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Bioelectrochemical Denitrification by *Pseudomonas* sp. or Anaerobic Bacterial Consortium

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Abstract In a bacterial denitrification test with *Pseudomonas* sp. and anaerobic consortium, more nitrates and less substrate were consumed but less metabolic nitrite was produced under an anaerobic H_2 condition rather than under N_2 condition. In a bioelectrochemical denitrification test with the same organisms, the electrochemically reduced neutral red was confirmed to be a substitute electron donor and a reducing power like H_2. The biocatalytic activity of membrane-free bacterial extract, membrane fraction, and intact cell for bioelectrochemical denitrification was measured using cyclic voltammetry. When neutral red was used as an electron mediator, the electron transfer from electrode to electron acceptor (nitrate) via neutral red was not observed in the cyclic voltammogram with the membrane-free bacterial extract, but it was confirmed to gradually increase in proportion to the concentration of nitrate in that of the membrane fraction and the intact cell of *Pseudomonas* sp.

Key words: Anaerobic bacterial consortium, bioelectrochemistry, cyclic voltammetry, neutral red, nitrate reduction, *Pseudomonas* sp.

Bacterial denitrification is a dissimilatory nitrate reduction, which involves four metabolic steps where nitrate is sequentially reduced to nitrogen via nitrite, nitric oxide, and nitrous oxide [1, 8]. The dissimilatory nitrate reduction is influenced by the concentration of electron donor and acceptor since the ratio of carbon to nitrogen (C/N ratio) has to be more than about 3 [3, 16]. This is a reason why organic compounds such as methanol or acetate have been added to the wastewater treatment bioreactor for denitrification [13, 18]. H_2 can be used as a substitute electron donor in the wastewater treatment system for denitrification but is difficult to handle and potentially dangerous because it is explosive. Electric energy, therefore, has been of continuous interest as a substitute for H_2 in various bioreactors such as bacterial desulfurization of benzothiophene [9], succinate fermentation [10], and amino acid fermentation [2]. Biological oxidation of H_2 [14] is coupled to the reduction of electron carriers, including NAD^+ [16], cytochromes [17], and quinones [4]. For bioelectrochemical denitrification, electrons must be transferred from the electrode to electron carriers such as NAD^+, quinone, and cytochromes. However, the biological membrane prevents electron transfer between the electrode and bacterial electron carriers. Investigation of the oxidation-reduction relationship between the redox dye and the biological system by the electrochemical technique [15] is useful for understanding bioelectrochemical denitrification. Cyclovoltammetry has been used to measure electron transfer between electrode and redox enzymes such as cytochrome c, electrode and redox compounds such as neutral red, or from electrode to redox enzyme via neutral red [10, 11]. Cyclic voltammograms show information about electron transfer between electrode and redox compounds such as redox dyes, redox enzymes, and modified intact cells with redox dyes, as a current peak. An oxidation peak shows that electrons transfer from redox compounds to the electrode, but a reduction peak shows that electrons transfer from the electrode to the redox compounds. Redox dyes for bioelectrochemical systems must easily react with both electrode and biological electron carriers such as nitrite reductase [21] and nitrate reductase [22]. Neutral red is a better redox dye than others, such as methyl viologen, benzyl viologen, or thionine, for electron transfer between electrode and bacterial intact cells modified with redox dyes because it has special function as an electronophore [10, 11].

In this study, we describe how neutral red can be a substitute electron donor for bacterial denitrification instead of H_2, and propose a possibility that cytoplasmic membrane isolated from *Pseudomonas* may catalyze the electrochemical reduction of nitrate or nitrite with neutral red as an electron mediator, by using the cyclovoltammetric technique *in vitro*.

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MATERIALS AND METHODS

All data were means based on values obtained in triplicate experiments and were within 1 standard deviation of each other.

Organisms and Medium

Pseudomonas sp. or an anaerobic bacterial consortium was the biocatalyst for denitrification. *Pseudomonas* sp. was obtained from Graduate School of Biotechnology in Korea University. Supernatant of an anaerobic digestive sludge was used as an anaerobic bacterial consortium. *Pseudomonas* sp. and an anaerobic bacterial consortium cultivated in phosphate buffered basal medium (PBBM) [5] prepared with 10 g/l of sodium lactate and 3.5 g/l of potassium nitrate were used as an inoculum for the denitrification test, respectively. The medium was prepared without phosphate, brought to pH 7.2 with NaOH, boiled, sparged with N_2-CO_2 (8:2), dispensed into 158-ml serum vials, sealed with butyl rubber stoppers, and finally, autoclaved. Phosphate, vitamin solution, and 2 atm N_2-CO_2 (8:2) were added after autoclaving. All procedures for medium preparation, inoculation, and cultivation were performed under anaerobic N_2 condition.

Bacterial Growth for Denitrification

Five % (v/v) culture of *Pseudomonas* sp. or anaerobic bacterial consortium was inoculated into and grown in 158-ml serum vials containing 80 ml of PBBM medium with 714 mg/l of potassium nitrate (100 mg/l as nitrogen), respectively. H_2 (99.8%; 2 atm) was pressed into serum vials, while serum vials with N_2 (99.5%; 2 atm) instead of H_2 were prepared for the control test.

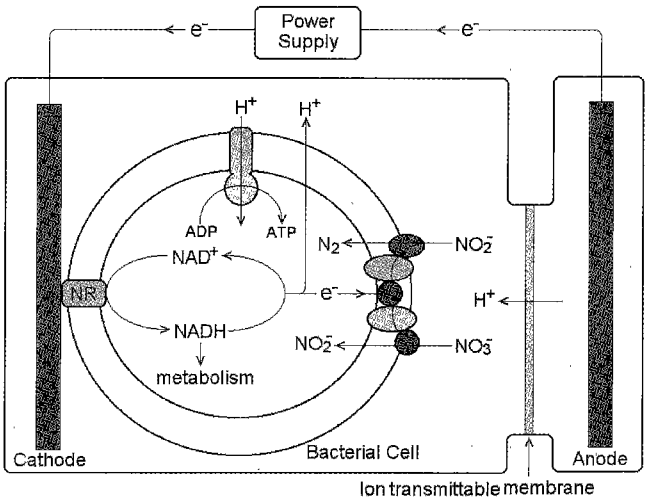

Fig. 1. Schematic mechanism of bioelectrochemical denitrification, and schematic diagram of an electrochemical system for the bacterial denitrification.
The cathode compartment was separated from the anode compartment by an ion transmittable membrane. Culture medium and 100 mM phosphate buffer (pH 7.0) with 100 mM NaCl were used as a catholyte and anolyte, respectively.

Electrochemical Reactor System

Electrochemical reactors specially designed for autoclaving, maintaining anaerobic conditions, and growing bacteria were made from Pyrex glass. The electrochemical system was separated into anode and cathode compartments by an ion transmittable membrane (Fig. 1). Chemicals and metabolite cannot be transferred across the membrane. Both the anode and cathode were made from finely woven graphite felt (10 mm thick; 0.47 m^2g^{-1} available surface area, Electrosynthesis, New York, U.S.A.). The weight of both anode and cathode was 4.5 g. The current and voltage between the anode and cathode were measured with a precision multimeter (Fluke model 8842A, Washington, U.S.A.).

Bioelectrochemical Denitrification

Five % (v/v) *Pseudomonas* sp. or anaerobic bacterial consortium was inoculated into and grown in the cathode compartment of electrochemical bioreactor systems containing 300 ml of PBBM medium with 714 mg/l of potassium nitrate (100 mg/l as nitrogen) and 100 μM of neutral red, respectively, under anaerobic N_2 condition (99.5%, 1 atm). Neutral red is one of the useful electron mediators for electron transfer between electrode and bacterial intact cells [10, 11, 12]. The culture medium (PBBM with nitrate and neutral red) and 100 mM of phosphate buffer (pH 7.0) with 100 mM of NaCl were used as a catholyte and anolyte, respectively. The potential between anode and cathode was adjusted to 1.5 volt, enough for electrochemical reduction of neural red, and the current was a variable from 3 to 8 mA that is dependent on biomass, nitrate concentration, or substrate concentration.

Nitrate and Nitrite Analysis

Nitrate and nitrite were analyzed using a auto-analyzer (Altken Model Slow Solution O^{IV}, Chicago, U.S.A.) and the concentration was calculated using a standard calibration curve that was previously made.

Lactate Analysis

Lactate concentration was analyzed by using HPLC (Waters Model 510, Milford, U.S.A.) equipped with a RI detector and Aminex HPX-87H ion exchange column (Bio-Rad, Burlington, U.S.A.). Bacterial culture was centrifuged at 20,000 ×g for 30 min and filtrated using membrane filter (pore sized, 0.22 mm), which was used as a sample for analysis. The concentration was calculated using a standard calibration curve that was previously made.

Isolation of Membrane from *Pseudomonas* sp.

The membrane of *Pseudomonas* sp. was isolated at 4°C under anaerobic N_2 atmosphere as described previously

[11]. The purified membranes were obtained from the cell extracts by centrifugation at 100,000 ×g for 90 min. The supernatant was decanted and saved as the membrane-free extract. The clear brown precipitate was washed twice with 50 mM phosphate buffer (pH 7.0) and resuspended in the same buffer by homogenization. The precipitate resuspended in 50 mM of phosphate buffer was used for bioelectrochemical denitrification as an isolated membrane.

Cyclic Voltammetry

The cyclic voltammetry was performed as described previously [6]. A 3-mm diameter glassy carbon working electrode (BAS), a platinum wire counter electrode (BAS), and Ag/AgCl reference electrode (BAS) were used in an electrochemical cell with a working volume of 3 ml. Cyclic voltammetry was performed with a cyclic voltammetric potentiostat (BAS model CV50W, U.S.A.). The cell was closed with a Teflon cap through which the solution could be flushed with purified nitrogen.

Results and Discussion

Sugar, alcohol, organic acid, or H_2 are electron donors for nitrate respiration [18]. The electron donors are metabolically oxidized, by coupling to reduction of NAD^+ to NADH or other electron carriers such as $FADH_2$. Nitrate or nitrite are reduced to N_2 via the electron transport system with NADH as an electron donor. The NADH/NAD^+ ratio in cytoplasm affects respiration because NADH is a primary electron donor for most of the electron transport systems. A microbial hydrogenase [23] catalyzes H_2 oxidation coupled to reduction of NAD^+ to NADH [20]. By adding H_2 to a bioreactor containing H_2-oxidizing bacteria, an extra reducing power can be produced, and metabolism for consuming the extra reducing power can be activated. In cultivation of *Peudomonas* with H_2 as an extra electron donor, the nitrate consumption, bacterial growth, metabolic nitrite production, and lactate consumption were 42% higher, 45% higher, 52% lower, and 15% lower than in cultivation without H_2 (Figs. 2A and 2B), respectively. In cultivation of anaerobic bacterial consortium with H_2, the nitrate consumption, metabolic nitrite production were 35% higher, 40% lower and 9% lower than in cultivation without H_2 (Fig. 3A and 3B), respectively. Theoretically, the respiration can be activated under high NADH/NAD^+ ratio because an electron acceptor such as nitrate is reduced by coupling to oxidation of NADH to NAD^+. Neutral red can be electrochemically reduced and reoxidized by coupling to a reducing electron carrier such as NAD^+ in cytoplasm [10]. The NADH/NAD^+ ratio was reported to increase by extracellular reducing agents such as electrochemically reduced neutral red [11] or biologically reduced [7] agents. To confirm the function of neutral red on bacterial denitrification, we tested a

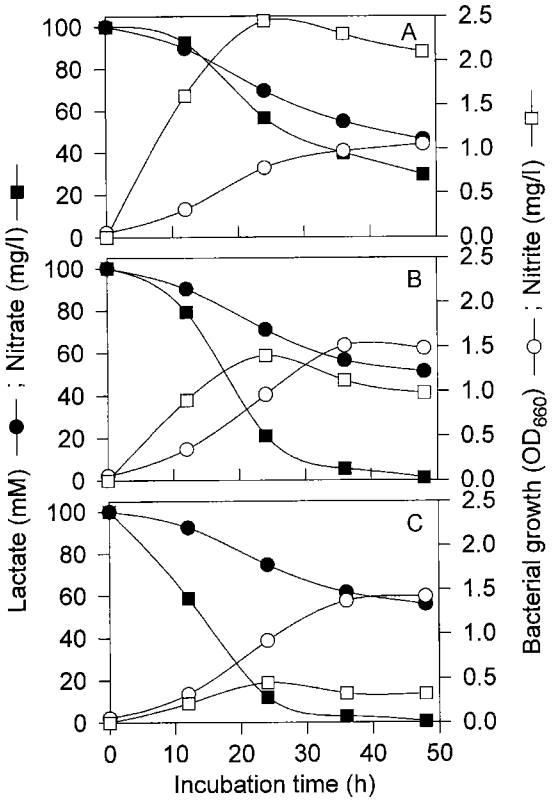

Fig. 2. Effects of electrochemically reduced H_2 and NR on metabolic nitrate reduction of *Pseudomonas* sp. Nitrite was produced by bacterial metabolism but was not added to the reactor.

N_2 was used to keep the anaerobic condition of reactor (A), and electrochemically reduced H_2 (B) and NR (C) were additional reducing power for bacterial denitrification. The N_2 and H_2 were continuously supplied to reactors for maintenance of 15 psi gas pressure by an automatic gas supplier.

possibility that reduced neutral red, like H_2, may be a substitute electron donor for bacterial denitrification. In bioelectrochemical cultivation of *Pseudomonas* with neutral red as an extra electron donor, the nitrate consumption, bacterial growth, metabolic nitrite production, and lactate consumption were 35% higher, 38% higher, 84% lower, and 20% lower than in cultivation in normal condition without neutral red, respectively (Fig. 2C). In bioelectrochemical cultivation of the anaerobic consortium with neutral red as an extra electron donor, the nitrate consumption, metabolic nitrite production, and lactate consumption were 60% higher, 95% lower, and 55% lower than in growth without neutral red (Fig. 3C). From these results, we can conclude that the electrochemically reduced neutral red, like H_2, can be an electron donor for denitrification, and an energy source for free energy production, which was bioelectrochemically verified by cyclic voltammetry. The possibility of electrons transferring from the electrode to the membrane-free cell extract, membrane, or intact cells of *Peudomonas* sp. via neutral red was tested with cyclic voltammetry. In

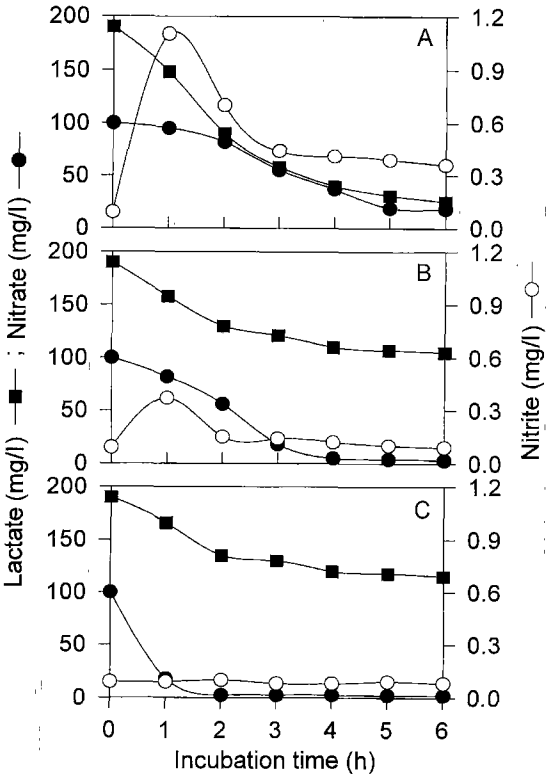

Fig. 3. Effects of electrochemically reduced H_2 and NR on metabolic nitrate reduction (A), nitrite reduction (B), and COD variation (C).

The anaerobic digestive sludge was used as a biocatalyst. Nitrite was produced by bacterial metabolism but was not added to the reactor. N_2 was used to keep the anaerobic condition in the reactor. H_2 and NR electrochemically reduced were additional reducing power for bacterial metabolism. The N_2 and H_2 were continuously supplied to the reactor for maintenance of 15 psi gas pressure by an automatic gas supplier.

cyclic voltammetry (Fig. 4), the lower and upper peaks located between −0.4 and −0.6 volt indicated the oxidation and reduction potential (versus Ag/AgCl) of neutral red, respectively. The upper and lower peaks indicated reduction and oxidation peaks, respectively. Theoretically, the reduction peak can be produced when electrons are transferred from the electrode to neutral red, whereby neutral red is reduced, but the oxidation peak can be produced when electrons are transferred from the reduced neutral red to electrode. The reduced neutral red can be reoxidized by being coupled to reduce NAD to NADH, and oxidized neutral red can be reduced by the electrode. Some bacteria can use H_2 as an electron donor and H_2 can be oxidized by being coupled to bacterial hydrogenase catalysis of NAD to NADH. This shows that reduced neutral red can function as an electron donor, like H_2. Without a biocatalyst and an electron acceptor such as nitrate, the reduced neutral red can not be reoxidized and electron transfer from electrode to neutral red can not be performed. The electron transfer from electrode to neutral red or neutral red to electrode can be converted

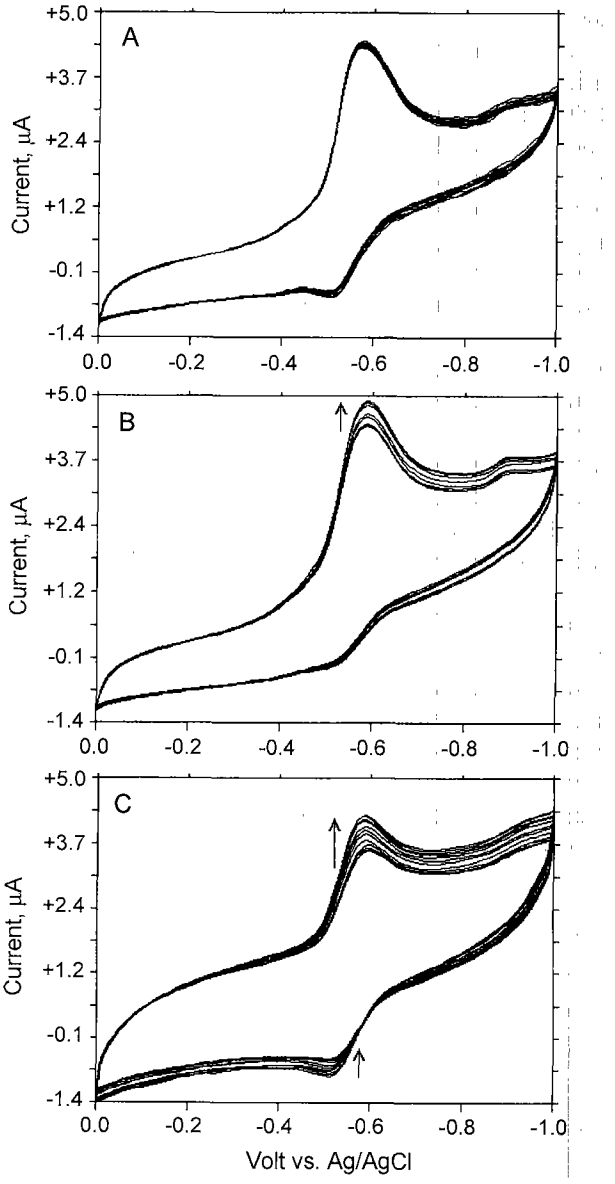

Fig. 4. Cyclic voltammograms measured with glassy carbon electrode during 30 successive cycles of reactants containing 50 mM phosphate buffer (pH 7.0), 100 μM NR, and 5 mM NaCl on either membrane-free extract (A, 0.82 mg/ml protein), purified membrane (B, 0.86 mg/ml protein), or intact cell suspension of *Pseudomonas* sp. (C, OD_{660}, 2.8).

Glassy carbon, platinum wire, and Ag/AgCl were the working electrode, counter electrode, and reference electrode, respectively. The total working volume was 3.0 ml and scan rates were 25 mV/s. Arrow marks indicate that the current was increased after the electron acceptor (nitrate 100 mM) or NAD^+ (10 μM) was added to the reactants. The current in CV of membrane-free extract was not increased even with NAD^+ and that in CV of whole cell was increased even without NAD^+, but that in CV of purified membrane was increased only with NAD^+.

to a signal, which is recorded on the cyclic voltammogram. NO peaks in the cyclic voltammogram or no change of peak height means that no electrons can be transferred from the electrode to the bacterial cell or electron acceptor.

By using cyclic voltammetry, the redox potential of specific compounds can be measured and electron transfer efficiency in a specific reaction, such as denitrification, dehydrogenation, oxidation, and reduction, can be estimated. The increase of upper peak height means that electrons were transferred from the electrode to the biocatalyst-nitrate complex (enzyme-substrate complex) via neutral red. The upper peak height in cyclic voltammetry of the membrane-free cell extract was not shifted by adding nitrate (Fig. 4A), but the upper peak height in cyclic voltammetry of membranes (Fig. 4B) and intact cells (Fig. 4C) of *Peudomonas* gradually increased by adding nitrate. This indicates that nitrate reductase of *Peudomonas* sp. can catalyze nitrate reduction with electrochemical reducing power both *in vitro* and *in vivo*. The significance of this observation is that membrane-bound proteins can catalyze only the reduction of nitrate to nitrite by using the reducing power of neutral red instead of H_2, but it can not catalyze the oxidation of nitrite to nitrate. As shown in Fig. 5A, electrons can not transfer from electrode to nitrate via neutral red without enzymes, and they can only transfer from electrode to nitrate via neutral red by the catalytic reactions of enzymes, such as respiration enzymes and nitrate reductase which are membrane-bound proteins (Figs. 5B and 5C). The increasing electron transfer (current) from electrode to nitrate increased the peak height in the cyclic voltammograms, as shown in Figs. 4B and 4C. Decrease of the lower peak height in cyclic voltammetry of intact cell suggests that some reduced bacterial compounds were oxidized on electrode coupling with oxidation of neutral red. The conclusion obtained from this study is that neutral red can be an electron donor in substitution of other substrates such as lactate and H_2, and bacterial nitrate reduction can be electrochemically activated with neutral red [9, 10].

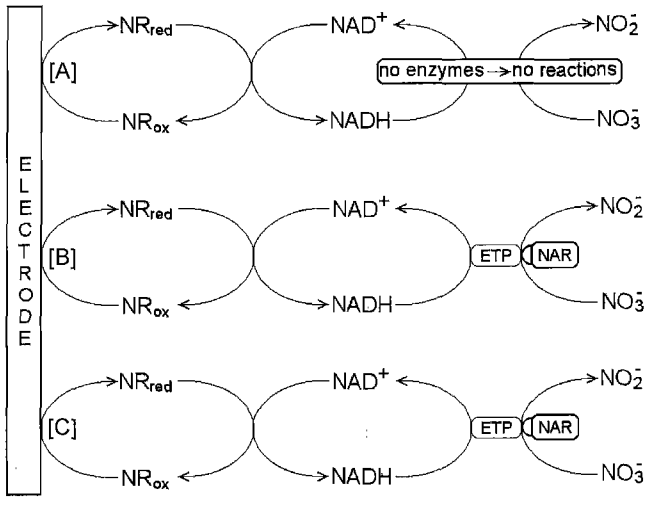

Fig. 5. Schematic diagram of electron transfer from electrode to nitrate reductase via neutral red, by which nitrate can be reduced to nitrite, and electrons can be continuously transferred from electrode to nitrate.

Since the membrane-free cell extract does not have nitrate reductase (NAR) and enzymes for the electron transport system (ETP), nitrate can not be reduced to nitrite and electrons cannot transfer from electrode to nitrate (A), but electrons can transfer from electrode to nitrate via neutral red (B and C), which shows the different cyclic voltammetric peaks as shown Figs. 4A, 4B, and 4C. NR_{ox} and NR_{red} are oxidized neutral red and reduced neutral red, respectively.

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