ENVIRONMENTAL BIOTECHNOLOGY

Anaerobic humus and Fe(III) reduction and electron transport pathway by a novel humus-reducing bacterium, *Thauera humireducens* SgZ-1

Chen Ma · Zhen Yu · Qin Lu · Li Zhuang · Shun-Gui Zhou

Abstract In this study, an anaerobic batch experiment was conducted to investigate the humus- and Fe(III)-reducing ability of a novel humus-reducing bacterium, *Thauera humireducens* SgZ-1. Inhibition tests were also performed to explore the electron transport pathways with various electron acceptors. The results indicate that in anaerobic conditions, strain SgZ-1 possesses the ability to reduce a humus analog, humic acids, soluble Fe(III), and Fe(III) oxides. Acetate, propionate, lactate, and pyruvate were suitable electron donors for humus and Fe(III) reduction by strain SgZ-1, while fermentable sugars (glucose and sucrose) were not. UV-visible spectra obtained from intact cells of strain SgZ-1 showed absorption peaks at 420, 522, and 553 nm, characteristic of *c*-type cytochromes (cyt *c*). Dithionite-reduced cyt *c* was reoxidized by Fe-EDTA and HFO (hydrous ferric oxide), which suggests that cyt *c* within intact cells of strain SgZ-1 has the ability to donate electrons to extracellular Fe(III) species. Inhibition tests revealed that dehydrogenases, quinones, and cytochromes *b/c* (cyt *b/c*) were involved in reduction of AQS (9, 10-anthraquinone-2-sulfonic acid, humus analog) and oxygen. In contrast, only NADH dehydrogenase was linked to electron transport to HFO, while dehydrogenases and cyt *b/c* were found to participate in the reduction of Fe-EDTA. Thus, various different electron transport pathways are employed by strain SgZ-1 for different electron acceptors. The results from this study help in understanding the electron transport processes and environmental responses of the genus *Thauera*.

Keywords *Thauera* · Humus · Fe(III) species · Electron transport pathway · *c*-type cytochrome

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Introduction

Microbial humus and Fe(III) reduction have been widely studied because of their environmental significance and practical applications (Wu et al. 2010; Liu et al. 2011a; Martinez et al. 2013). Knowledge of the electron transport chains (ETC) in microbes capable of extracellular respiration should help in understanding how they react with humus and Fe(III) in their environment (Lovley et al. 1996; Straub et al. 2005).

It is known that electron transport for extracellular respiration in gram-negative bacteria occurs across the cytoplasmic membrane (CM), the periplasmic space, and the outer membrane (OM). Thus, investigating the electron carriers and associated *c*-type cytochromes (cyt *c*) composing the ETC may shed light on the extracellular respiration process (Myers and Myers 1992; Woznica et al. 2003). The ETC of humus and Fe(III) reduction in the model bacteria *Shewanella* sp. and *Geobacter* sp. has been investigated extensively (Carlson et al. 2012; Shi et al. 2012). Studies with respiratory inhibitors and genetic/transcriptional analysis revealed that various ETCs may be employed by the model bacteria in electron transport to soluble (e.g., oxygen, nitrate) or insoluble (e.g., electrodes, metals) electron acceptors (Myers and Myers 1992; Bretschger et al. 2007; Rosenbaum et al. 2012). However, much remains to be learned about the electron transport pathways used by non-model bacteria and the different ETCs operating during bacterial growth on different electron acceptors (Bird et al. 2011).

Thauera species are noted for their ability to metabolize various aromatic compounds under denitrifying conditions and for using simple compounds (e.g., hydrogen and acetate) for growth (Liu et al. 2013). Consequently, *Thauera* has emerged as an important genus in the investigation of metabolic versatility and remediation of environmental pollutants (Liu et al. 2013). Recently, a novel humus-reducing bacterium, *Thauera humireducens* SgZ-1 (KACC 16524=CCTCC M2011497), was isolated from the anode biofilm of a sediment microbial fuel cell in our laboratory (Yang et al. 2013). The bacterium is capable of growing by oxidizing various organic compounds anaerobically, using a humus analog and nitrate as terminal electron acceptors. Thus, strain SgZ-1 has vast potential for application in environmental remediation. To date, there have been few reports on electron transport pathways employed by the genus *Thauera*. The electron transport pathway of selenate respiration by *Thauera selenatis* has been elucidated (Macy et al. 1993; Lowe et al. 2010), but little is known about respiratory pathways for humus and Fe(III) reduction in *Thauera* species. Owing to the potential environmental and metabolic importance of strain SgZ-1, in this study, we have investigated anaerobic humus and Fe(III) reduction and electron transport pathways. The results enhance our understanding of the electron transport processes and environmental responses of the genus *Thauera*.

Materials and methods

Use of electron donors and acceptors in humus and Fe(III) reduction

To investigate the use of various electron donors in humus and Fe(III) reduction, six organic substrates (acetate, propionate, pyruvate, lactate, glucose, and sucrose) were tested with 9, 10-anthraquinone-2,6-disulfonic acid (AQDS, humus analog) and Fe-EDTA as model electron acceptors. The experiment was conducted in 20.0 mL basal medium ($NaHCO_3$ 2.50 g L^{-1}, NH_4Cl 0.25 g L^{-1}, $NaH_2PO_4 \cdot 2H_2O$ 0.68 g L^{-1}, KCl 0.10 g L^{-1}, pH 7.0) in 25.2 mL serum bottles. Electron donor was added to a final concentration of 10.0 mmol L^{-1}. AQDS and Fe-EDTA were used as electron acceptor with a final concentration of 1.0 mmol L^{-1} and 2.0 mmol L^{-1}, respectively. Cell suspension, prepared as described previously (Wu et al. 2010), was added with a final concentration of 1.0×10^7 CFU mL^{-1}. In experiments to measure bacterial growth, the initial concentration was 1.0×10^5 CFU mL^{-1} with pyruvate or acetate as electron donor (each at 5.0 mmol L^{-1}). Colony number was determined by aerobic plate count (colony forming units, CFU mL^{-1}). All the bottles were purged with 80/20 % (v/v) N_2/CO_2 for 15 min, then immediately stoppered with butyl rubber bungs and crimped with aluminum caps. All the treatments with different electron donors

were conducted in triplicate. The bottles were incubated in the dark at 30 °C.

To test the ability of strain SgZ-1 to reduce various electron acceptors, similar procedures were conducted and pyruvate was used as the electron donor. Electron acceptors tested included AQDS and 9, 10-anthraquinone-2-sulfonic acid (AQS), each at 1.0 mmol L^{-1}; sigma humic acids (sigma-HA) and Elliott soil humic acid standard (ES-HA, International Humic Substances Society, 1S102H), each at 2.0 g L^{-1}; Fe-citrate and Fe-EDTA, each at 2.0 mmol L^{-1}; and hydrous ferric oxide (HFO) and goethite, each at 10.0 mmol L^{-1}. Effects of electron shuttles on microbial reduction of Fe(III) oxides were also investigated when the insoluble HFO and goethite were used as electron acceptors. Electron shuttles employed were AQDS (0.5 mmol L^{-1}), AQS (0.5 mmol L^{-1}), and sigma-HA (2.0 g L^{-1}).

Characterization of cyt *c* in intact cells

Cells of SgZ-1 were grown aerobically for 16 h in LB medium, collected by centrifugation at $10,000 \times g$ for 5 min, washed twice, and resuspended in 10.0 mmol L^{-1} HEPES (pH 7.0) to a final cell density of $OD_{600}=2.5$. Spectra of dithionite-reduced and air-oxidized cyt *c* were recorded as previously described (Collins and Niederman 1976). Reduction of samples was achieved by adding an excess of sodium dithionite. Oxidized samples were prepared by sparging with oxygen. To determine whether reduced cyt *c* in intact cells was able to donate electrons to Fe-EDTA or HFO, cell suspension in a cuvette was fully reduced using sodium dithionite in an N_2 atmosphere. Then, Fe-EDTA or HFO at a concentration of 10.0 mmol L^{-1} was added to the cuvette immediately. Reoxidation of cyt *c* was monitored by recording difference spectra using a TU-1901 spectrophotometer (Purkinje General, Beijing, China) (Lowe et al. 2010). The strains used for positive and negative control experiments were *Shewanella onedensis* MR-1 (ATCC 700550, a model bacterium) and *Bacillus pseudofirmus* MC02 (CGMCC 4771), respectively.

SDS-PAGE detection of cyt *c* was undertaken as follows. Cells grown for 16 h in LB medium were harvested by centrifugation at $12,000 \times g$ for 5 min at 4 °C, and suspended in 20.0 mmol L^{-1} Tris–HCl (pH 7.0) containing 1.0 mL lysis solution (lysozyme [100.0 μg mL^{-1}] and phenylmethanesulfonyl fluoride [PMSF] [1.0 μg mL^{-1}]). The cells were then lyzed with a JY92-I ultrasonic liquid processor (Scientz, Ningbo, China) at 300 W every 10 s for 60 cycles. The supernatant was separated on SDS-PAGE gels (20 μg protein per lane) and stained for whole protein with Coomassie blue R250 or for cyt *c* using the 3,3′,5,5′-tetramethylbenzidine (TMBZ)-hydrogen peroxide method (Thomas et al. 1976).

Inhibition experiments

Four electron acceptors (AQS, Fe-EDTA, HFO, and oxygen) were used. Experiments were conducted in 20.0 mL basal medium containing 5.0 mmol L^{-1} pyruvate and 1.0 mL cell suspension (1.0×10^7 CFU mL^{-1}). When AQS, Fe-EDTA, and HFO were used as the electron acceptors, the experiment was performed under anaerobic conditions with the acceptor present at concentrations of 1.0 mmol L^{-1}, 5.0 mmol L^{-1}, or 5.0 mmol L^{-1}, respectively. When oxygen was the electron acceptor, the experiment was conducted aerobically in tubes at ambient atmosphere. Inhibitors of the electron transport chain or ATP synthase and uncouplers of the periplasmic membrane used in this study included DCCD (*N, N′*-dicyclohexylcarbodiimide) (dissolved in acetone), dicumarol (dissolved in 0.5 mmol L^{-1} NaOH), quinacrine (dissolved in H_2O), and CCCP (carbonyl-cyanide-m-chlorophenyl hydrazine)/antimycin A/capsaicin (dissolved in 95 % ethanol) (Knight and Blakemore 1998; Woznica et al. 2003). Any inhibition effect was expressed as the percentage inhibition in electron transport to different electron acceptors (i.e., percent decrease in reduction product) compared with that without inhibitor (control) (Knight and Blakemore 1998; Woznica et al. 2003). When oxygen was the electron acceptor, percentage inhibition was calculated as the percent decrease in microbial aerobic growth, determined by $OD_{600 \ nm}$.

Analytical techniques

Reduced humus analogs of AQDS and AQS were quantified with a UV-visible spectrophotometer (TU1800-PC, Beijing, China) at 450 nm and 410 nm, respectively (Liu et al. 2007; Wu et al. 2013). Fe(II), the reduction product of Fe(III), was first mixed with 0.5 mol L^{-1} HCl for 1.5 h; the acid ensured that Fe(II) adsorbed on the surface of iron oxides was released, and reoxidation of Fe(II) to Fe(III) was prevented. Fe(II) was then determined colorimetrically with 1, 10-phenanthroline (Fredrickson and Gorby 1996). HA reduction was monitored as described by Lovley et al. (1996) with modifications. As reduced HA (RHA) cannot be directly quantified, it is indirectly quantified by the maximum Fe(III) it can reduce (Lovley et al. 1996; Wu et al. 2013). All analyses were performed in triplicate.

**Results**

Humus and Fe(III) reduction by strain SgZ-1

Figure 1 shows the production of reduced AQDS (AHDS) and Fe(II) by *T. humireducens* strain SgZ-1 with various compounds as electron donors. There was almost no AHDS or Fe(II) production in abiotic controls (<0.05 mmol L^{-1} AHDS, data for Fe[II] production not shown). In the treatments with bacterial cells, the concentrations of AHDS at the end of the experiment were 0.32 mmol L^{-1}, 0.29 mmol L^{-1}, 0.43 mmol L^{-1}, 0.06 mmol L^{-1}, 0.08 mmol L^{-1}, and 0.40 mmol L^{-1} for acetate, propionate, pyruvate, glucose, sucrose, and lactate, respectively (Fig. 1a). The concentrations of Fe(II) at the end of the experiment were 0.06 mmol L^{-1}, 0.03 mmol L^{-1}, 0.35 mmol L^{-1}, 0.44 mmol L^{-1}, 0.47 mmol L^{-1}, and 0.34 mmol L^{-1} for glucose, sucrose, acetate, lactate, pyruvate, and propionate, respectively (Fig. 1b). Thus, it seems that strain SgZ-1 used organic acids (acetate, propionate, pyruvate, and lactate) as electron donors in preference to fermentable sugars such as glucose and sucrose.

Figure 2 shows reduction of humus analog and humic acids (HA) by strain SgZ-1. The concentrations of reduced AQS and AQDS after a 7-day incubation period were 0.38 mmol L^{-1} and 0.36 mmol L^{-1}, respectively (Fig. 2a). The quantity of RHA increased with time in treatments containing bacterial cells, while almost no RHA was detected in abiotic treatments (Fig. 2b). After a 17-day incubation period, the concentration of RHA reached 0.28 mmol L^{-1} and 0.31 mmol L^{-1} (expressed in terms of Fe[II]) in treatments with sigma-HA and ES-HA, respectively. To investigate the ability of strain SgZ-1 to reduce various Fe(III) species, soluble Fe(III) (Fe-citrate and Fe-EDTA) and insoluble Fe(III) (HFO and goethite) were tested (Fig. 2c, d). After an 18-day incubation period, the concentrations of Fe(II) were 1.3 mmol L^{-1} and 0.4 mmol L^{-1} in the Fe-EDTA and Fe-citrate treatments, respectively (Fig. 2c). The concentrations of Fe(II) were 0.33 mmol L^{-1} and 0.03 mmol L^{-1} in the HFO and goethite reductions, respectively, after a 30-day incubation period (Fig. 2d). Figure 2e shows the effects of electron shuttles on HFO reduction, such molecules facilitate electron transport from the bacteria to insoluble Fe(III) species. Fe(II) concentration declined a little in the latter stages of the incubation. This may have been owing to a decrease in microbial activity owing to lack of nutrients or to formation of secondary minerals from Fe(II) with PO_4^{3-} or CO_3^{2-} in the buffer solution (Liu et al. 2011b). The zero-order kinetic constants (k, mmol L^{-1} d^{-1}) for Fe(II) production over the first 12 days of HFO reduction were: no mediator added, 0.009; sigma-HA added, 0.0148; AQDS added, 0.0355, and AQS added, 0.0362. Figure 2f shows the curves for goethite reduction in different electron shuttle-added systems, stimulation effects were relatively similar to those for HFO reduction.

Figure 3 shows the extent of anaerobic bacterial growth in different conditions, with AQDS, AQS, Fe-EDTA, or HFO as electron acceptor and pyruvate or acetate as electron donor. When pyruvate was the electron donor, the number of bacterial cells in all treatments increased during the first 60 h (Fig. 3a). The resulting colony numbers were: AQS, 520×

Fig. 1 AQDS and Fe-EDTA reduction by *Thauera humireducens* strain SgZ-1 with various electron donors (each at 5.0 mmol L^{-1}) in 1 mL cell solution (1.0×10^7 CFU mL^{-1}). **a** AQDS reduction (1.0 mmol L^{-1} AQDS). **b** Fe-EDTA reduction (2.0 mmol L^{-1} Fe-EDTA)
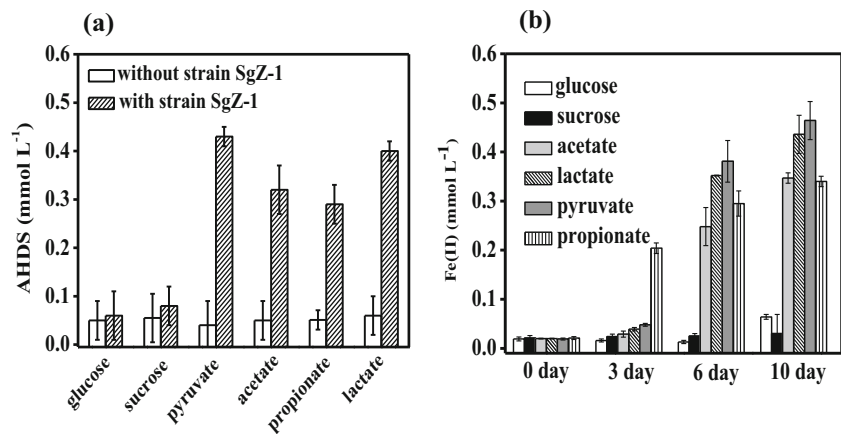
10^5 CFU ml^{-1}; AQDS, 420×10^5 CFU ml^{-1}; Fe-EDTA, 370×10^5 CFU ml^{-1}; and HFO, 230×10^5 CFU ml^{-1}. When acetate was the electron donor, there was almost no growth with HFO as terminal electron acceptor, but the quantity of bacterial cells increased in the first 60 h in the other treatments (Fig. 3b).

Total cell growth was lower with acetate as the electron donor compared with pyruvate. This indicated that strain SgZ-1 was able to grow anaerobically with pyruvate or acetate as its energy source when AQDS, AQS, or Fe-EDTA was the electron acceptor. However, when HFO was the

Fig. 2 Anaerobic reduction of humus and Fe(III) by strain SgZ-1 with 5.0 mmol L^{-1} pyruvate as electron donor and 1 mL cell solution (1.0×10^7 CFU mL^{-1}). **a** Reduction of humus analogs (AQDS and AQS, each at 1.0 mmol L^{-1}). **b** Reduction of humic acids (sigma-HA and ES-HA, each at 2.0 g L^{-1}). **c** Reduction of soluble Fe(III) (Fe-EDTA and Fe-citrate, each at 2.0 mmol L^{-1}). **d** Reduction of insoluble Fe(III) (HFO and goethite, each at 10.0 mmol L^{-1}). **e** Effects of electron shuttles on reduction of 10.0 mmol L^{-1} HFO. Electron shuttles employed were 0.5 mmol L^{-1} AQDS, 0.5 mmol L^{-1} AQS, and 2.0 g L^{-1} sigma-HA. **f** Effects of electron shuttles on reduction of 10.0 mmol L^{-1} goethite. Electron shuttles employed were 0.5 mmol L^{-1} AQDS, 0.5 mmol L^{-1} AQS, and 2.0 g L^{-1} sigma-HA
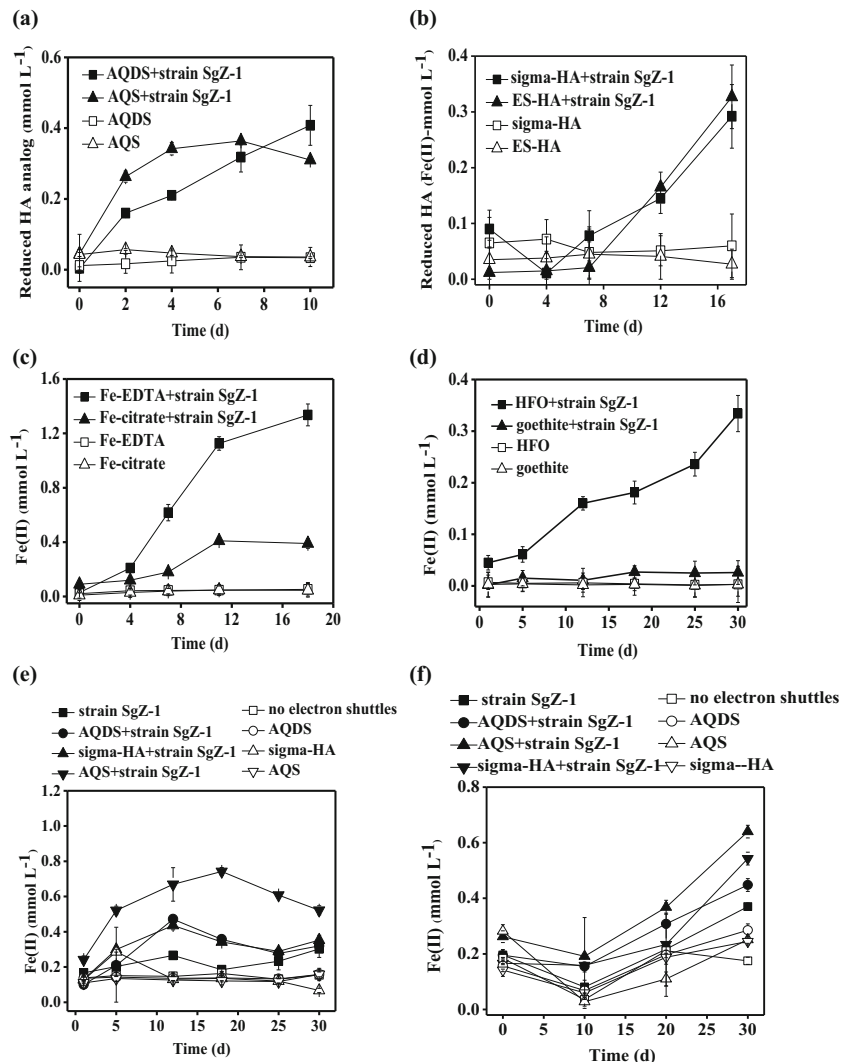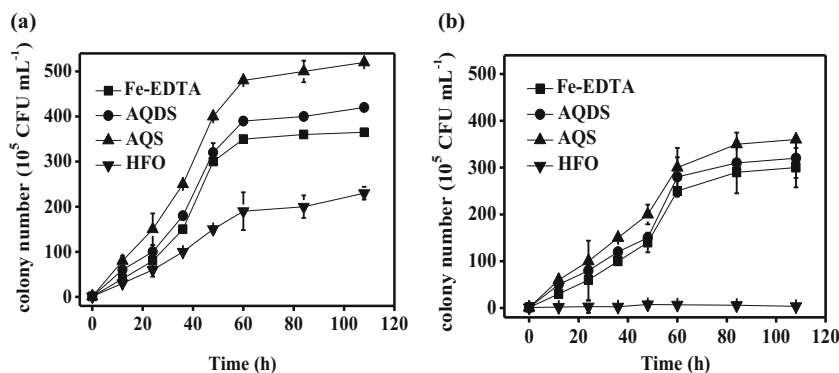
Fig. 3 Growth of strain SgZ-1 under different conditions: AQDS, AQS, Fe-EDTA, or HFO as electron acceptor, with pyruvate or acetate as electron donor, in 1 mL cell solution (1.0× 10^5 CFU mL^{-1}). **a** 5.0 mmol L^{-1} pyruvate as electron donor. **b** 5.0 mmol L^{-1} acetate as electron donor

electron acceptor, strain SgZ-1 could grow anaerobically on pyruvate but not on acetate.

UV-visible difference absorption spectra and SDS-PAGE analysis of cyt c in whole cells

Figure 4 shows UV-visible absorption spectra and SDS-PAGE analysis of cyt c in cells of strain SgZ-1 and *S. onedensis* MR-1 (a species rich in c-type cytochromes). Cell pellets of both strain SgZ-1 (Fig. 4a, 1 in the inset figure) and strain MR-1 (Fig. 4a, 2 in the inset figure), obtained from culture by centrifugation at 10,000×g, were red. In comparison, the negative control *B. pseudofirmus* MC02 (which contains little cytochrome) was light yellow after pelleting (Fig. 4a, 3 in the inset figure). This suggests that strain SgZ-1 may contain cytochromes in its intact cells as strain MR-1 does.

Dithionite-reduced and air-oxidized spectra obtained from intact cells of strain SgZ-1 were also typical of cyt c. The reduced spectrum displayed absorption maxima at 420 nm (Soret band), 522 nm (β-band), and 553 nm (α-band) (Fig. 4b, inset figure: shown as the enlarged figure of reduced cyt c

spectrum). The oxidized spectrum showed a shift in the Soret band to 410 nm and the peaks decreased in intensity (Fig. 4b). Fig. 4c and d show electrophoretically resolved cyt c and the total soluble cellular proteins, respectively, from strain SgZ-1 and strain MR-1. Heme-staining was used to identify cyt c and Coomassie blue R250 staining for total proteins. Five bands were detected on the heme-stained SDS-PAGE of strain SgZ-1 (~28, ~22, ~19, ~17, and ~15 KDa) (Fig. 4c). The differences between the heme- and Coomassie-stained SDS-PAGE indicated that the cyt c expressed in strain SgZ-1 represents only a small portion of the total soluble cellular proteins (Fig. 4c, d).

Intact cells of strain SgZ-1 were tested for their ability to donate electrons to extracellular electron acceptors (i.e., Fe-EDTA, HFO) in anaerobic conditions. The upper curve in Fig. 5 is the spectrum of dithionite-reduced cyt c after subtraction of the spectrum of air-oxidized cyt c in intact cells (i.e., it is the reduced *minus* oxidized difference spectrum). The second curve (from the top) is the air-oxidized form of cyt c. The oxidized spectrum showed a shift in the Soret band to 410 nm and the peaks decreased in intensity. Similar spectra to
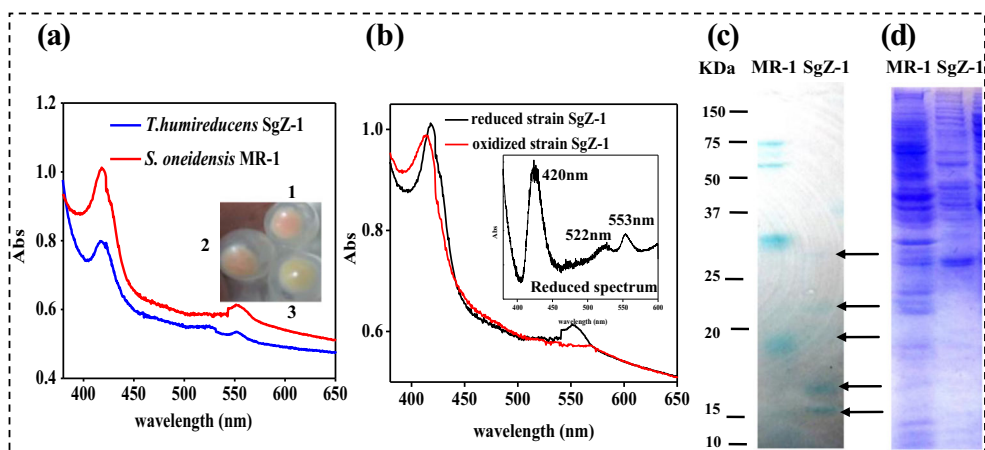

Fig. 4 UV-visible absorption spectra and SDS-PAGE of cyt c in cells of strain SgZ-1 (OD_{600}=2.5). **a** UV-visible absorption spectra of SgZ-1 cells and *Shewanella onedensis* MR-1 cells; *inset figure*: *1*, strain MR-1; *2*, strain SgZ-1; *3*, strain MC02. **b** Spectra of dithionite-reduced and air-oxidized cyt c in intact cells of strain SgZ-1; *inset figure*, enlarged figure of reduced cyt c spectrum. **c** Heme-stained SDS-PAGE of soluble proteins from strain SgZ-1 and strain MR-1. **d** Coomassie blue-stained SDS-PAGE of soluble cellular proteins from strain SgZ-1 and strain MR-1

that of the air-oxidized cyt c were obtained after the addition of Fe-EDTA or HFO (the lower two curves in Fig. 5) to the solution of dithionite-reduced cyt c, suggesting that initially dithionite-reduced cytochromes were oxidized by the addition of Fe-EDTA or HFO, and thus, cyt c may be involved in electron transport to these compounds.

Effects of inhibitors on the reduction of various electron acceptors

Figure 6 shows the influence of inhibitors of the electron transport chain or ATP synthase, and uncouplers of the periplasmic membrane on the reduction of oxygen, Fe-EDTA, AQS, and HFO by strain SgZ-1. The higher the inhibitor concentration, the greater the inhibition effect observed. For oxygen reduction, inhibition percentages by quinacrine, DCCD, CCCP, antimycin A, and capsaicin at high concentrations were 85, 71, 87, 70, and 69 %, respectively, while dicumarol showed a relatively low inhibition effect (38 %) (Fig. 6a). In the reduction of Fe-EDTA, percentage inhibition by CCCP and antimycin A at high concentrations reached 50 and 90 %, respectively, while those by quinacrine and capsaicin were ~38 and 42 %, respectively. Fe-EDTA reduction was only slightly inhibited by dicumarol and DCCD (inhibition
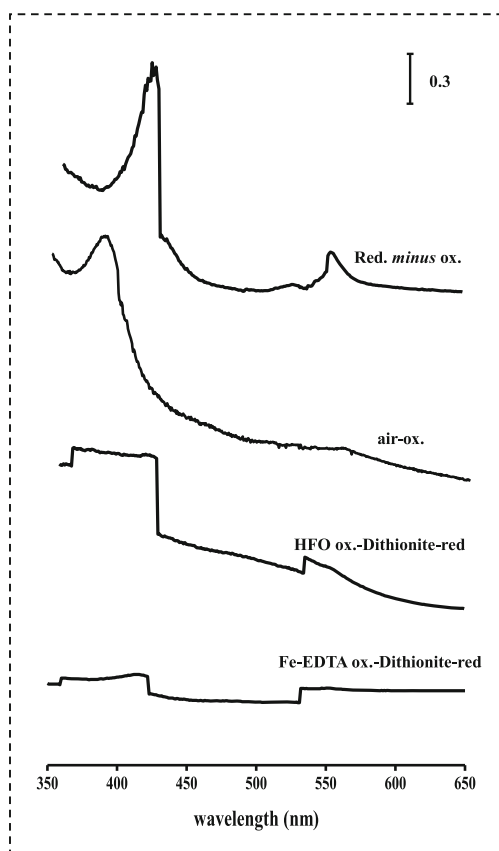

Fig. 5 Absorption changes of dithionite-reduced cyt c in intact cells of strain SgZ-1 on addition of 10.0 mmol L^{-1} Fe-EDTA or 10.0 mmol L^{-1} HFO (*Red* reduced, *Ox* oxidized)

only 10–14 and 7 %, respectively) (Fig. 6b). AQS reduction was inhibited by dicumarol (51 %), DCCD (52 %), antimycin A (60 %), and capsaicin (70 %), but not by CCCP (2 %) (Fig. 6c). When HFO acted as electron acceptor, only CCCP (61 %) and rotenone (40–50 %) inhibited the Fe(III) reduction, while dicumarol, quinacrine, DCCD, antimycin A, and capsaicin had no inhibitory effect (Fig. 6d; data for antimycin A and capsaicin not shown).

Discussion

Humus and Fe(III) reduction

In our experiments, *T. humireducens* strain SgZ-1 was able to use organic acids (acetate, propionate, pyruvate, and lactate) but not fermentable sugars (glucose and sucrose) as electron donors in AQDS and Fe(III) reduction. Previous studies demonstrated that many species in the genus *Thauera* are able to use acetate in the reduction of various electron acceptors, for example, in the reduction of selenite by *T. selenatis* (Lowe et al. 2010). *Thauera aminoaromatica* MZ1T can metabolize acetate to methane (Jiang et al. 2012). To our knowledge, there are only a few bacteria possessing the ability to oxidize acetate for humus and Fe(III) reduction, e.g., the iron-reducing bacterium *Geobacter* sp. (Bird et al. 2011), the sulfate-reducing bacterium *Desulfobacter postgatei* (Ingvorsen et al. 1984), the Fe(III)/Mn(IV)/Cr(VI)-reducing bacterium *Pantoea agglomerans* (Francis et al. 2000), and the humus-reducing bacterium *Corynebacterium humireducens* (Wu et al. 2013). The genera *Thauera* and *Geobacter* share some metabolic features. Both are capable of mineralizing aromatic compounds (Jiang et al. 2012) and contain abundant cyt c (Lowe et al. 2010). However, *Thauera* species are facultatively anaerobic while *Geobacter* species are obligately anaerobic. Because of its metabolic versatility and capacity for extracellular respiration, strain SgZ-1 is a good model bacterium for studies of extracellular electron transport.

Table 1 shows the zero-order kinetic constants (k) for HFO reduction mediated by the electron shuttles AQDS, AQS, and sigma-HA in this study, and the corresponding redox potentials, percent transmittance in FT-IR spectra, and the electron accepting capacity (EAC) for the electron shuttles obtained from Wolf et al. (2009) and Wu et al. (2013). As the literature of Wu et al. (2013) reported, the absorption at about 1650 and 1630 cm^{-1} in the FT-IR spectrum can be assigned to the C = O stretching of quinones (D'Orazio and Senesi 2009). The intensity of a band can be measured as the percent transmittance of the FT-IR radiation, which can represent the amount of quinones groups in the redox-active compounds. The higher the percent transmittance, the lower the amount of quinones groups are. It is evident that k (mmol L^{-1} d^{-1}) correlated

Fig. 6 Effects of respiratory inhibitors, ATP synthase inhibitors, and uncouplers on the reduction of various electron acceptors by strain SgZ-1: **a** Oxygen reduction. **b** Fe-EDTA reduction. **c** AQS reduction. **d** HFO reduction. Concentrations of the inhibitors and uncouplers are as follows: 20, 200, 400, and 500 μmol L^{-1} dicumarol; 20, 100, 200, and 400 μmol L^{-1} quinacrine; 20, 40, and 80 μmol L^{-1} DCCD; 10, 20, and 40 μmol L^{-1} CCCP; 150 and 300 μmol·L^{-1} Antimycin A; 500 and 1000 μmol L^{-1} capsaicin; 40, 100, and 200 μmol L^{-1} rotenone; shown in *different colors* from left to right in the figures
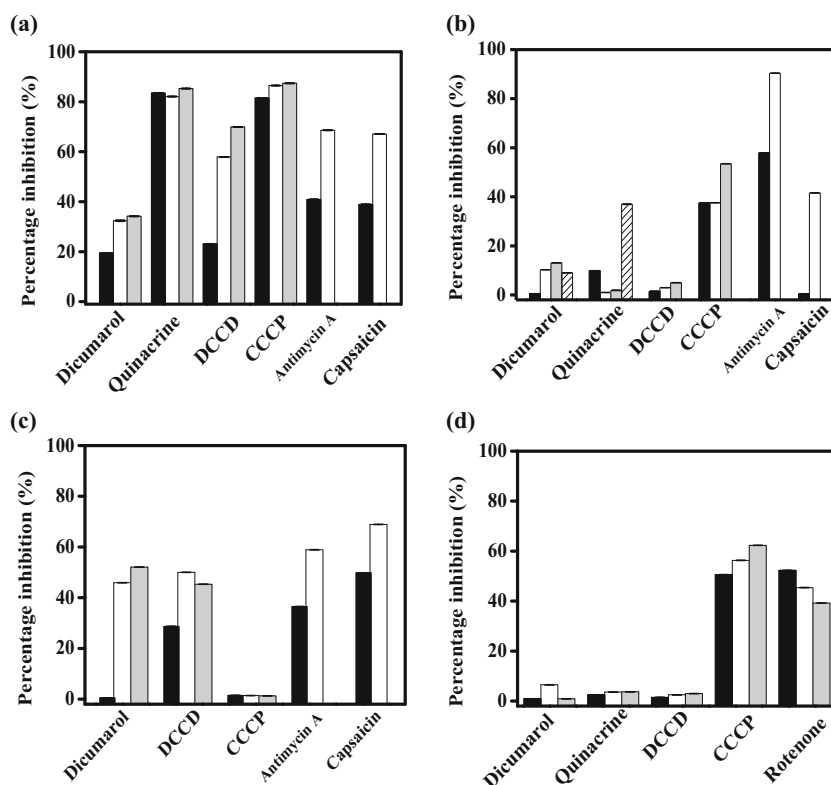
negatively with the redox potential and the percent transmittance in FT-IR spectra of the electron shuttles (positively related with the amount of quinones groups). The order of the EAC was AQS>AQDS>HA, which is positively related to k (Yuan et al. 2011). The reducing capability (electron accepting capacity) of humus analogs (AQDS and AQS) and their stimulation effects on Fe(III) reduction were much higher than those of HA. The humus analogs have higher electron accepting capacity (EAC) and lower redox potential (shown as in Table 1). Moreover, AQDS and AQS are significantly smaller than HA, enabling readier transfer of electrons at the interfaces of Fe(III) oxides (Van Trump et al. 2006). In the experimental condition of pH 7.0, the solubility of AQDS or AQS is higher than that of HA, and HA is more soluble under alkaline conditions (Van Trump et al. 2006).

In addition to quinones in humus, non-quinone (NQ) redox-active functional groups in HA samples also affect the electron accepting capacity of HA. Ratasuk and Nanny (2007) demonstrated by examining eight quinone compounds and 14 HA and fulvic acids (FA) samples that the electron-carrying capacity (ECC) of NQ moieties represents 21–56 % of the total ECC of HA and FA. Hernandez-Montoya et al. (2012) reported that NQ redox-active functional groups (e.g., nitrogenous and sulfurous redox mediating functional groups) in HA samples are responsible for 25–44 % of the total electron-transferring capacity. Furthermore, they reported that humus-reducing microorganisms, e.g., *G. sulfurreducens,* are capable of reducing both quinone and NQ redox-active functional groups in different HA samples. In our study, it was demonstrated that the quinones in humus were responsible for an important fraction of the electron accepting capacity of HA.

Table 1 Zero-order kinetic constants (k, mmol L^{-1} d^{-1}) for HFO reduction mediated by various electron shuttles (AQDS, AQS, and sigma-HA) and chemical features of the electron shuttles [redox potential, contents of quinone groups (expressed as percent transmittance on FT-IR spectrum) and electron accepting capacity (EAC)]

Electron shuttle	k^a (mmol L^{-1} d^{-1})	Redox potential[b] (mV)	Percent transmittance[b] (%)	EAC[b] μmol$_e$–(gC)$^{-1}$
AQS	0.0362	−225	0.09	2261
AQDS	0.0355	−184	0.22	1481
Sigma-HA	0.0148	−120	0.47	680

[a] This study

[b] From Wolf et al. (2009) and Wu et al. (2013)

However, additional experiments are required on the relationship of NQ groups in the HA samples and their electron accepting capacity.

Preliminary models for electron transport pathways with various electron acceptors

Based on the effects of inhibitors, we propose electron transport chains in strain SgZ-1 for four different conditions (Fig. 7). Inhibition sites of the seven respiratory and ATP synthase inhibitors and uncouplers used are shown in Fig. 7a (Knight and Blakemore 1998; Woznica et al. 2003): capsaicin and rotenone inhibit the activity of NADH dehydrogenase, quinacrine inhibits the activity of FAD dehydrogenase, dicumarol is an inhibitor of the quinone loop, antimycin A inhibits the cytochrome b/c complex, DCCD acts as an inhibitor of ATP synthase, and CCCP dissipates the proton motive force (PMF).

The electron transport components involved in reduction of oxygen were similar to those in the traditional bacterial electron transport chain, including dehydrogenases (NADH/FAD), a quinone loop, cyt b/c (and, linked chemiosmotically, ATP synthase) (Fig. 7a). The results also indicate that oxygen reduction was only partly inhibited by dicumarol (about 38 %), thus the quinone loop may not be an essential component in oxygen reduction. The inhibitory effects of DCCD and CCCP suggest that oxygen reduction was linked to the formation of PMF and energy conservation. According to the literature (Bird et al. 2011), oxygen reductase is located in the inner part of the cytoplasmic membrane (Fig. 7a).

For Fe-EDTA reduction, the proposed electron transport chain involves dehydrogenases (NADH/FAD) and cyt b/c (Fig. 7b). Classically, energy available from electron transport produces an electrochemical PMF that drives ADP phosphorylation (coupled to proton retranslocation) via membrane-

Fig. 7 Proposed electron transport chains for different electron acceptors in strain SgZ-1. a Oxygen. b Fe-EDTA. c AQS. d HFO. *CM* cytoplasmic membrane, *OM* outer membrane, *PMF* proton motive force
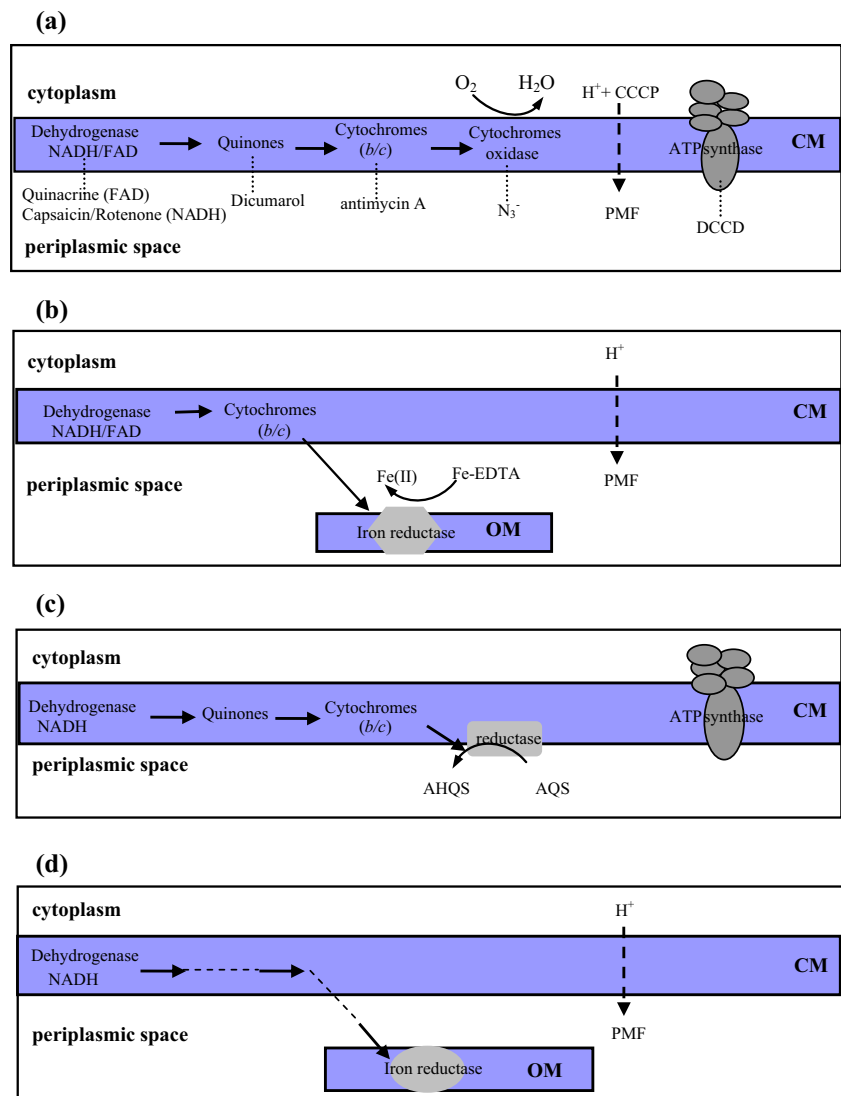
bound ATP synthase. However, ATP production with Fe(III) as electron acceptor primarily depends upon substrate level phosphorylation in *S. putrefaciens*, *Escherichia coli*, etc. (Gorby and Lovley 1991; Park and Kim 2001; Hunt et al. 2010). In our study, electron transport for Fe-EDTA reduction in strain SgZ-1 was inhibited by CCCP, but not by DCCD. Therefore, ATP was probably not produced through the respiratory electron transport chain derived PMF on reduction of Fe(III) to Fe(II), but protons were transferred across the cytoplasmic membrane and consumed during Fe(III) reduction (Gorby and Lovley 1991; Park and Kim 2001). However, the results shown in Fig. 3 indicate that strain SgZ-1 was able to grow anaerobically with pyruvate or acetate as its energy source in the presence of Fe-EDTA. Thus, additional experiments are required to fully explain these observations. Additionally, the location of the terminal soluble Fe(III) reductase is unclear (Kaufmann and Lovley 2001; Bird et al. 2011). UV-visible absorption spectra in our study indicate that cyt c in intact cells can donate electrons to extracellular Fe-EDTA (Fig. 5). Therefore, the terminal Fe-EDTA reductase of strain SgZ-1 may be located in the outer membrane (Fig. 7b).

When AQS acted as terminal electron acceptor, the electron transport chain was composed of NADH dehydrogenase, quinone, and cyt b/c; ATP synthase was probably also functional in ADP phosphorylation (Fig. 7c). However, surprisingly, AQS reduction by strain SgZ-1 was inhibited by DCCD (an ATP synthase inhibitor), but not by CCCP (which dissipates the proton motive force). It is also unclear at present where the terminal AQS reductase is located. We propose that the terminal AQS reductase is in the cytoplasmic membrane (Fig. 7c).

When HFO was the electron acceptor, only NADH dehydrogenase was found to participate in the electron transport pathway (Fig. 7d). Similar to Fe-EDTA reduction, HFO reduction was inhibited by CCCP, but not by DCCD. This result suggests that electron transport for HFO reduction involved proton translocation, and that the protons were probably consumed during Fe(III) reduction to Fe(II), rather than by ATP synthase. Figure 3 shows that strain SgZ-1 grows anaerobically with pyruvate as its energy source, but is unable to grow anaerobically on acetate in the presence of HFO. Thus, HFO probably serves merely as an electron sink in the anaerobic growth of strain SgZ-1, which indicated that strain SgZ-1 did not conserve energy by oxidative phosphorylation when using HFO as electron acceptor. Cyt c in intact cells donated electrons to extracellular HFO (Fig. 5), thus the terminal HFO reductase of strain SgZ-1 is probably located in the outer membrane (Fig. 7d).

Overall, respiratory chain components involved in oxygen and AQS reduction were relatively similar, consisting of dehydrogenases, a quinone loop, and cyt b/c (and ATP synthase consuming the PMF). The electron transport chains with Fe-EDTA or HFO as electron acceptor involved

dehydrogenase(s) and were linked to translocated protons. Furthermore, there were differences in the electron transport chains to soluble (oxygen, Fe-EDTA, and AQS) and insoluble (HFO) electron acceptors. Electron transport pathways with various electron acceptors in strain SgZ-1 are similar to those in the model bacterium *Shewanella* (Hunt et al. 2010; Bird et al. 2011). In other bacteria, the quinone loop has been confirmed to participate in microbial Fe(III) reduction (Knight and Blakemore 1998; Carlson et al. 2012). However, in our study, the quinone loop was probably not an essential component in Fe(III) reduction. Our results demonstrate that the composition of microbial electron transport chains is genus or species dependent and electron acceptor specific.

To date, very few studies have been conducted on electron transport mechanisms in the genus *Thauera*. The electron transport chains in nitrate reduction and selenate respiration by *T. selenatis* were only characterized recently (Lowe et al. 2010). The ETC of *T. selenatis* during anaerobic growth on selenate contained dehydrogenases, a quinone loop in the cytoplasmic membrane and cyt c_4 or selenate reductase in the periplasmic space (Lowe et al. 2010). Lowe et al. (2010) reported that there is more than one route by which electrons can be transferred to selenate reductase: via the quinol-cytochrome c oxidoreductase (QCR), which is inhibited by myxothiazol and antimycin A, or via another type of quinol dehydrogenase which is inhibited by HQNO. Our study on electron transport chains in *T. humireducens* SgZ-1 indicated that the reduction of AQS, Fe-EDTA, and HFO was only partially inhibited by the tested inhibitors. Thus, it is expected that there are other yet to be identified routes for electron transport to various electron acceptors in strain SgZ-1.

Our study mainly investigated the anaerobic humus and Fe(III) reduction by *T. humireducens* SgZ-1 and the associated electron transport pathways. Strain SgZ-1 used acetate, propionate, lactate, and pyruvate for the anaerobic reduction of humus and Fe(III) species. Results of inhibition tests and cytochromes c analysis indicated that various different electron transport pathways are employed by strain SgZ-1 when oxygen, AQS, Fe-EDTA, and HFO serve as electron acceptors. At present, electron transport chains with different electron acceptors in *Thauera* species have not been well characterized and further investigation is required.

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