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ENVIRONMENTAL BIOTECHNOLOGY

Effects of electron donors and acceptors on anaerobic reduction of azo dyes by *Shewanella decolorationis* S12

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Abstract *Shewanella decolorationis* S12 was able to reduce various azo dyes in a defined medium with formate, lactate, and pyruvate or H_2 as electron donors under anaerobic conditions. Purified membranous, periplasmic, and cytoplasmic fractions from strain S12 analyzed, respectively, only membranous fraction was capable of reducing azo dye in the presence of electron donor, indicating that the enzyme system for anaerobic azoreduction was located on cellular membrane. Respiratory inhibitor Cu^{2+}, dicumarol, stigmatellin, and metyrapone inhibited anaerobic azoreduction by purified membrane fraction, suggesting that the bacterial anaerobic azoreduction by strain S12 was a biochemical process that oxidizes the electron donors and transfers the electrons to the acceptors through a multicompound system related to electron transport chain. Dehydrogenases, cytochromes, and menaquinones were essential electron transport components for the azoreduction. The electron transport process for azoreduction was almost fully inhibited by O_2, 6 mM of NO_3^-, and 0.9 mM of NO_2^-, but not by 10 mM of Fe^{3+}. The inhibition may be a result from the competition for electrons from electron donors. These findings impact on the understanding of the mechanism of bacterial anaerobic azoreduction and have implication for improving treatment methods of wastewater contaminated by azo dyes.

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Introduction

Azo dyes are almost all xenobiotic compounds, which are characterized by containing one or more azo groups (–N=N–). They are the largest and most versatile class of dyes and are widely used in textiles, leather, plastics, cosmetics, and food industries (Selvam et al. 2003). Because they are toxic, highly persistent, and ubiquitously distributed in the environment (Pearce et al. 2003; Maguire 1992; Zollinger 1991; Selvam et al. 2003), azo dyes are environmental pollutants (Stolz 2001). The discharge in open waters not only presents an aesthetic problem, but also causes a toxic impact on aquatic life and eventually affect human health (Brown and DeVito 1993; Chung and Cerniglia 1992).

Normally, upon reductive cleavage of the azo bond, azo dyes are reduced and amines are formed. Bacteria can subsequently mineralize some aromatic amines aerobically (Stolz 2001; Pearce et al. 2003; Haug et al. 1991). A variety of microorganisms can reduce azo dyes, including purely anaerobic strains (e.g., *Bacteroides* sp., *Eubacterium* sp., and *Clostridium* sp.) (Rafii et al. 1990; Bragger et al. 1997), facultative anaerobic strains (e.g., *Sphingomonas* sp. strain BN6, *Pseudomonas luteola* sp., *Proteus vulgaris*, and *Streptococcus faecalis*) (Kudlich et al. 1997; Hu 1994; Dubin and Wright 1975; Scheline et al. 1970), and some intestinal anaerobes (Brown 1981; Chung et al. 1978). The most generally accepted hypothesis is that anaerobic

bacterial azoreduction is a nonspecific reduction process by redox mediator shuttling electrons from bacteria to azo dyes (Keck et al. 1997; Rau and Stolz 2003; Rau et al. 2002). It is generally considered that azoreduction by bacteria was occurred under anaerobic condition (Stolz 2001; Kudlich et al. 1997; Rau and Stolz 2003). Anaerobic azo dye reduction was considered an important reaction in the process of degradation of azo dyes. Therefore, how to improve the rate of anaerobic azoreduction is a key factor to the treatment of wastewater contaminated by azo dyes.

Because various electron donors and alternative electron acceptors are almost always present in the environment (He and Sanford 2003; Jan et al. 1999), it is important to study the effects of different electron donors and acceptors on bacterial azoreduction. However, there have been few studies in which azoreduction by pure cultures under different electron donor and acceptor conditions was examined. In this paper we describe that *S. decolorationis* S12, previously isolated from activated sludge of a textile-printing wastewater treatment plant in Guangzhou, China (Xu et al. 2005), can reduce azo dyes with various organic substances and H_2 as electron donors. Moreover, the azoreduction of strain S12 was affected by several typical electron acceptors. These findings are significant for understanding the mechanism of bacterial anaerobic azo-reduction in-depth and for gaining sufficient control over azoreduction process based on the activity of strain S12 when it is used in situ or off site for the treatment of soil or water contaminated by azo dyes.

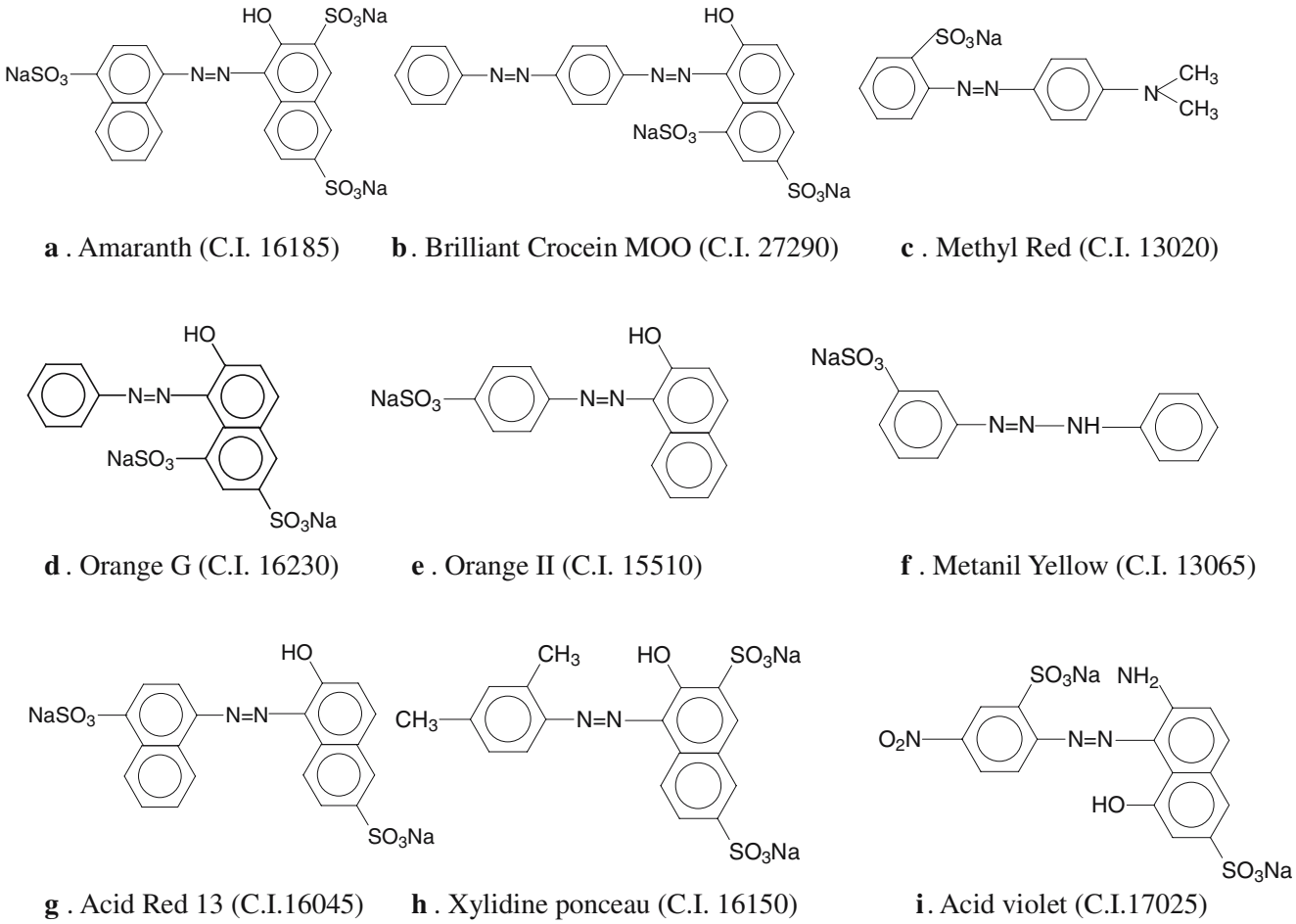
Materials and methods

Azo dyes

Amaranth, a typical azo dye used in this study, was purchased from Sigma-Aldrich. Other azo dyes were purchased from a dyestuff factory in Guangzhou, China. Chemical structures of azo dyes are depicted in Fig. 1.

Organism, media, and cultivation

S. decolorationis S12 (Xu et al. 2005), a new species of the genus *Shewanella*, was isolated from activated-sludge of textile-printing wastewater treatment plant, Guangzhou, China. The strain S12 was facultative anaerobic capable of



a . Amaranth (C.I. 16185) **b** . Brilliant Crocein MOO (C.I. 27290) **c** . Methyl Red (C.I. 13020)

d . Orange G (C.I. 16230) **e** . Orange II (C.I. 15510) **f** . Metanil Yellow (C.I. 13065)

g . Acid Red 13 (C.I.16045) **h** . Xylidine ponceau (C.I. 16150) **i** . Acid violet (C.I.17025)

Fig. 1 Chemical structures of azo dyes used in this study

growing under both aerobic and anaerobic conditions. Luria–Bertani (LB) medium was used for aerobic cultivation at 32°C with shaking at 150 rpm. The bacteria were cultivated under anaerobic condition in the defined medium (pH 7.5) containing the following (mM): succinate (10), Na_2HPO_4 (5.7), KH_2PO_4 (3.3), NH_4Cl (18.0), $MgSO_4$ (1.01), L-cysteine (20 μg ml^{-1}), vitamin solution, and trace element solution (Wolin et al. 1963). A modified Hungate technique (Miller and Wolin 1974) was used throughout the study for anaerobic cultivation. All batch experiments were conducted in 50-ml serum bottles. The medium was prepared by adding all the components from concentrated stock solutions in O_2-free distilled water and equilibrated the preparation with N_2–CO_2 (4:1), which was passed through a filter to remove bacteria. After flushed with the 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 with 101 kPa unless otherwise noted. The initial concentration of cells was $3.0\sim3.8\times10^5$ CFU ml^{-1} unless indicated otherwise.

Electron donor experiments

Aerobically grown cells in LB medium were harvested by centrifugation, washed twice, and resuspended in the fresh defined medium (initial pH 7.5) containing 1 mM of azo dye and supplying 10 mM of different organic substance or H_2 as electron donors, then dispensed into 50-ml serum bottles (25 ml per bottle), and incubated as described above. Control experiments were conducted to test the reduction of azo dyes in the absence of external electron donors or using heat killed cells (incubated at 95°C for 30 min). All cultures were incubated statically under anaerobic condition at 32°C.

Electron acceptor experiments

For experiments in which azoreduction by strain S12 was studied in the presence of different electron acceptors, different volumes of sterile aqueous stock solutions of KNO_3, $NaNO_2$, and ferric citrate were added to 30 ml of cultures, resulting in final concentrations of 3, 6, and 9 mM of NO_3^-; 0.6, 0.9, and 1.2 mM of NO_2^-; and 5 and 10 mM of Fe^{3+}. The experiments were conducted in the defined medium (initial pH 7.5) supplemented with 1.0 mM of amaranth and 20 mM of formate at 32°C. To study the effects of O_2 on azoreduction, 30 ml of the defined medium supplemented with 1.0 mM of amaranth and 20 mM of formate was incubated for 33 h under an aerobic condition with shaking at 150 rpm for 33 h and followed anaerobic incubation for 2 h.

Membranous, cytoplasmic, and periplasmic preparation of proteins

The buffer used throughout was 50 mM of Tris–HCl buffer (pH 8.0), containing 2 mM of sodium dithionite (buffer A). 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 sonicated in an ice bath (3 s, 40% output, ×80) using a sonification unit (SONICS VC-505, USA), with monitoring of the cells' breaking progress under the microscope. Unbroken cells were removed by centrifugation at 10,000×*g* for 15 min. The crude extract was then centrifuged at 150,000×*g* for 2.0 h. The supernatant contained cytoplasmic proteins and the pellet was membrane. The membrane fraction was resuspended in buffer A. The periplasmic fraction was prepared using a modified method of Osborn and Munson (1974). Protein concentrations were determined by using Bradford reagent (Bradford 1976), with bovine serum albumin as standard.

Analysis methods

Formate, nitrate, and nitrite concentration was measured by ion chromatograph analyzer (Metrohm 761 Compont IC) equipped with a polysulfonate ion exclusion column (Metrosep A Supp 5). The eluent contained the following: 3.2 mM of Na_2CO_3, 0.8 mM of $NaHCO_3$, and 3% MeOH. The experiment was performed under the condition at 25°C, 7.3 MPa. Fe(II) in the samples was analyzed by using the HCl extraction ferrozine assay as previously described (Lovley and Phillips 1988).

The double bond of the azo linkage together with the conjugated-bond system of the aromatic compounds constitutes the chromophore of azo dyes. If the double bond of the azo compounds was reduced, the azo compounds were decolorized. So the azoreduction was quantified according to the change of OD value measured at a specific wavelength. The azoreduction activity was calculated according to the formulation as follows at maximum absorbance, and all assays were done in duplicate.

$$\text{Azoreduction extent}\,(\%) = \frac{(A-B)}{A}\times 100$$

A − initial absorbance

B − observed absorbance

Results

Effect of electron donors on azoreduction

Several organic substances and H_2 were chosen to determine their effects on reduction of azo dyes. To study

the effect of different electron donors on azoreduction, cells of strain S12 were inoculated in the defined medium supplemented with 10 mM of organic substance or H_2 and 1 mM of azo dyes under anaerobic condition. Experimental results showed that H_2, formate, lactate, and pyruvate were able to serve as sole electron donor for reduction of various azo dyes by *S. decolorationis* S12, but acetate, propionate, salicylate, glycerin, ethanol, citrate, and succinate were not effective electron donors for azoreduction (Table 1). Single azo bond azo dye amaranth, methyl red, orange G, orange II, acid red 13, and xylidine ponceau were almost completely reduced within 36 h with H_2, formate, and lactate as electron donor in this experiment, but the extent of reduction of acid violet and metanil yellow was lower than other single azo bond azo dyes under the same condition. The extents of reduction of double azo bond azo dye brilliant crocein MOO with H_2, formate, and lactate as electron donor were 68.4, 75.3, and 80.4%, respectively. The difference of reducing rate may be resulted from the different structures of azo dyes. When the cells of S12 were incubated at 95°C for 30 min or electron donors was omitted, almost no reduction could be measured over a period of 36 h.

To quantify the electron transfer from electron donor to azo dyes, the oxidation of formate and the reduction of amaranth were determined simultaneously in the incubation containing 5 mM of formate and 1 mM of amaranth. Within 40 h, complete disappearance of 1.0 mM of amaranth was accompanied stoichiometrically consuming 2.0 mM of formate over time. When 1.0 mM of amaranth was reduced completely, the consumption of formate was stopped simultaneously (Fig. 2a). The result in Fig. 2b revealed there was a linear relation between consumption of formate and reduction of amaranth with excess amaranth (2.0 mM) and limiting formate. When formate was exhausted, the reduction of amaranth stopped. These results clearly showed that azoreduction by strain S12 completely depend on the consumption of formate. The ratio of the number of moles of formate oxidized to the number of moles of amaranth reduced was 1.92. Because one molecular formate can provide two electrons and one molecular amaranth (one azo bond) can accept four electrons for reduction, the ratio is 2.0 in theory. It can be concluded that the amount of electrons accepted by amaranth were almost completely supplied from formate. Formate consumption and amaranth reduction was in agreement with the reaction according to: $Ar_1 - N = N - Ar_2 + 2COOH^- + 2H_2O \rightarrow Ar_1 - NH_2 + H_2N - Ar_2 + 2HCO_3^- + 2H^+$.

Effect of O_2 on azoreduction

To investigate the effect of different electron acceptors on azoreduction by strain S12, amaranth and formate were chosen as model azo dye and electron donor, respectively. O_2 is a very effective electron acceptor for energy conservation due to its high redox potential. To evaluate the effect of O_2 on azoreduction, two experiments under aerobic and anaerobic condition were performed. Within 33 h, 1.0 mM of amaranth was reduced under anaerobic condition with formate as electron donor in the defined

Table 1 Reduction of azo dyes by *S.decolorationis* S12 with different organic substances or H_2 as electron donor under anaerobic condition

Electron donor (ED)	Extent of azoreduction (%)[a]								
	A[b]	B[b]	C[b]	D[b]	E[b]	F[b]	G[b]	H[b]	I[b]
No ED	2.5±0.2	1.4±0.1	4.8±0.5	2.6±0.2	3.5±0.4	1.9±0.1	2.7±0.3	4.5±1.9	0.9
H_2	98.5	68.4±8.3	98.4	99.3	98.2	64.3±4.3	97.5±0.2	97.6±0.6	5.2±1.3
Formate	99.6	75.3±3.9	98.0	99.5	97.4±0.2	58.5±2.6	99.1	96.5±0.3	7.9±1.5
Lactate	99.4	80.4±4.6	97.3±0.1	99.5	99.3	76.4±1.8	99.9	99.7	8.2±0.9
Pyruvate	72.1±5.8	52.2±5.7	86.2±2.2	79.3±10.2	91.3±0.7	58.3±3.3	78.2±4.2	82.2±3.7	4.6±0.4
Acetate	3.3±0.5	1.6	3.4±0.4	2.6±0.2	3.4±0.3	1.5±0.1	2.8	3.8±0.8	1.3
Succinate	4.3±0.4	2.7±0.2	4.6±0.3	2.6±0.4	4.5±0.2	2.2±0.2	2.7±0.2	2.5±0.6	2.7±0.2
Glycerin	3.2±0.3	1.1	3.7±0.3	3.1±0.1	2.9±0.1	0.7	2.6±0.4	3.9±0.3	0.28
Salicylate	4.4±0.4	2.4±0.3	4.0±0.2	3.8±0.3	4.5±0.3	1.2	3.5±1.3	5.8±0.4	1.2
Propionate	2.7±0.2	1.4	3.4±0.5	2.1±0.2	3.4	2.3±0.1	1.7	4.4±0.3	0.8
Citrate	2.8±0.2	1.7±0.1	4.0±0.2	2.4±0.2	4.7±0.5	1.4	3.8±0.8	2.6±0.2	0.6
Ethanol	2.3±0.2	1.6	3.5±0.2	2.7±0.4	2.2	1.7	3.0±0.5	3.3±0.3	1.0

The experiments were performed at 32°C by the addition of 1 mM of azo dye as electron acceptor and 10 mM of organic substance or H_2 as electron donor under anaerobic condition. Data were obtained from duplicate separated experiments.

[a] Extent of azoreduction was measured spectrophometrically after 36 h of incubation according to the formulation: extent of azoreduction (%)=$(A-B)/A\times100\%$, with A as the initial absorbance and B the observed absorbance. SD was not shown if it was lower 0.1.

[b] The maximal wavelengths at which they determined the decolorization of the azo dyes A, B, C, D, E, F, G, H, and I are 520, 510, 430, 483, 488, 414, 507, 520, and 489.5 nm, respectively.

A Amaranth, *B* brilliant crocein MOO, *C* methyl red, *D* orange G, *E* orange II, *F* metanil yellow, *G* acid red 13, *H* xylidine ponceau, *I* acid violet

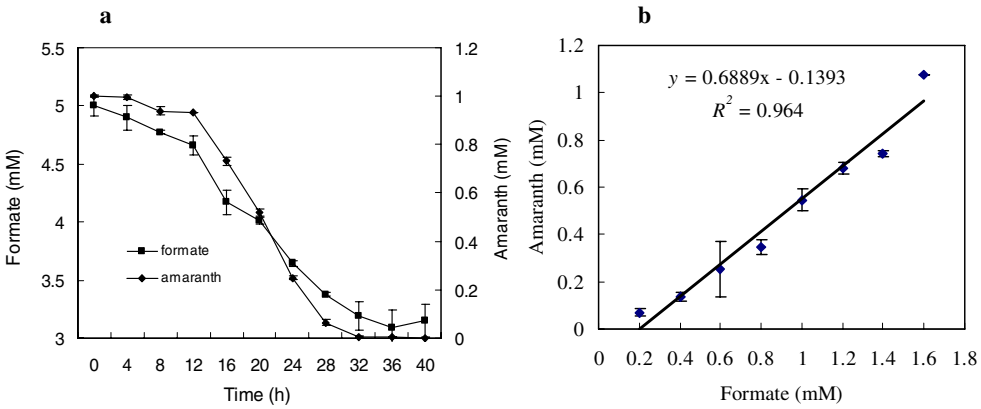


Fig. 2 Stoichiometry of azoreduction coupled to the oxidation of electron donor by *S. decolorationis* S12. Samples were incubated at 32°C for 36 h. **a** The consumption of formate and reduction of amaranth were simultaneously determined. **b** Reduction of amaranth under different concentration formate. Data are averages from duplicate experiments. Error *bars* represent standard deviations of duplicate incubations

medium, but almost no reduction was observed from the culture under aerobic condition at 32°C with shaking at 150 rpm. The result indicates that azoreduction was strongly inhibited by oxygen. After 33 h of cultivation, the biomass produced in aerobic condition was around tenfold more than that produced in anaerobic condition. If the aerobic culture was switched to anoxic static condition, color disappeared within 2 h due to fast consumption of the DO by the large biomass previously built up under aerobic condition (Fig. 3), indicating that anaerobic condition would be essential to achieve an effective azoreduction. The biomass was an essential but not a sufficient condition for azoreduction by strain S12.

Effect of nitrate and nitrite on anaerobic azoreduction

The impact of different concentrations of nitrate and nitrite on reduction of amaranth was also studied. After 36 h incubation, 1 mM of amaranth was completely reduced in the culture containing no NO_3^-, but the extent of azoreduction was only 50% in the culture containing 3 mM of nitrate at 36 h. This result showed that the reduction of amaranth was obviously inhibited by 3 mM of NO_3^-. When the concentration of nitrate was more than 6 mM, azoreduction was inhibited completely within 36 h (Fig. 4a). The amounts of NO_3^- and amaranth were measured simultaneously in the anaerobic culture containing 6 mM of NO_3^- and 1 mM of amaranth. Until around 90% of NO_3^- was consumed after 60 h of incubation, azoreduction began to occur and was completed within 20 h. The inhibition by NO_3^- can be relieved if the concentration of NO_3^- drops to lower than 1.5 mM (Fig. 5a).

The effect of nitrite on anaerobic azoreduction was similar to the effect of nitrate on the reduction; 0.6 mM of nitrite had no obvious impact on the rate of anaerobic azoreduction, while 0.9 and 1.2 mM of nitrite strongly inhibited the reduction. Within 36 h, no reduction could be detected in the culture added with 0.9 mM of NO_2^- under anaerobic condition (Fig. 4b). The amounts of NO_3^- and amaranth were measured simultaneously in the anaerobic culture containing 1 mM of NO_2^- and 1 mM of amaranth. The result in Fig. 5b shows that amaranth was not reduced until the concentration of nitrite dropped to 0.2 mM.

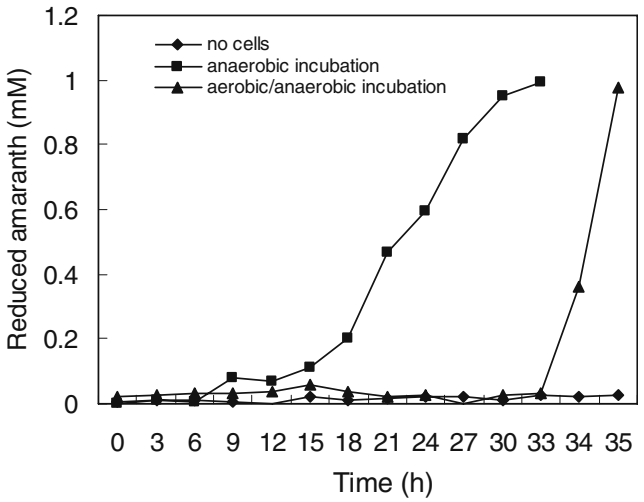


Fig. 3 Effect of O_2 on azoreduction by *S. decolorationis* S12. *Diamonds*, aerobic incubation without cells of S12. *Squares*, anaerobic incubation for 33 h. *Triangles*, after aerobic incubation for 33 h, then anaerobic incubation for 2 h. The experiments were performed at 32°C by adding 1 mM of amaranth and 10 mM of formate

Effect of Fe^{3+} on anaerobic azoreduction

The result from Fig. 4c revealed that the rate of azoreduction by strain S12 was improved at ratio about 20% in the presence of 5 or 10 mM of Fe^{3+} citrate. Because the typical rate of Fe^{3+} reduction by *S. decolorationis* S12 would have resulted in depletion of Fe^{3+} within several hours, we performed an experiment in which reduction of amaranth and reduction of Fe^{3+} were measured simultaneously. The result showed that azoreduction and Fe^{3+} reduction occurred simultaneously in the culture containing 10 mM of Fe^{3+} and 1 mM of amaranth (Fig. 5c). There is weak competition for accepting electrons between Fe^{3+} and azo dyes.

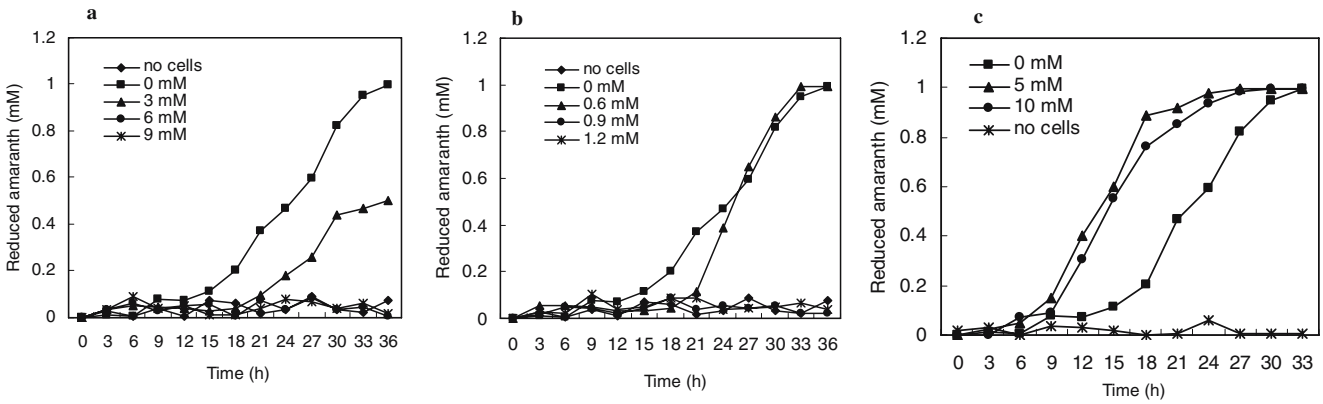


Fig. 4 Effect of NO_3^- (**a**), NO_2^- (**b**), and Fe^{3+} (**c**) on azoreduction by *S. decolorationis* S12 under anaerobic condition. The experiments were performed in the defined medium supplemented with 1 mM of amaranth and 20 mM of formate at 32°C

Location of the system for anaerobic azoreduction and effect of respiratory inhibitors on the azoreduction

To locate the enzymic system for anaerobic azoreduction, periplasmic, cytoplasmic, and membranous proteins of S12 were prepared. Different fractions with equal protein concentration were resuspended in equal volumes of buffer A containing 10 mM of formate as electron donors and 1 mM of amaranth. Figure 6a showed that the proteins or enzymes from the cytoplasm or periplasm had no activity of azoreduction, but the membranous fraction was capable of efficiently catalyzing azoreduction with formate as electron donors. The specific azoreduction activity of membranous fraction was 12.5 μM min^{-1} mg^{-1} protein. These results demonstrated the system of anaerobic azoreduction of strain S12 is located on the cellular membrane (Fig. 6b). The effect of respiratory inhibitors on the azoreduction by membranous fraction of S12 is shown in Fig. 6b. The reduction by membranous fraction was strongly inhibited by 5 μM of CuP^{2+} ions, 200 μM of dicumarol, 100 μM of stigmatellin, and 100 μM of metyrapone. CuP^{2+} ions, a membrane-impermeable dehydrogenase inhibitor (Fernandez et al. 1989; Louie and Mohn 1999), inhibit anaerobic azoreduction when HB_{2B} or formate serve as electron donor, indicating that hydrogenase and formate dehydrogenase are important components for electron transfer. Stigmatellin, which are quinone analogs that are able to bind to the cytochrome b (Gerencsér et al. 2004; Ouchane et al. 2002), inhibited anaerobic azoreduction, suggesting that a low-potential cytochrome b is involved in the electron transport from electron donor to azo dyes. It is likely that the cytochrome b shuttle electrons between primer dehydrogenases and menaquinone (MK). Dicumarol, which was thought to inhibit electron transport of MK in bacteria (Ghiorse and Ehrlich 1976; Arnold et al. 1986), also inhibited azoreduction. This observation supports that MK is an essential compound of electrons transport for azoreduction. Furthermore, anaerobic azoreduction was sensitive to metyrapone, a specific cytochrome P_{450} inhibitor (Williams et al. 2004), indicating that P_{450} cytochrome plays important roles in the anaerobic azoreduction of strain S12.

Discussion

Bacterial anaerobic azoreduction was explored by a number of investigators and previously reviewed in documents (Stolz 2001; Bumpus 1995; Chung et al. 1992), but the

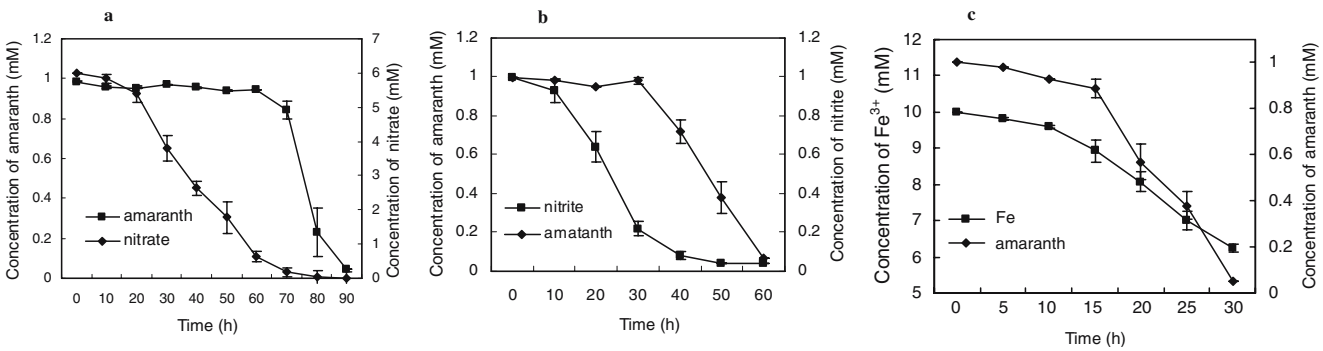


Fig. 5 Reduction of amaranth by *S. decolorationis* S12 in the presence of NO_3^- (**a**), NO_2^- (**b**), and Fe^{3+} (**c**). The initial concentrations of NO_3^-, NO_2^-, and Fe^{3+} are 6, 1, and 10 mM, respectively

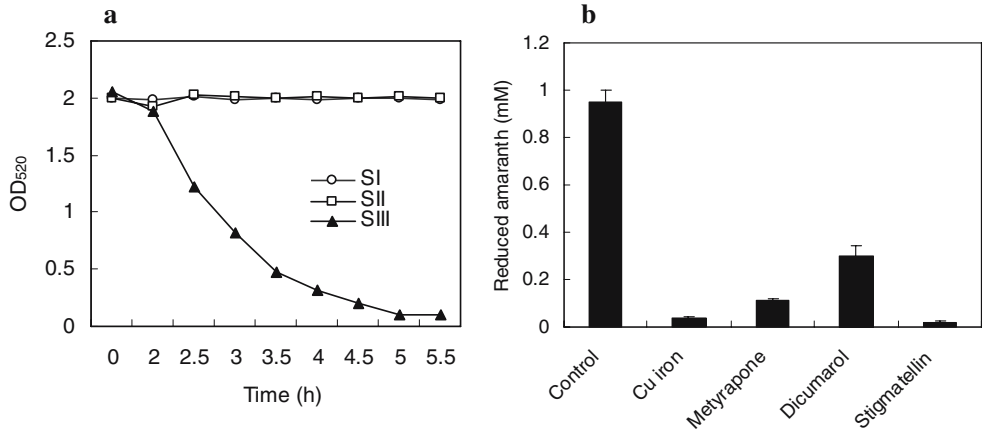


Fig. 6 Anaerobic azoreduction by membranous vesicle of *S. decolorationis* S12 and the effect of respiratory inhibitors on the azoreduction. The experiments were performed in the defined medium under anaerobic condition at 32°C with formate as electron donors. **a** Anaerobic azoreduction by proteins from cytoplasm, periplasm, and membrane of cells. *SI* Periplasmic fraction, *SII* cytoplasmic fraction, *SIII* membranous fraction. **b** Anaerobic azoreduction by membranous vesicle in the presence of various respiratory inhibitors: 5 μM of Cu^{2+}, 100 μM of metyrapone, 200 μM of dicumarol, and 100 μM of stigmatellin. All measurements were obtained by calculating the average values for two independent incubations and error *bars* represent standard deviations of duplicate incubations

mechanism of the subject is not completely known. It was generally considered that bacterial anaerobic azoreduction is a nonspecific reduction process, either a direct enzymatically catalyzed reaction or a reaction with enzymatically reduced redox mediator (Stolz 2001). To date, the correlation between primary electron donors and azo dyes is not clear. In this paper, we describe that azoreduction of strain S12 is a process of transferring the reducing equivalents originating from the oxidation of organic substance or H_2 to the azo dyes under anaerobic condition. Moreover, the azoreduction is accompanied by stoichiometric consumption of electron donors. Pure culture microorganism that is capable of oxidizing formate, lactate, pyruvate, or H_2 coupled to azoreduction has apparently not been described previously. Only mixed culture in anaerobic granular sludge studies have been shown, indicating that azoreduction can be stimulated when volatile fatty acids were present as electron donor (Van der Zee et al. 2001).

Because azo dyes containing a sulfonate group is hydrophilic, it is difficult for the dyes to enter into cells across cell membrane (Kudlich et al. 1997). For intact cells, a membrane transport system would be more suited to the reduction of azo dyes. Our study showed that the azoreduction enzyme system of strain S12 is completely located on cellular membrane fraction. Periplasmic and cytoplasmic fractions of S12 have no azoreduction activity. Similar studies suggest that azoreduction by whole cells of *Sphingomonas* sp. strain BN6 was mainly related to membrane fraction (presumably an NADH–ubiquinone oxidoreductase), but azoreduction activity was also found in the cytoplasm (a soluble FAD-dependent enzyme) (Kudlich et al. 1997). According to an azoreduction character of strain S12, we presume that anaerobic azoreduction by strain S12 was catalyzed by a multicompound system linking to electron transport chain. Respiratory inhibitor experiments confirm the deduction. Dehydrogenase, cytochrome b, MK, P_{450} type cytochrome, and a deduced terminal azoreductase are important components in the multicompound system for azoreduction. These evidences indicate that the bacterial anaerobic azoreduction is an electron transport process depending on electron transport chain system. Azo compounds can serve as electron acceptor of accepted electrons transported through an electron transport chain from a primary electron donor. Whether this electron transport from electron donors to terminal electron acceptor azo dyes can conserve energy for growth is a more interesting subject to be illustrated in the future.

S. decolorationis S12 displays remarkable anaerobic respiratory plasticity, which can conserve energy using a variety of terminal electron acceptors, including O_2, NO_3^-, NO_2^-, and Fe^{3+} (Xu et al. 2005), it provides a unique opportunity to study the competitive effects of different electron acceptors on azoreduction in a single organism. In the simultaneous presence of O_2 and azo dyes, electrons prioritize to transport to O_2 because of its high redox potential (+820 mV); therefore, the azoreduction was fully inhibited by O_2. The reduction of amaranth by S12 was also fully inhibited by 3 mM of NO_3^- and 0.9 mM of NO_2^-. Possibly because redox potential of NO_3^- (+360 mV) and NO_2^- (+440 mV) is higher than amaranth (−180 mV) (Kudlich et al. 1997), electrons are favorable to flow toward NO_3^- or NO_2^-, resulting in the inhibition. Moreover, the inhibition by NO_3^- or NO_2^- could be the result of the production of NO as a product of NO_3^- and NO_2^- reduction. NO is capable of binding to heme and iron-sulfur to form metal–nitrosyl complexes and to inhibit the ability of the

metalloprotein to transfer electrons (Satoh 1984; Zumft 1993). Because dehydrogenase and cytochrome contain iron-sulfur and heme, it is possible that NO inhibits the activity of dehydrogenase and cytochrome to transfer electrons. In addition, it is very interesting that Fe^{3+} can stimulate azoreduction because its redox potential (+770 mV) is also higher than amaranth. The stimulation may be due to azoreduction and Fe^{3+} reduction using different electron transport pathway; therefore, there might be weak competition between azoreduction and Fe^{3+} reduction. The mechanism needs to be further investigated in depth.

This study is significant for the treatment of wastewater contaminated by azo dyes. Normally, azo dyes are reduced under anaerobic condition and the reducing products are subsequently mineralized under aerobic condition (Stolz 2001; Haug et al. 1991). Therefore, the anaerobic/aerobic treatment is a very effective method to decolorize azo dye-containing wastewaters (Seshadri et al. 1994; Chang and Lin 2000). Based on our study, the conditions of electron donors and acceptors have a remarkable effect on azo-reduction. Therefore, the concentration of electron donors and acceptors is an important factor for anaerobic treatment of azo dye-containing wastewaters. Under a general condition, there is abundance of organic substance in the active sludge, so azoreduction can occur with some organic substance as electron donors. But if there is a lack of electron donor in the treatment reactor, the azoreduction will not occur. Therefore, adding an exogenous electron donor is essential to realize the effective reduction of azo dyes. Because of the competition for electrons, electron acceptors can inhibit the anaerobic azoreduction. Especially if a high concentration of NO_3^- and NO_2^- is present in the environment, microbial anaerobic azoreduction may be greatly repressed. So how to decrease the concentration of NO_3^- and NO_2^- is a very important strategy to effectively decolorize azo dyes under anaerobic condition.

In summary, the results of this study with *S. decolorationis* S12 have demonstrated that azo dyes can be used as terminal electron acceptor accepting electrons from primary electron donors through electron transport chain. This reduction was affected by other electron acceptors that can be used by strain S12. This strain provides a novel model organism with which to study organic substances or H_2 metabolism coupled to azoreduction under a defined condition.

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