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Microbial Utilization of Electrically Reduced Neutral Red as the Sole Electron Donor for Growth and Metabolite Production

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Electrically reduced neutral red (NR) served as the sole source of reducing power for growth and metabolism of pure and mixed cultures of H_2-consuming bacteria in a novel electrochemical bioreactor system. NR was continuously reduced by the cathodic potential (−1.5 V) generated from an electric current (0.3 to 1.0 mA), and it was subsequently oxidized by *Actinobacillus succinogenes* or by mixed methanogenic cultures. The *A. succinogenes* mutant strain FZ-6 did not grow on fumarate alone unless electrically reduced NR or hydrogen was present as the electron donor for succinate production. The mutant strain, unlike the wild type, lacked pyruvate formate lyase and formate dehydrogenase. Electrically reduced NR also replaced hydrogen as the sole electron donor source for growth and production of methane from CO_2. These results show that both pure and mixed cultures can function as electrochemical devices when electrically generated reducing power can be used to drive metabolism. The potential utility of utilizing electrical reducing power in enhancing industrial fermentations or biotransformation processes is discussed.

Several microorganisms (e.g., *Escherichia* and *Actinobacillus*) grow with H_2 as the electron donor and reduce fumarate into succinate in an anaerobic respiration process (13, 16, 24). These bacteria obtain free energy and reducing power from the electron driving force generated by the E_o' difference between the coupled oxidoreduction half-reactions of [$2H^+/H_2$] and [fumarate/succinate].

Methanogens are strict anaerobic bacteria that can couple H_2 or HCOOH oxidation to CO_2 reduction into methane (26). Methanogenesis produces less free energy than other anaerobic respiration processes (e.g., fumarate, nitrate, or sulfate reduction) because the E_o' difference between the oxidation-reduction half-reactions of [$2H^+/H_2$] and [CO_2/CH_4] is −0.17 V, from which about −34 kJ of $\Delta G^{\circ\prime}/H_2$ is produced (23).

Hydrogen oxidation by microbial hydrogenases (25) can be coupled to reduction of different biological electron carriers, including NAD^+ (22), cytochromes, and quinones (8, 14, 29), or to certain artificial redox dyes, such as methyl-viologen and neutral red (NR) (2, 10). The influence of redox dyes, with or without electrochemical reduction systems, on altering metabolite patterns and H_2 production has been examined in several microbial processes, including the glutamate (7), butanol (4, 11), and butyrate (20) fermentations. These investigations did not determine whether electrically reduced dyes could serve as the sole electron donor for driving growth and cellular metabolism.

The energetics of living systems is driven by electron transfer processes (18). Electrons are transferred from a substrate that becomes oxidized to a final acceptor that becomes reduced. This observation implies that it may be possible to control or alter metabolism by plugging biochemical processes into an external electrochemical system. Previously, an electrochemical system (17) was used to generate reduced iron for *Thiobacillus ferrooxidans* growth on electrical reducing power. We have previously shown (19) that electrically reduced NR could serve as an extra electron donor and enhance growth and succinate production from glucose by *Actinobacillus succinogenes*. We also reported (19) that this organism contained membrane-bound hydrogenase and fumarate reductase and that electrically reduced NR could chemically reduce NAD^+ and replace menaquinone as the electron donor for fumarate reductase.

The purpose of the present report is fourfold: first, to show the utility of this novel electrochemical reactor for replacing H_2 with electrically reduced NR as the sole electron donor for microbial growth and metabolite production; second, to show that an *A. succinogenes* mutant that lacks key oxidoreductases and that cannot grow on fumarate alone can grow on either H_2 plus fumarate or on electrically reduced NR plus fumarate; third, to show that in mixed methanogenic cultures electrically reduced NR can replace H_2 in CO_2 reduction to methane; and finally, to discuss the potential industrial utility of this novel electrochemical reactor system for enhancing microbial fermentation and transformation processes.

MATERIALS AND METHODS

***A. succinogenes* growth and metabolic analysis.** *A. succinogenes* strains 130Z and FZ-6 were obtained from our culture collection at Michigan Biotechnology Institute International (Lansing, Mich.). Bacteria were grown in 158-ml serum vials with butyl rubber stoppers containing 40 ml of medium with fumarate under a N_2 gas phase (100%; 20 lb/in^2). Traces of oxygen were removed by passing the gas over heated (370°C) copper filings. Growth medium A contained the following (per liter of double-distilled water): yeast extract, 5.0 g; $NaHCO_3$, 10.0 g; $NaH_2PO_4 \cdot H_2O$, 8.5 g; and Na_2HPO_4, 15.5 g. The pH was adjusted to 7.0 after autoclaving. Fumarate (final concentration, 50 mM) was added aseptically to the medium after autoclaving. Na_2S (final concentration, 0.02%) was added to establish strict anoxic conditions. Media were inoculated with 3.0% (by volume) samples of cultures grown in the same medium and incubated at 37°C for 24 (strain 130Z) or 48 (strain FZ-6) h. The culture samples were aseptically and anaerobically removed with 3-ml syringes. Glucose, fumarate, succinate, acetate, ethanol, and formate concentrations in the cultures were determined by high-performance liquid chromatography (5). The components were analyzed chromatographically by elution with 0.006 M H_2SO_4 from a cation-exchange resin in the hydrogen form.

Enzymatic analysis. All procedures for bacterial cultivation, cell extract preparation, and enzyme activity measurement were done under a strict anaerobic N_2

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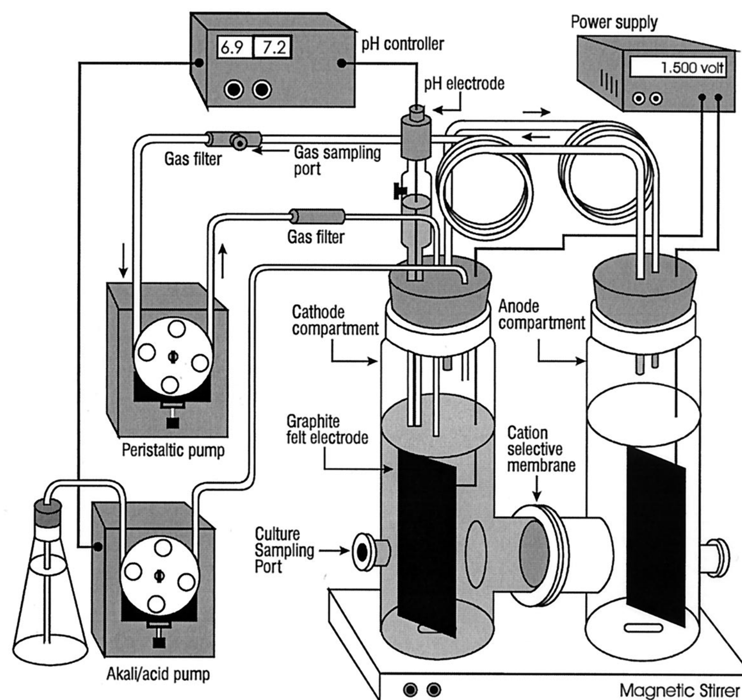

FIG. 1. Schematic of the electrochemical bioreactor system used (19). Cells were placed in the cathode compartment. In electricity-dependent succinogenesis, the gassing system was not used and the pH was held constant. For electricity-dependent methanogenesis, a sulfide reducing agent was added in the anode compartment. The electrical potential resulted in the following half-reactions in the anode and cathode compartments: succinogenesis, $H_2O \rightarrow 2H^+ + 2e^- + 1/2O_2$ (anode reaction), $2NR^+ + 2H^+ + 2e^- \rightarrow 2NRH$ (cathode reaction), $2NRH + 2X^+ \rightarrow 2NR^+ + 2XH$ (electron mediation), $2XH$ + fumarate $\rightarrow 2X^+$ + succinate (succinogenesis); and methanogenesis, $S^{2-} \rightarrow SO_4^{2-} + 8e^- + 8H^+$ (anode reaction), $8NR^+ + 8e^- + 8H^+ \rightarrow 8NRH$ (cathode reaction), $8NRH + 8X^+ \rightarrow 8NR^+ + 8XH$ (electron mediation), $8XH + CO_2 \rightarrow 8X^+ + CH_4 + 2H_2O$ (methanogenesis). X, biological electron carrier(s).

atmosphere as described previously (24). Dithiothreitol was used as a chemical reductant. *A. succinogenes* 130Z and FZ-6 were anaerobically cultivated on medium A (5) containing 2% glucose in a 4-liter carboy under a N_2-CO_2 (80:20) atmosphere. Cells grown for 16 h were harvested by centrifugation at 5,000 × *g* at 4°C for 30 min and washed three times with 100 ml of 50 mM phosphate buffer (pH 7.2). The washed cells were resuspended in the same buffer and disrupted with a French press at 2,000 lb/in^2 and 4°C. The cell debris was removed by centrifuging the cells twice at 40,000 × *g* and 4°C for 30 min. The clear, light-brown supernatant was used as an extract for enzyme assays. D-Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12; NAD^+ dependent) activity was measured as previously described (5). Fumarate reductase (EC 1.3), formate dehydrogenase (EC 1.2), hydrogenase (EC 2.12.2.1), and malate dehydrogenase (EC 1.1.1.37) (24); pyruvate dehydrogenase (EC 1.2.2.2) (21); and pyruvate formate-lyase (EC 2.3.1.54) (12) activities were measured as described previously. The oxidation and reduction reactions of pyridine nucleotide were measured spectrophotometrically at 340 nm (ε_{334} = 6.23 mM^{-1} cm^{-1}). Methyl viologen and benzyl viologen reductions were spectrophotometrically measured at 578 nm. The millimolar extinction coefficients (ε_{578}) of methyl viologen and benzyl viologen were 9.78 and 8.65 mM^{-1} cm^{-1}, respectively.

Electrobiochemical reactor systems. *A. succinogenes* 130Z and FZ-6 were grown in an electrochemical bioreactor system containing 40 ml of medium A (24) with 50 mM fumarate, 100 μM NR, and a N_2 headspace (99.5%; 1 atm). All procedures for medium preparation, inoculation, and cultivation were the same as those for vial cultures except that Na_2S was not used because the medium was electrically reduced. The current and potential between the anode and cathode were 0.3 to 0.35 mA and 1.5 V. Anaerobic culture samples were aseptically removed with 3-ml syringes. Cells grew suspended in the liquid medium and self-immobilized on the cathode. Cell growth in the liquid was determined spectrophotometrically by measuring the optical density at 660 nm. The growth of cells adsorbed on the cathode was calculated by measuring bacterial protein concentration. The protein concentration was converted to optical density by using a predetermined calibration curve (bacterial density = protein concentration [in milligrams per milliliter] × 1.7556). Bacterial cells that were adsorbed on the cathode were washed three times with 100 ml of phosphate buffer (50 mM; pH 7.0) for 30 min to remove medium compounds. A bacterial lysate was obtained from the electrodes after alkaline treatment at 100°C for 10 min in 1 N NaOH. The protein concentration of the bacterial lysate was determined by using a calibration curve (protein concentration [in milligrams per milliliter] = A_{595} × 1.3327) with the Bradford reagent (Bio-Rad, Hercules, Calif.) after cell debris was removed from the lysate by centrifugation at 10,000 × *g* and 4°C for 30 min.

The electrochemical bioreactor system (ECBS) was specially designed for cultivating strictly anaerobic bacteria (Fig. 1). It was made from Pyrex glass by the Chemistry Department, Michigan State University (East Lansing). The ECBS was separated into anode and cathode compartments by a cation-selective membrane septum (Nafion; diameter, 22 mm; 3.5 Ω cm^{-2} when measured in 0.25 N NaOH; Electrosynthesis, Lancaster, N.Y.). No chemicals or metabolites can be transferred across the Nafion membrane except protons or cations. The anode and cathode were made from fine woven graphite felt (6 mm thick; 0.47 m^2 g^{-1} available surface area) (Electrosynthesis). The electrodes were connected to the power supply (model 1825; Cole-Parmer, Vernon Hills, Ill.) with platinum wire (diameter, 0.5 mm; <1.0 Ω cm^{-2}; Sigma, St. Louis, Mo.). The platinum wire was connected to the graphite felt electrodes with graphite epoxy (<1.0 Ω cm^{-2}; Electrosynthesis). The electrical resistance between anode and cathode was <10.0 Ω. The total volume in each ECBS compartment was 70 ml, and the liquid volume was 40 ml. The weight of both electrodes was adjusted to 0.4 g (surface area, 0.188 m^2). The voltage and current between the anode and cathode were measured with a precision multimeter (model 45; Fluka, Everett, Wash.) and were adjusted to 1.5 V and 0.4 to 1.5 mA, respectively. For *Actinobacillus* experiments, the anode and cathode compartment headspaces were separated and filled with O_2-free N_2. For the methanogenesis experiments, the two CO_2-filled (initially 99.5%; 1.2 atm) compartment headspaces were connected by two stainless steel tubing sets (each 4.0 mm inside diameter; 40 cm long), and the gas phases were equilibrated by continuous circulation at a 25 ml/h flow rate. The cells were grown in the cathode compartment. Medium A with fumarate (15) was used for *A. succinogenes* FZ-6, and phosphate-buffered basal medium (PBBM) (9) was used for methanogenic granules. The culture medium was used as the catholyte, and 100 mM Na-phosphate buffer (pH 6.0) with 100 mM NaCl was used as the anolyte. Reducing agent was added to the anode compartment only for growth of methanogenic granules. The NR concentration was 100 μM. The electrode potential in the cathode generated a half-reaction for NR reduction. Hydrogen was not detected in the cathode because it cannot be produced at an E_o' of −0.325 V.

Methanogenic granules growth and metabolic analysis. Methanogenic granules containing mixed cultures of fatty acid-degrading syntrophiles and methanogens were obtained from a bench scale anaerobic sludge reactor fed on a mixture of 50 mM [each] acetate, butyrate, and propionate at Michigan Biotechnology Institute International (27, 28). Methanogenic granules were cultivated in

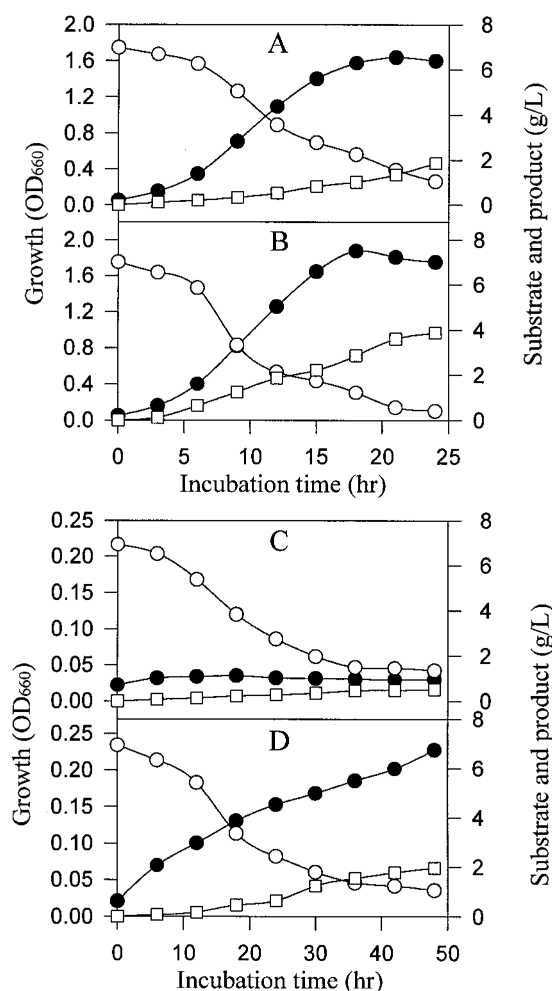

FIG. 2. Growth and succinate production by the *A. succinogenes* type strain 130Z on fumarate-N_2 (A) and on fumarate-H_2 (B) and by mutant strain FZ-6 on fumarate-N_2 (C) and on fumarate-H_2 (D). ●, cell growth; ○, fumarate; □, succinate; OD_{660}, optical density at 660 nm.

PBBM prepared without organic compounds (9). The medium was prepared without phosphate, brought to pH 7.2 with NaOH, boiled, sparged with N_2-CO_2 (80:20%) or H_2-CO_2 (80:20%), dispensed into 158-ml Wheaton serum vials, sealed with butyl rubber stoppers, and autoclaved. Phosphate, sulfide (0.01%), N_2-CO_2 (80:20%) or H_2-CO_2 (80:20%), and vitamin solution were added after autoclaving. The medium volume was 40 ml, and the initial headspace gas pressure in the serum vials was adjusted to 30 lb/in^2. The media were inoculated with 3.0% (by volume; protein concentration, 1.995 mg/ml) methanogenic granules and incubated at 37°C. All procedures for medium preparation, inoculation, and cultivation were the same as those used for vial cultures except that Na_2S was not added because the medium was electrically reduced. Na_2S (2%) was added to the anode compartment as a reducing agent to remove the O_2 generated. NR (100 μM) was added to the cathode compartment as an electron mediator. The current and potential between the anode and cathode were 0.4 mA and 2.0 V. CO_2 and CH_4 were analyzed with a gas chromatograph equipped with a carbosphere column and flame-ionized detector. The injector and column temperatures were 50 and 150°C, respectively, and the carrier (N_2) flow rate was 45 ml/min. Gas samples were removed with a pressure lock syringe. CO_2 consumption and CH_4 production are shown as the percentage of total gas composition in the headspace.

RESULTS AND DISCUSSION

We previously reported that *A. succinogenes*, sp. nov., wild-type strain 130Z grew and produced succinate by fermentation of H_2 and fumarate (24). Mutant FZ-6 is a succinate-overproducing mutant selected by fluoroacetate resistance that produces low levels of acetate and formate (6). Figure 2 compares the growth and succinate production of wild-type 130Z to those of mutant FZ-6 on fumarate plus or minus H_2. The wild-type strain 130Z grew equally well on fumarate plus or minus H_2, whereas strain FZ-6 grew on fumarate only in the presence of H_2 as an electron donor.

To produce free energy with fumarate as the sole anaerobic energy source, fumarate reduction to succinate must be coupled to the oxidation of a reductant generated by fumarate metabolism to acetate plus formate. We compared oxidoreductase levels in *A. succinogenes* 130Z and FZ-6 to determine the metabolic basis for strain FZ-6's inability to grown on fumarate without H_2 (Table 1). While the strains had equivalent levels of glyceraldehyde-3P dehydrogenase, hydrogenase, fumarate reductase, and malate dehydrogenase activities, strain FZ-6 lacked pyruvate formate lyase and formate dehydrogenase activities. The absence of pyruvate-metabolizing enzymes also explains the inability of the mutant strain to grow on fumarate or pyruvate alone (data not shown).

Experiments were initiated to test if electrically reduced NR could replace H_2 for strain FZ-6 growth on fumarate. These studies were performed in an ECBS (Fig. 1) in the presence of NR, which served as an electron mediator, or electronophore. Figure 3 demonstrates that strain FZ-6 can grow by reducing fumarate to succinate with electrically reduced NR as the electron donor. The organism did not grow on fumarate alone without cathodic reduction of NR. NR serves as an electron mediator to transfer electrons from the cathode to the cellular electron transport chain. Table 2 summarizes the electrical dependence of growth and succinate production by strain FZ-6 on fumarate. At the end of growth, succinate was the only detectable fermentation product, and 92% of the starting carbon in fumarate was recovered in the succinate and cells produced. Growth and succinate production by strain FZ-6 were higher when electrically reduced NR served as the energy source (Table 2) in place of H_2 (Fig. 2).

Experiments were initiated to test whether electricity could replace H_2 for microbial CO_2 reduction to methane. These studies were performed in the reactor system described above (Fig. 1). The reactor contained methanogenic granules comprised of mixed cultures of syntrophic fatty acid-degrading bacteria and methane-producing bacteria (27, 28). Figure 4 shows that CO_2 reduction to methane was dependent on either H_2 or electricity. Notably, electrically reduced NR served as a better electron donor for CO_2 reduction than H_2. Determination of total cell growth after 120 h indicated a twofold increase in cells grown on electrically reduced NR versus a 1.4-fold increase on H_2-CO_2.

To our knowledge, this is the first report to demonstrate that

TABLE 1. Oxidoreductase levels in the *A. succinogenes* wild-type strain 130Z versus those in mutant strain FZ-6[a]

Enzyme	Sp act [nmol min^{-1} (mg of protein)$^{-1}$]	
	130Z	FZ-6
D-Glyceraldehyde-3P-dehydrogenase	4,575	4,289
Hydrogenase	1,824	1,564
Fumarate reductase	174	242
Malate dehydrogenase	1,582	1,502
Formate dehydrogenase	76	<1
Pyruvate dehydrogenase	<1	<1
Pyruvate formate lyase	257	<1

[a] Cell extracts were prepared from glucose-grown cells, and activities were measured by standard assays.

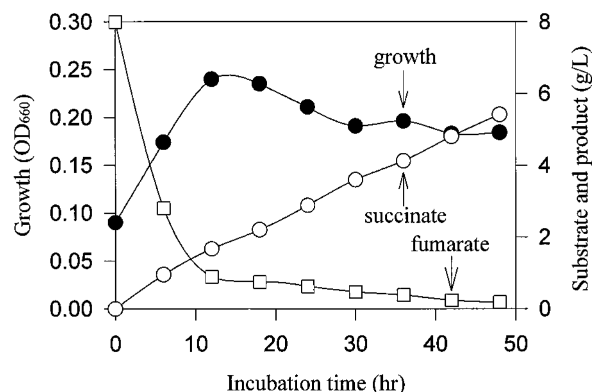

FIG. 3. Electricity-dependent growth and succinate production of the *A. succinogenes* mutant strain FZ-6 on fumarate. The cathode compartment contained medium A, fumarate, and 100 μM NR. The potential and current between anode and cathode were 1.5 V and 0.4 to 1.5 mA. Although cell growth also takes place on the cathode, it was only measured as cells were released into the liquid medium, thus underestimating the total growth. OD_{600}, optical density at 600 nm.

H_2-utilizing microbes can utilize reducing power from the electrically reduced electron donor NR as the sole electron donor for growth and for either fumarate reduction to succinate or CO_2 reduction to methane. Previous work on electrochemically reduced redox dyes (i.e., electroenergized systems) in microbial fermentations (3, 7) focused only on altering fermentation product patterns, and the systems used were not the carefully controlled electrochemical systems used here. In these systems, bacteria were not necessarily using the reduced dye as reducing power because the high voltage supplied produced H_2, which in turn could be used as an electron donor.

Figure 5A summarizes our hypothesis explaining how electrically reduced NR can replace H_2 as an energy source for microbial growth and succinate production. In respiratory succinate metabolism linked to fumarate reduction (13), electron carriers such as quinones are reduced by an electron donor (e.g., H_2 and NADH), and the reduced quinone is subsequently oxidized. This oxidation is coupled to the production of a proton motive force (PMF) that is dependent on the electron driving force. Similarly, in respiratory methane production linked to CO_2 reduction (Fig. 5B), electron donors (e.g., H_2) may be linked to a proton-translocating electron

TABLE 2. Fermentation parameters of the *A. succinogenes* mutant strain FZ-6 grown on electrical reducing power, with NR as electron mediator and fumarate as electron acceptor[a]

Fermentation parameters	Value	
	Initial	Final
Fumarate concentration [g/liter (mM)]	8.0 (50)	0.18 (1.125)
Fumarate consumption [g/liter (mM)]		7.82 (48.9)
Succinate production [g/liter (mM)]		5.4 (38.8)
Total growth (OD_{660})[b]	0.0901	0.3016
Other products		None detected
Carbon recovery[c]		0.92
Electron recovery[d]		1.19

[a] Analyses were performed at the beginning and at the end of the experiment shown in Fig. 2.
[b] OD_{600}, optical density at 600 nm.
[c] Cell carbon was calculated with $CH_2O_{0.5}N_{0.2}$.
[d] Electron recovery was calculated by using the difference between substrate and product hydrogen contents.

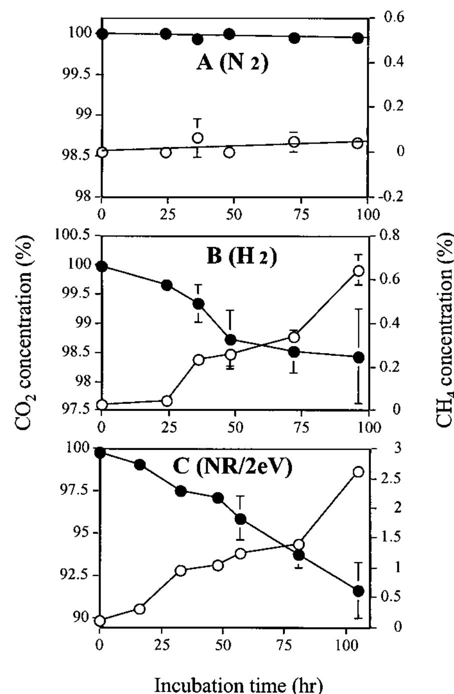

FIG. 4. Influence of electron donors on CO_2 reduction to methane by methanogenic granules. (A) N_2-CO_2 control; (B) H_2-CO_2; (C) electrical reduction. For electrical reduction, the cathode compartment contained PBBM and 100 μM NR. The potential and current between the anode and cathode were 1.5 V and 0.3 to 0.35 mA, respectively. The data values represent the averages of three replicate experiments. ●, CO_2; ○, CH_4.

transport chain which includes cytochrome *b* (8). Electrons can also be transferred from the electrode to these two different microbial electron carrier chains through electrical (i.e., cathodic) reduction of NR and its subsequent microbial oxidation. The E_o' of reduced NR (−0.325) is similar to that of NADH, and its microbial oxidation generates an electron driving force coupled to the production of a PMF to account for energy conservation.

In short, NR functions as an electronophore, or artificial electron carrier, enabling electricity to indirectly supply the electron driving force needed to generate a PMF for energy conservation and the electrons needed for growth and metabolite production. Much remains to be learned about the exact biochemical mechanism(s) that accounts for the ability of NR to function in electron transfer and about how PMF and energy conservation are driven by electrically reduced NR in microbes. It will also be of interest to study further, from both fundamental and applied perspectives, the use of electrically reduced NR as a reductant to drive different metabolic processes, such as photosynthesis, fermentative production of organic alcohols or acids, biotransformation of organic compounds into higher-value drugs and specialty chemicals, and anaerobic transformations that are rate limited by reducing equivalents (e.g., aromatic-compound reductive dechlorination or oil desulfurization).

Previously, a different electrochemical reactor system was used to grow *T. ferrooxidans* indirectly on electrically reduced iron as the immediate electron donor (29). Our electrochemical system is quite different; it works with pure and mixed cultures, and it is more versatile, since it is not limited to metal- or H_2-oxidizing microbes because NADH is formed directly from electrically reduced NR.

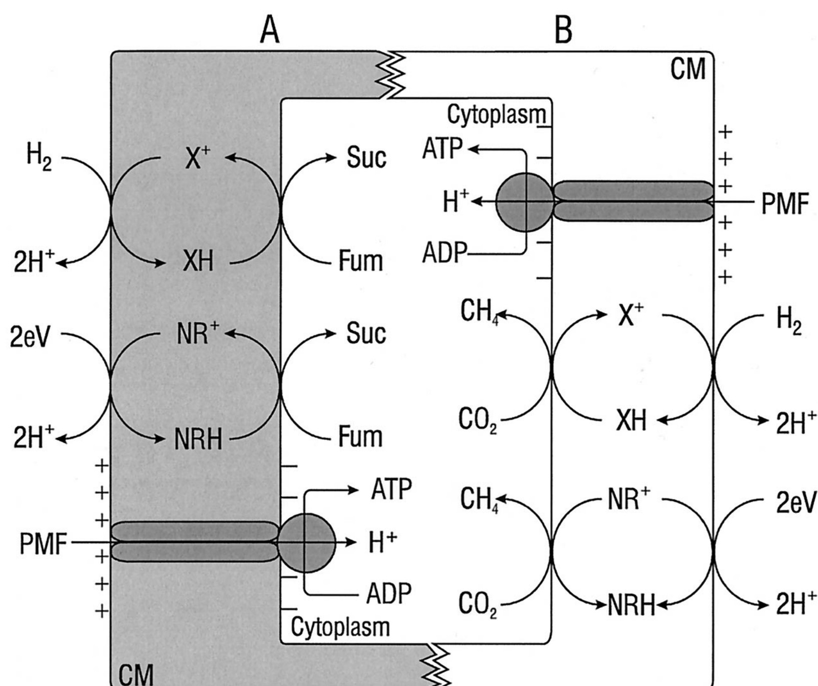

FIG. 5. Hypothetical models for microbial energy conservation with H_2 and with electrically reduced NR as sources of reducing power for fumarate reduction to succinate (A) and CO_2 reduction to methane (B). The electrical reduction of NR, which serves as an electronophore, or of H_2 depends on the reduction of normal cellular electron carriers (XH). Electron carrier reduction is coupled to the generation of a PMF that drives ATP synthesis and charge separation across the cell membrane. CM, cytoplasmic membrane; 2eV, electrical reducing power.

In light of these results, perhaps living cells can be more easily viewed as bioelectrical devices. That is, cells can function by maintaining charge separation across their membranes and by passing currents through their electron transport chains when utilizing light, chemicals, or electricity itself in the presence of electrically reduced NR as the source of reducing power. Actually, microbial fuel cells containing *Proteus vulgaris* and redox dyes were placed in the anode of an electrochemical bioreactor to generate an electrical current produced from the microbial metabolism of carbohydrates (1). Thus, it appears that the utilization or production of an electric current is reversible in microbial systems as long as an appropriate electron mediator is present.

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