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Energy Generation Coupled to Azoreduction by Membranous Vesicles from *Shewanella decolorationis* S12

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Previous studies have demonstrated that *Shewanella decolorationis* S12 can grow on the azo compound amaranth as the sole electron acceptor. Thus, to explore the mechanism of energy generation in this metabolism, membranous vesicles (MVs) were prepared and the mechanism of energy generation was investigated. The membrane, which was fragmentized during preparation, automatically formed vesicles ranging from 37.5–112.5 nm in diameter under electron micrograph observation. Energy was conserved when coupling the azoreduction by the MVs of an azo compound or Fe(III) as the sole electron acceptor with H_2, formate, or lactate as the electron donor. The amaranth reduction by the vesicles was found to be inhibited by specific respiratory inhibitors, including Cu^{2+} ions, dicumarol, stigmatellin, and metyrapone, indicating that the azoreduction was indeed a respiration reaction. This finding was further confirmed by the fact that the ATP synthesis was repressed by the ATPase inhibitor *N,N'*-dicyclohexylcarbodiimide (DCCD). Therefore, this study offers solid evidence of a mechanism of microbial dissimilatory azoreduction on a subcell level.

Keywords: Membranous vesicles, azoreduction, energy conservation, *Shewanella decolorationis* S12

The contamination of drinking water, groundwater, and surface water by azo dyes has already been recognized as a serious health risk [5, 8, 18, 23]. Although the microbial-mediated reduction of azo dyes has already been established for more than 50 years and is now considered an environmentally friendly treatment option [3, 6, 7, 24], little is known about this metabolism process.

Thus, to elucidate the mechanism of microbial azoreduction, extensive studies were recently conducted by the current authors [14], and it was discovered that *S. decolorationis* S12, a new species of the genus *Shewanella* isolated from activated-sludge from a textile-printing wastewater treatment plant [27], conserves energy for growth by coupling the dissimilatory azoreduction process to the oxidation of various electron donors. This finding not only expands the known potential electron acceptors for microbial energy generation, but also explains the mechanism of microbial anaerobic azoreduction. Furthermore, the results are also significant for the bioremediation of environments contaminated by azo dyes. However, despite the confirmation of a new form of bacterial anaerobic respiration-azorespiration, the mechanism of the energy generation from this metabolism process is still unknown.

Isolated bacterial cytolpasmic membrane vesicles (MVs) have already been proven as a particularly useful model system for studies of energy generation and electron transport [9, 13, 25]. Previous studies have shown that such vesicles are essentially devoid of cytoplasmic enzymes and constituents [1, 16]; thus, any coupling between respiration and energy generation must take place within the membrane itself. Therefore, MVs are more advantageous than intact cells when studying bacterial respiration.

Accordingly, to understand the mechanism of dissimilatory azoreduction more deeply, this study used an ultrasonic method to prepare cytoplasmic MVs from strain *S. decolorationis* S12, and then investigated the energy generation coupled to the azoreduction by the MVs. As a result, evidence is presented on the mechanism of microbial dissimilatory azoreduction on a subcell level.

Materials and Methods

Strain and Cultivation

Shewanella decolorationis S12^T (CCTCC M 203093, IAM 15094) was isolated from activated sludge from a textile-printing wastewater treatment plant in Guangzhou, China [27]. The strain was

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facultative anaerobic and capable of growing under both aerobic and anaerobic conditions. An LB medium was used for aerobic cultivation at 32°C with shaking at 150 rpm.

Chemical Regents and Enzymes

The amaranth, used as the model azo compound, and *N*,*N*′-dicyclohexylcarbodiimide (DCCD) were purchased from Aldrich (Milwaukee, U.S.A.) at the highest purity commercially available. The glucose-6-phosphate dehydrogenase and hexokinase were products of Sigma (St. Louis, U.S.A.).

Preparation of MVs from *S. decolorationis* S12

The membrane isolation and vesicle preparation were performed using the method of Sapra *et al.* [25] with some modifications. Buffer A [50 mM Tris-HCl buffer (pH 8.0), containing 2 mM sodium dithionite (DT)] was used throughout. The cell-free extracts of *S. decolorationis* S12 were prepared by suspending 5 g (wet weight) of frozen cells in 50 ml of buffer A. The cell suspension was then sonicated in an ice bath (3 s, 40% output, 80× SONICS VC-505; U.S.A.), and the cell breakage monitored by examining the cells under a microscope. The unbroken cells were removed by centrifugation at 10,000 ×*g* for 15 min, and the crude extract was centrifuged at 150,000 ×*g* for 2.0 h. Thus, the resulting pellet contained the cell membranes, whereas the cytoplasmic proteins remained in the supernatant. The membrane fraction was resuspended in buffer A to allow the spontaneous formation of vesicles, which was confirmed by transmission electron microscopy. The protein concentrations were determined using the method of Bradford with bovine serum albumin as the standard [4].

Assay of Azoreduction

The membranous vesicles were resuspended in buffer A containing 1 mM amaranth, and 10 mM formate, lactate, or H_2 was supplied as electron donors. The incubations were performed under anaerobic conditions at 32°C. A modified Hungate technique [19] was used throughout the study for the anaerobic cultivation. All the batch experiments were conducted in 10-ml serum bottles. The medium was prepared by adding all the components from concentrated stock solutions to O_2-free distilled water, equilibrating the preparation with N_2-CO_2 (4:1), and passing it through a filter to remove any bacteria. After being flushed with O_2-free gas for 5 min, the serum bottles were sealed with butyl rubber stoppers and incubated in an anaerobic station (Ruskinn C0105). H_2 was provided at 101 kPa unless otherwise noted.

The azoreduction activity was then calculated according to the maximum spectrophotometrical absorbance at 520 nm. One unit of activity of azoreduction was defined as the reduction of 1 μM amaranth per minute per milligram of protein.

Kinetic Studies Employing Specific Respiratory Inhibitors

The kinetics of specific respiratory inhibitor-inhibited azoreduction were used to determine the components participating in the electron transport during the process. Cu^{2+}, dicumarol, stigmatellin, and metyrapone were chosen as the specific respiratory inhibitors, and their concentrations were 15, 200, 100, and 100 μM, respectively. The azoreduction rate in the presence of each inhibitor was recorded for each electron donor used. The kinetic data were obtained with formate, lactate, and hydrogen as the electron donors, and the results plotted as the specific activity of azoreduction versus the concentration.

Measurement of Oxidative Phosphorylation

The anaerobic energy coupling was assessed with the MVs by determining the amount of ATP synthesized [9]. The reaction buffer (5 ml) consisted of 15 mM $MgCl_2$, 0.5 mM ADP, 5 mM KPO_4H_2, and 50 mM Tris-HCl (pH 7.3). This buffer, when supplemented with appropriate alternative electron donors, 5 mM of formate or lactate, 0.5 mM of amaranth, and freshly prepared MVs, was able to measure the amount of ATP synthesized. The assays were run at 30°C in a N_2 atmosphere, and the incubation time was 40 min. The esterification of phosphate in the oxidative phosphorylation experiments was determined spectrophotometrically at 340 nm as the NADPH formation using a mixture of 2 U of hexokinase, 1 U of glucose-6-phosphate dehydrogenase, 10 mM glucose, and 0.5 mM NADP.

RESULTS

Characteristics of MVs Prepared from *S. decolorationis* S12

To investigate the bioenergetics in the process of dissimilatary azoreduction, MVs were prepared from *S. decolorationis* strain S12. The electron micrograph structures shown in Fig. 1 are the MVs obtained from strain S12 that were fragmentized using an ultrasonic method, followed by extensive washing and differential centrifugation. The circular structures consisted of segmental unit membrane-bond sacs, varying from about 37.5 to 112.5 nm in diameter. The sacs were empty and had no apparent internal structure, and most were surrounded by a single 80–100 Å membrane, whereas a few vesicles were surrounded by two or three membrane layers. The vesicles were between $1/5^{th}$ and $1/20^{th}$ the size of the intact *S. decolorationis* S12 cells, and were 0.6–1.0 μm in width and 1.0–4.0 μm in length. In addition, the structures presented in Fig. 1 suggest that the vesicles were closed, which was necessary for their physiological functions.

Azoreduction Coupled to Oxidation of Electron Donor by MVs

As intact cells of strain S12, the MVs were also able to reduce azo compounds coupled with the oxidation of an

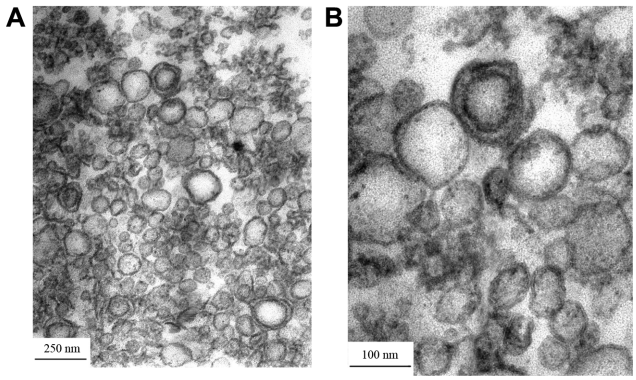

Fig. 1. Electron micrograph images of membranous vesicles prepared from *S. decolorationis* S12.
A. ×30,000; **B**. ×100,000.

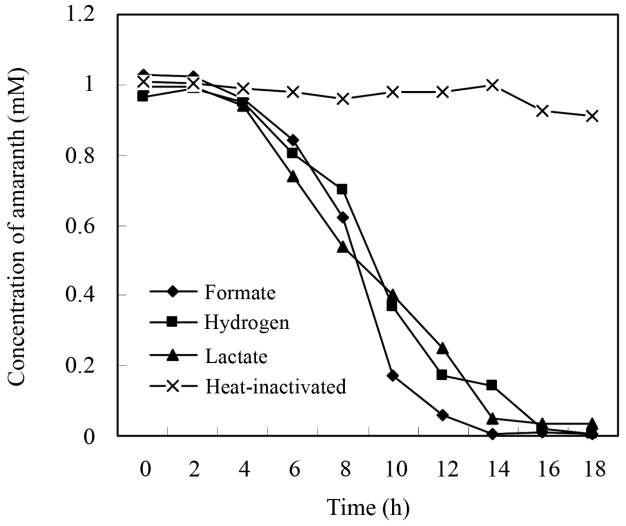

Fig. 2. Azoreduction by MVs of *S. decolorationis* S12 coupled to oxidizing electron donors under anaerobic conditions.
The initial MV concentration was approximately 13 μg/ml.

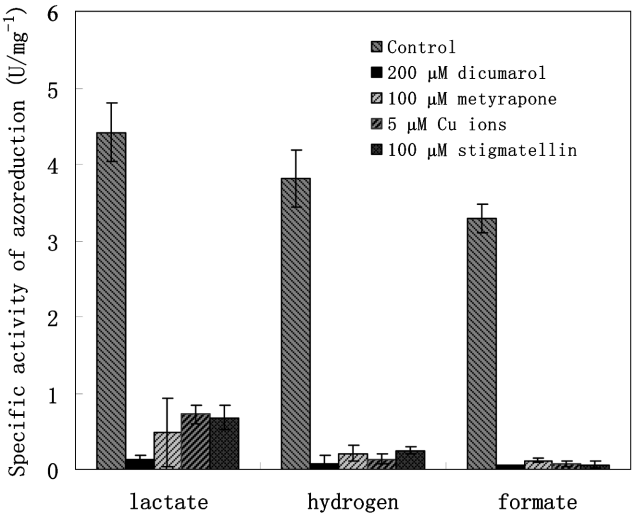

Fig. 3. Anaerobic azoreduction by membranous vesicles of *S. decolorationis* S12 and effect of respiratory inhibitors on azoreduction.
The experiments were performed in the defined medium under anaerobic conditions at 32°C, with lactate, H_2, or formate as the electron donor. All the measurements were obtained by calculating the average values for three independent incubations, and the error bars represent the standard deviations of triplicate cultures.

electron donor under anaerobic conditions (Fig. 2). More than 98% of the initial 1 mM amaranth was reductively converted in the presence of formate, lactate, or H_2 after anaerobic incubation for 14 h. In contrast, the control using heat-inactivated MVs did not show any decrease in the amaranth concentration throughout the experiment (18 h), suggesting the reaction was enzymatic.

Effect of Respiratory Inhibitors on Azoreduction of MVs

Studying the effect of specific respiratory inhibitor-inhibited azoreduction was used to determine the components participating in the process of electron transport, and the experimental results are shown in Fig. 3.

(i) Inhibition with Cu^{2+}. The Cu^{2+} ion is a membrane-impermeable dehydrogenase inhibitor that is able to destroy [4Fe-4S] clusters of hydrogenase and other dehydrogenases, thereby negatively affecting the electron transfer from the H-activating site to the electron acceptor [10, 17]. The azoreduction by the MVs was strongly inhibited by 5 μM Cu^{2+} when lactate, formate, or H_2 served as the electron donor, and the inhibitory rates were 83.4%, 96.3%, and 97.6%, respectively, indicating that the lactate dehydrogenase, formate dehydrogenase, and hydrogenase were important components of the MVs for electron transfer.

(ii) Inhibition with stigmatellin. Stigmatellin is a quinone analog and interferes with electron transport by blocking the binding ability to cytochrome *b* [11, 22]. In the presence of 100 μM stigmatellin, the azoreduction by the MVs was strongly suppressed, and the inhibitory rates with lactate, formate, or H_2 as the electron donor were 85%, 93%, and 98%, respectively, suggesting that a low-potential cytochrome *b* was involved in the electron transport from the electron donor to the azo dyes in the MVs. Thus, it would seem that the cytochrome *b* shuttled the electrons between the primer dehydrogenases and the menaquinone (MK).

(iii) Inhibition with dicumarol. Dicumarol is believed to inhibit the electron transport of menaquinone in bacteria [2, 12], and 200 μM dicumarol was found to inhibit the azoreduction by the MVs, where the inhibitory rates with lactate, formate, or H_2 as the electron donor were 97%, 98%, and 98%, respectively. Therefore, this observation confirmed that MK was an essential compound for electron transport in the azoreduction by the MVs.

(iv) Inhibition with metyrapone. The azoreduction by the MVs was also sensitive to metyrapone, where the inhibitory rates with lactate, formate, or H_2 as the electron donor were 89%, 94%, and 98%, respectively. Thus, since metyrapone is a specific cytochrome P_{450} inhibitor [26], it would seem that a cytochrome P_{450} played an important role in the anaerobic azoredution by the MVs.

Therefore, the overall results revealed that the anaerobic azoreduction by the MVs was catalyzed by a multicompound system, where dehydrogenase (hydrogenase), cytochrome *b*, MK, and P_{450}-type cytochrome were all involved in the process of electron transport to an azo bond. In addition, the respiratory inhibitor results also indicated a chemiosmotic model of dissimilatory azoreduction in *S. decolorationis* S12 at the subcell level.

ATP Generation Coupled to Azoreduction by MVs

The synthesis of ATP coupled to the formate-amaranth oxidation-reduction in the MVs prepared from *S. decolorationis*

Table 1. Phosphorylation coupling formate oxidation with azoreduction.

Reaction mixture	Amt. of ATP formation (nmol/mg membrane protein)
Complete	280±32
Complete plus DCCD	12±3
Without ADP	9±2
Without electron donor	18±6
Without amaranth	12±4
Without amaranth plus Fe(III)	320±28

The reaction mixture was supplemented with 1 mM Fe(III), and the ATP formation was determined as described in Materials and Methods.

S12 cells was also investigated (Table 1). The rate of ATP synthesis was 280±32 nmol/mg protein. No ATP synthesis was observed when the freshly prepared MVs were incubated with DCCD before the addition of amaranth. As expected, in the absence of ADP, no ATP synthesis occurred. Moreover, no ATP synthesis was observed in the absence of amaranth. However, when Fe(III) was used instead of amaranth as the final electron acceptor, the ATP synthesis rates were higher than those with amaranth.

Discussion

Previous studies by the current authors already showed that the bacterial anaerobic azoreduction by strain S12 is a biochemical process that oxidizes the electron donors and transfers the electrons to the acceptors through an electron transport chain, thereby conserving energy. Thus, the anaerobic azoreduction by S12 is a form of microbial respiration, defined as azorespiration, and since this respiration can achieve rapid and extensive azoreduction, microbial azorespiration would seem to play a significant and direct role in azo dye degradation [27]. Thus, to further elucidate and characterize the novel concept of azorespiration, MVs were prepared and their mechanism of energy generation coupled to azoreduction was investigated.

The present results reconfirmed that bacterial MVs are a perfect model system for studying energy generation and electron transport. Previous studies have shown that bacterial MVs are mosaics as regards their orientation, being a mixture of right-side-out and inside-out vesicles. In particular, when smaller in size, the percentage of inside-out vesicles is high [1, 16]. Thus, since an ultrasonic method was used to prepare the MVs in this study, the MVs of strain S12 were small in size and presumed to have a more inside-out orientation. However, whether the MV orientation has an affect on the azoreduction and energy generation remains unclear and requires further investigation.

The experimental results indicated that the MVs of S12 were able to reduce azo compounds, just like the intact cells. In addition, studies with respiratory inhibitors demonstrated that dehydrogenase (hydrogenase), MK, cytochrome *b*, and P_{450}-type cytochrome were all important components involved in the process of electron transport to the azo bond. Therefore, since the present results are consistent with those from the previous study with intact cells [27], this further clarifies that the azoreduction by S12 is an electron transport process catalyzed by a multienzyme system located in the cellular membrane.

The energy generation mechanism of the MVs also explains the measured growth yields of *S. decolorationis* S12 with the azo compound amaranth as the sole terminal electron acceptor. Moreover, since the vesicles were essentially devoid of cytoplasmic enzymes and constituents, the coupling of the respiration and energy generation must have taken place within the membrane itself. The present findings also correspond to the premises of Mitchell's chemiosmotic hypothesis [20, 21].

Strain S12 is also able to reduce Fe(III) and humic substances, and other studies have demonstrated an interactive relationship between dissimilatory humic reduction and azoreduction [15]. The MVs from strain S12 were able to generate ATP when using Fe(III) as the sole electron acceptor, suggesting the existence of an intact enzyme system for Fe(III) in the MVs. Moreover, it is possible that the electron transport system for Fe(III) and azoredution may share some electron transporters in the cell membrane.

Consequently, this study elucidated the energy generation mechanism of MVs from strain S12, and the results offer solid evidence of a mechanism of microbial dissimilatory azoreduction in *S. decolorationis* S12 on the subcell level.

Acknowledgments

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