grown with the EU-NR-electrode (A, C) differed slightly from that grown with the ER-NR-electrode (B, D). Three ammonium-oxidizing bacteria were enriched in the ER-NR-electrode and one ammonium-oxidizing bacterium was maintained in the EO-NR electrode, based on the results of a DNA sequence homology analysis. The nitrite-oxidizing bacteria were not detected by the TGGE technique. Nitrite is oxidized chemically to nitrate *via* catalysis of the NR-electrode, on which the nitrite-oxidizing bacteria are likely to be selectively eliminated from the bioreactor as the result of energy source (nitrite) exhaustion.

Cyclic Voltammetry

An NR capable of mediating electrons between the electrode and bacterial cell may function as an electron donor [34]. Electron transfer from the electrode to bacterial cells was measured *via* cyclic voltammetry, based on the upper or lower peak height variation. The bacterial community utilized for cyclic voltammetry was obtained from a bioreactor operated continuously for 8 weeks. As is shown in Fig. 6, the upper peak height was increased in the reaction containing the aerobic bacterial community and ammonium, but was not increased in the other reactions. The increase in the upper peak indicates that electrons are transferred from the electrode to ammonium-oxidizing bacteria, but not to nitrite-oxidizing bacteria functioning in combination with NADH regeneration. The cyclic voltammograms obtained by the addition of nitrite to the reaction conditions with and without an aerobic bacterial community did not differ noticeably. These results present some possibilities: namely, that the electrode may react directly with nitrite; that the nitrite-oxidizing bacteria may not have been enriched in the continuous bioreactor; or that electrons were not transferred from the electrode to the nitrite-oxidizing bacteria.

Discussion

The redox potentials for the oxidation of ammonium to nitrite and the oxidation of nitrite to nitrate are +440 mV and +420 mV, respectively [10]. Ammonium is more

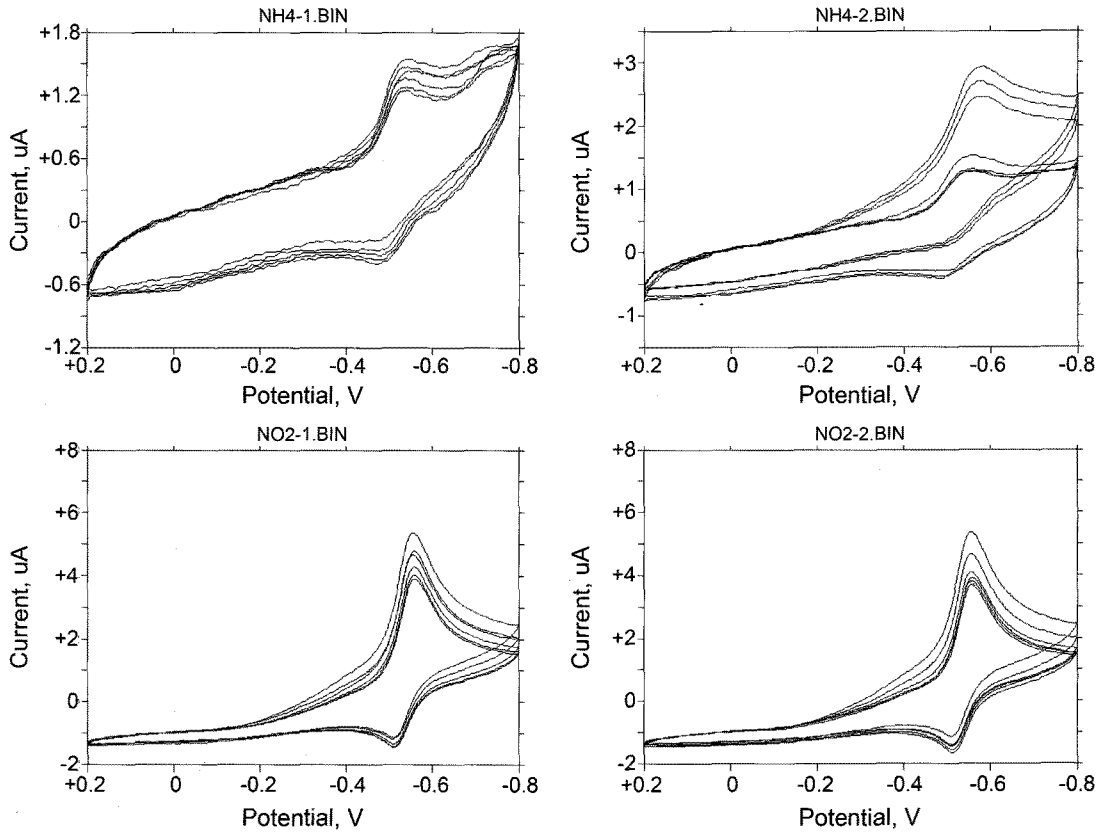

Fig. 6. Cyclic voltammograms to measure the electrochemical reactions between electrode and ammonium (NH4-1), electrode and nitrite (NO2-1), electrode and ammonium-oxidizing bacteria (NH4-2), and electrode and nitrite-oxidizing bacteria (NO2-2).
At the moment of switching from the 6th to 7th cycle, 100 mg/l of ammonium or nitrite was added to the reaction mixture without (NH4-1 and NO2-1) and with (NH4-2 and NO2-2) the aerobic bacterial community.

stable than nitrite, based on its redox potential. Nitrite has been previously reported to be chemically oxidized to nitrate [30]; however, the chemical oxidation of ammonium has not been thus far reported. Nitrite is a type of radical, and is thus naturally unstable. In the standard methods, combined treatment consisting of heating (autoclave) and chemical oxidant treatment have been used to nitrify ammonium and organic nitrogen [12], which suggests that extreme conditions involving high temperature and strong oxidation potential are requirements for the oxidation of ammonium. On the basis of this information, we applied a specially designed NR-electrode to the bioreactor in order to chemically or bioelectrochemically nitrify ammonium to nitrate using an aerobic bacterial consortium.

Ammonium-oxidizing bacteria are chemolithotrophic organisms, which oxidize ammonia to nitrite to generate ATP and reducing power (NADH) for carbon fixation *via* the Calvin cycle [38]. Ammonium oxidation is the first step, and an unusually rate-limiting reaction in the nitrification process, and functions as a critical link in the ecological nitrogen cycle [6]. Nitrite oxidation is the second step, and is absolutely dependent on the ammonium oxidizing activity and nitrite concentration, as nitrifying bacteria tend to be rather sensitive to variations in the concentration of the substrate [21, 22, 28]. In a bioreactor containing ammonium as the sole energy source, nitrite generated by ammonium-oxidizing bacteria must function as the sole energy source for nitrite-oxidizing bacteria. The chemical oxidation of nitrite may be a limiting factor in the growth and survival of nitrite-oxidizing bacteria. The biochemical oxidation of ammonium and nitrite proceeds *via* oxygen-dependent respiration, in which ATP is regenerated by the forward electron transport system but NADH is regenerated by the reverse electron transport system [10]. Forward and reverse electron transport may be controlled by the NADH/NAD^+ and ATP/ADP balance. The NR-electrode functions as a solid electron mediator [15], by which electrons can be transferred from the electrode to bacterial cells under electrochemical reduction conditions, or from bacterial cells to the electrode under electrochemical oxidation conditions, in combination with NAD^+ reduction or NADH oxidation [19, 20]. Ammonium-oxidizing bacteria were shown to be unable to grow on the EO-NR-electrode, because the NADH regenerated by reverse electron transport could be electrochemically oxidized to NAD^+. On the other hand, the ER-NR-electrode was shown to induce the ammonium-oxidizing bacteria to regenerate NAD^+ to NADH. In this reaction, the forward electron transport system in the ammonium-oxidizing bacteria may be activated in combination with ATP regeneration. Electrochemical NADH regeneration makes it possible to induce biochemical ATP regeneration in chemolithotrophic bacteria to obtain free energy for the Calvin cycle, in which 6 moles of NADH and 9 moles of ATP are generated for every mole of

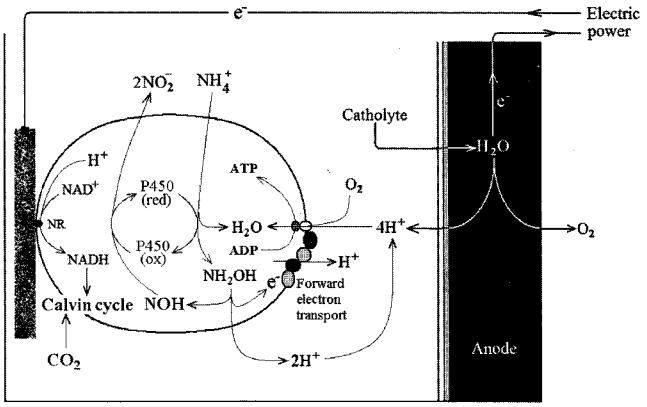

Fig. 7. The proposed mechanism of biochemical ammonium oxidation to nitrite *via* an ammonium-oxidizing bacterium immobilized on the NR-electrode.
NADH may be regenerated electrochemically by the ER-NR-electrode, and ATP may be regenerated biochemically *via* forward electron transport. Ammonium oxidation may be activated in combination with forward electron transport. This mechanism was designed on the basis of the metabolic oxidation of ammonium to nitrite, as described in a relevant text [10] and in previous experimental results.

triphosphate production [24]. It is likely that autotrophic carbon dioxide fixation is required for the control of NADH/NAD^+ balance and cytoplasmic redox balance. One of the functions of the Calvin cycle is to operate as an electron sink or an alternative electron acceptor, thus dissipating excess reducing power [23, 35, 36]. This presents

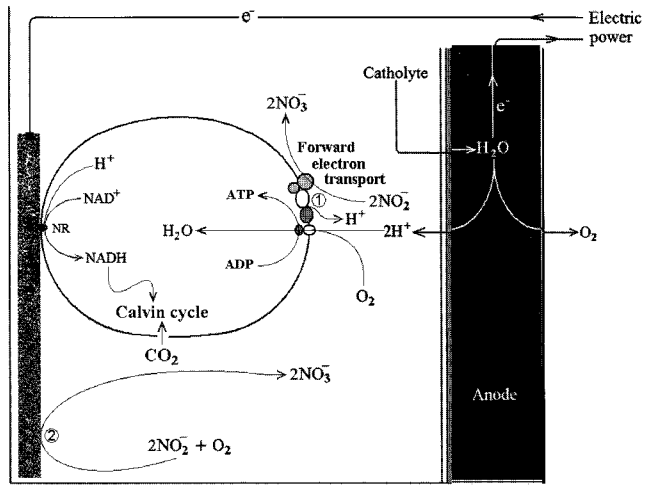

Fig. 8. Proposed mechanism of biochemical nitrite oxidation to nitrate by nitrite-oxidizing bacterium immobilized in the NR-electrode (1).
NADH may be regenerated electrochemically by the ER-NR-electrode and ATP is regenerated chemically *via* forward electron transport. This mechanism was designed on the basis of the metabolic oxidation of nitrite to nitrate, as described in a relevant text [10]. The majority of nitrite may be oxidized chemically to nitrate *via* catalysis of the NR electrode under aerobic conditions (2). This mechanism was designed on the basis of the metabolic oxidation of ammonium to nitrite as described in a relevant text [10] and by previous experimental results.

a logical basis for the explanation that the ammonium-oxidizing bacteria were enriched and the ammonium oxidation efficiency was increased in the ER-NR-electrode during continuous cultivation (Fig. 5), as is shown in Fig. 7.

The reaction from ammonium to nitrite is catalyzed by bacterial metabolism, and the reaction of nitrite to nitrate can be either biochemically or chemically catalyzed *via* bacterial metabolism and an NR electrode. However, the results of both TGGE and cyclic voltammetry suggest that it is more possible to oxidize nitrite to nitrate *via* an electrochemical reaction than a biochemical reaction, as is shown in Fig. 8. Biochemical nitrite oxidation could not be verifiably achieved by the electrochemical technique; however, nitrate was verifiably generated from ammonium by an aerobic bacterial community under conventional conditions during the maintenance period of a nitrifying reactor (data not shown). Accordingly, nitrite-oxidizing bacteria are expected to abrogate their biochemical functions on an ER-NR-electrode as the result of substrate exhaustion.

In this study, we discovered that biochemical ammonium oxidation could be activated *via* catalysis with an ER-NR-electrode, and that chemical nitrite oxidation could be activated by an EO-NR-electrode under aerobic conditions. This novel discovery may prove applicable in the development of green technologies for carbon dioxide fixation using solar energy under aerobic conditions.

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