Bioenergetics of the alkaliphilic sulfate-reducing bacterium *Desulfonatronovibrio hydrogenovorans*

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Energy metabolism of the alkaliphilic sulfate-reducing bacterium *Desulfonatronovibrio hydrogenovorans* strain Z-7935 was investigated in continuous culture and in physiological experiments on washed cells. When grown in chemostats with H₂ as electron donor, the cells had extrapolated growth yields [$Y_{\max}$, g dry cell mass (mol electron acceptor)⁻¹] of 5.5 with sulfate and 12.8 with thiosulfate. The maintenance energy coefficients were 1.9 and 1.3 mmol (g dry mass)⁻¹ h⁻¹, and the minimum doubling times were 27 and 20 h with sulfate and thiosulfate, respectively. Cell suspensions reduced sulfate, thiosulfate, sulfite, elemental sulfur and molecular oxygen in the presence of H₂. In the absence of H₂, sulfite, thiosulfate and sulfur were dismutated to sulfide and sulfate. Sulfate and sulfite were only reduced in the presence of sodium ions, whereas sulfur was reduced also in the absence of Na⁺. Plasmolysis experiments showed that sulfate entered the cells via an electroneutral symport with Na⁺ ions. The presence of an electrogenic Na⁺–H⁺ antiporter was demonstrated in experiments applying monensin (an artificial electroneutral Na⁺–H⁺ antiporter) and propylbenzylcholine mustard.HCl (a specific inhibitor of Na⁺–H⁺ antiporters). Sulfate reduction was sensitive to uncouplers (protonophores), whereas sulfite reduction was not affected. Changes in pH upon lysis of washed cells with butanol indicated that the intracellular pH was lower than the optimum pH for growth (pH 9.5). Pulses of NaCl (0.52 M) to cells incubated in the absence of Na⁺ did not result in ATP formation, whereas HCl pulses (shifting the pH from 9.2 to 7.0) did. Small oxygen pulses, which were reduced within a few seconds, caused a transient alkalization. The results of preliminary experiments with chemiosmotic inhibitors provided further evidence that the alkalization was caused by sodium–proton antiport following a primary electron-transport-driven sodium ion translocation. It is concluded that energy conservation in *D. hydrogenovorans* depends on a proton-translocating ATPase, whereas electron transport appears to be coupled to sodium ion translocation.

Keywords: growth yield, reversed proton gradient, sulfate transport, sodium–proton antiport, sodium ion translocation

INTRODUCTION

Desulfonatronovibrio hydrogenovorans was the first alkaliphilic sulfate-reducing bacterium isolated and described by Zhilina *et al.* (1997) among a series of novel

types of anaerobic alkaliphiles which show a fascinating phylogenetic and physiological diversity (Zhilina & Zavarzin, 1994; Jones *et al.*, 1998; Zavarzin *et al.*, 1999).

Alkaliphilic bacteria face the bioenergetical problem that they have to sustain an inverse transmembrane pH gradient (Krulwich, 1995). The intracellular pH is lower than that of their environment. In spite of this, it has been shown that some alkaliphiles can regenerate ATP

Abbreviations: PrBCM, propylbenzylcholine mustard.HCl; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TCS, tetrachlorosalicylanilide.

coupled to proton translocation (Prowe *et al.*, 1996). In this case a high membrane potential must be the driving force.

In many alkaliphiles growth depends on sodium ions, which are present in high concentrations in soda lakes and most alkaline environments. Sodium ions can have several functions. They may be the coupling ions of electron transport (Tokuda & Unemoto, 1982). In some anaerobic bacteria sodium-ion-translocating ATPases (Heise *et al.*, 1993) and sodium-driven motility have been detected (Chernyak *et al.*, 1993). Sodium-driven ATP conservation has also been shown for another new alkaliphilic sulfate reducer, *Desulfonatronum lacustre* (Pikuta *et al.*, 1998; Pusheva *et al.*, 1999, 2000). In contrast to this species, all neutrophilic sulfate reducers studied so far conserve ATP by proton translocation (Fitz & Cypionka, 1989; Kreke & Cypionka, 1994).

An inverse pH gradient appears less critical if energy conservation is coupled to sodium cycling. However, the cells have sodium-proton antiporters that tend to compensate for the proton versus the sodium-ion gradient (Hoffmann & Dimroth, 1991), and they have to regulate their intracellular pH (Krulwich *et al.*, 1997). Sodium ions are also symported during substrate uptake. Symport and antiport systems may function in an electroneutral or electrogenic manner, which is also of energetic relevance. In neutrophilic sulfate reducers, sodium-dependent transport of sulfate was found in marine, but usually not in freshwater, strains (Stahlmann *et al.*, 1991; Cypionka, 1995). Electrogenic $\text{Na}^+ - \text{H}^+$ antiporters have been detected in freshwater and marine sulfate reducers (Varma *et al.*, 1983; Kreke & Cypionka, 1994).

The present study was carried out in order to characterize the energy metabolism of *Desulfonatronovibrio hydrogenovorans* by means of physiological experiments. We used chemostats to determine growth yields and maintenance energy coefficients with sulfate and thiosulfate as electron acceptors. To study the transport of sulfate, the sodium-proton antiport mechanism and the coupling ions of chemiosmotic energy conservation, we used washed cells and chemiosmotic inhibitors.

METHODS

Organism and growth conditions. *Desulfonatronovibrio hydrogenovorans* strain Z-7935 was kindly provided by Dr Tatjana N. Zhilina and Professor George A. Zavarzin (Russian Academy of Sciences, Inst. for Microbiology, Moscow, Russia).

The medium (Zhilina *et al.*, 1997, modified) was prepared from four stock solutions (A–D) with the following final concentrations: (A) 100 mM NaCl, 2 mM NaCH_3COOH , 10 mM NH_4Cl , 0.5 g yeast extract l^{-1} , 0.25 mg resazurin l^{-1} and 1 ml trace element solution SL9 l^{-1} (Tschech & Pfennig, 1984); (B) 0.2 mM CaCl_2 and 2.3 mM MgCl_2 ; (C) 1 mM K_2HPO_4 ; and (D) 47 mM Na_2CO_3 and 90 mM NaHCO_3 . To avoid precipitations, the solutions, A–D, were autoclaved separately and combined after cooling under N_2 . Vitamin solution (1 ml l^{-1}) (Pfennig, 1978) and the electron acceptor sodium sulfate (15 mmol l^{-1}) or sodium thiosulfate (10 mmol l^{-1}) were

then added to the solution. The pH was adjusted to 9.4–9.6 with HCl or NaOH. The medium was reduced with small amounts of sodium dithionite until the redox indicator resazurin was decolourized.

Cells were grown in chemostats (Cypionka & Pfennig, 1986) with H_2 as electron donor and the electron acceptor (sulfate or thiosulfate) as the limiting factor. To avoid loss of NH_3 from the medium the inflowing gas was flushed through an ammonia solution (106 mM, pH 9.5).

Measurement of growth parameters. Growth was followed by measuring the OD at 436 nm with a Shimadzu UV-1202 photometer, as well as by determination of the protein concentration according to the method of Bradford (1976); cell dry mass was determined according to the method of Cypionka & Pfennig (1986). To prove electron acceptor limitation in chemostats, ion chromatography was used to detect sulfate and thiosulfate (Fuseler *et al.*, 1996).

Determination of the activities of washed cells. Cells were harvested by centrifugation of freshly grown cultures for 10 min at 15000 r.p.m. The pellet was resuspended, washed once in N_2 -saturated salt solution, and finally stored on ice in the same solution under H_2 . The composition of the salt solutions varied, as described below, depending on the type of experiment to be conducted.

Various cell activities were studied at 30 °C in a multi-electrode chamber (Cypionka, 1994), which allowed simultaneous measurement of O_2 , pH and sulfide. The reduction and disproportionation of sulfur compounds was studied with cells at a final concentration of 0.36 mg protein ml^{-1} in a H_2 - or N_2 -saturated salt solution with 320 mM NaCl, 80 mM KCl, 10 mM MgCl_2 , and 2 mM K_2CO_3 (pH 9.5). After preincubation for 30 min, sulfur compounds (pulses of 5–50 μM) were added from 10 mM stock solutions.

To analyse proton extrusion after pulses of sodium chloride, the cells were suspended in a solution of 350 mM KCl, 10 mM MgCl_2 and 200 mM KSCN (pH 9.2). The permeant thiocyanate was present to destroy the membrane potential. After 30 min equilibration, sodium chloride pulses were added out of a 4 M stock solution.

Proton translocation coupled to the reduction of oxygen was measured with cells (0.4 mg protein ml^{-1}) suspended in a H_2 -saturated solution of 300 mM NaCl, 20 mM KCl and 10 mM MgCl_2 (pH 9.5). Small oxygen pulses were added in the form of an O_2 -saturated salt solution (Fitz & Cypionka, 1989). The chemiosmotic inhibitors carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), tetrachlorosalicylanilide (TCS), valinomycin and monensin were dissolved in methanol (10–50 mM stock solutions) and applied at a concentration of 50 μM .

Estimation of intracellular pH. The intracellular pH was studied with cells (1.1 mg protein ml^{-1}) suspended in N_2 -saturated salt solution (320 mM NaCl, 80 mM KCl and 10 mM MgCl_2) adjusted to different pH values by NaOH or HCl. Butanol (4%) was then added to perforate the cell membranes; pH changes were followed as described by Scholes & Mitchell (1970).

Study of transport of various salts. Uptake of different ions was determined by measuring the changes in light scattering in cell suspensions (Mitchell & Moyle, 1969; Varma *et al.*, 1983; Kreke & Cypionka, 1994). Cells were suspended at OD₄₃₆ 1.0 (light path = 1 cm) in 2 ml 175 mM KCl with 10 mM MOPS buffer (pH 7.0). After preincubation until the optical density was constant, 150 μl 5 M salt solutions (350 mM) were added and the changes in light scattering were measured with a Shimadzu UV-1202 photometer.

Measurement of the dependency of ATP production on proton or sodium ions. Cells (0.725 mg protein ml⁻¹) were incubated on ice in a solution of 320 mM NaCl, 80 mM KCl and 10 mM MgCl₂ (pH 9.5). Twenty microlitres of 0.1 M HCl (resulting in a pH shift of 2 units), or pulses of NaCl or KCl (both 0.52 M), were then added; subsamples of 100 µl were analysed for ATP as described by Kreke & Cypionka (1994).

RESULTS

Growth parameters in the chemostat

D. hydrogenovorans was grown in a chemostat with H₂ as electron donor and limiting concentrations of sulfate, or thiosulfate, as electron acceptor. Since the addition of yeast extract (0.05 %, w/v) resulted in a significant

Fig. 1. Reciprocal plots of growth yields (Y) versus growth rates (μ) of *D. hydrogenovorans*. Cells were grown in a chemostat with H₂ as electron donor and sulfate (●) ($1/Y_{\text{SO}_4^{2-}}$) or thiosulfate (■) ($1/Y_{\text{S}_2\text{O}_3^{2-}}$) as the electron acceptor at limiting concentrations.

increase of the growth yield and prevented aggregation of the cells, it was added to the mineral chemostat medium. There was no growth with yeast extract in the absence of H₂. Real growth yields (Y) of up to 4.2 and 10.0 g cell dry mass were obtained per mol sulfate or thiosulfate reduced. The maximum growth rates (μ_{max}) determined in washout experiments were 0.0261 h⁻¹ (doubling time = 26.5 h) with sulfate and 0.0345 h⁻¹ (doubling time = 20.1 h) with thiosulfate. Plots of $1/Y_{\text{SO}_4^{2-}}$ or $1/Y_{\text{S}_2\text{O}_3^{2-}}$ versus $1/\mu$ gave straight lines (Fig. 1) and could be extrapolated according to Pirt (1982) to estimate maximum yields (Y_{max}) and maintenance energy coefficients (m) by equation (1):

$$1/Y = m/\mu + 1/Y_{\text{max}} \quad (1)$$

Y_{max} values for growth with sulfate and thiosulfate were 5.5 and 12.8 g cell dry mass (mol electron acceptor reduced)⁻¹, respectively. The maintenance coefficients were 1.9 and 1.3 mmol (g dry mass)⁻¹ h⁻¹ for growth on sulfate and thiosulfate, respectively.

Estimation of the intracellular pH

In salt solutions with pH values between 8.0 and 9.0, cell lysis with butanol according to Scholes & Mitchell (1970) led to an acidification, whereas at pH 6.9 a slight alkalization was observed. In control experiments without cells, butanol had no effect on the pH. These results confirmed that the cytoplasm of *D. hydrogenovorans* is more acidic than its environment during growth.

Reduction of different electron acceptors, and disproportionation or oxidation of sulfur compounds

Sulfate-grown washed cells reduced additions of 20 µM sulfate, sulfite and sulfur to equimolar amounts of sulfide with rates of 10–60 nmol min⁻¹ (mg protein)⁻¹.

Table 1. Reactions of the sulfur cycle catalysed by *D. hydrogenovorans*

Equation no.	Reaction	Free energy change (kJ mol ⁻¹) at:	
		pH 9*	pH 7*
Complete reduction of sulfur compounds:			
(2)	4 H ₂ + SO ₄ ²⁻ + H ⁺ → HS ⁻ + 4 H ₂ O	-140	-155
(3)	3 H ₂ + SO ₃ ²⁻ + H ⁺ → HS ⁻ + 3 H ₂ O	-161	-176
(4)	4 H ₂ + S ₂ O ₃ ²⁻ → 2 HS ⁻ + 3 H ₂ O	-173	-179
(5)	H ₂ + S → HS ⁻ + H ⁺	-39	-30
Disproportionation of sulfur compounds:			
(6)	4 SO ₃ ²⁻ + H ⁺ → 3 SO ₄ ²⁻ + HS ⁻	-224	-236
(7)	S ₂ O ₃ ²⁻ + H ₂ O → SO ₄ ²⁻ + HS ⁻ + H ⁺	-21	-25
(8)	4 S + 4 H ₂ O → SO ₄ ²⁻ + 3 HS ⁻ + 5 H ⁺	-15	+33
Oxidation of sulfur compounds†:			
(9)	HS ⁻ + 2 O ₂ → SO ₄ ²⁻ + H ⁺	-808	-797

* Free energy changes under standard conditions at pH 9 and pH 7 (ΔG^0) were calculated after Thauer *et al.* (1977). It was taken into account that at pH 7 H₂S and HSO₃⁻ are only partially dissociated.

† Fuseler *et al.* (1996).

Fig. 2. Dependency of sulfate reduction on sodium ions. Washed cells of *D. hydrogenovorans* ($0.36 \text{ mg protein ml}^{-1}$, assay volume 2.9 ml) were incubated in H_2 -saturated salt solution (350 mM KCl , 10 mM MgCl_2 and $2 \text{ mM K}_2\text{CO}_3$, $\text{pH } 9.5$) at 30°C . A pulse of 50 nmol sulfate did not result in sulfide formation before NaCl (200 mM) was added.

Reduction of sulfate and sulfite was accompanied by alkalization (disappearance of $1 \text{ H}^+ \text{ mol}^{-1}$) and reduction of sulfur was accompanied by acidification (production of $1 \text{ H}^+ \text{ mol}^{-1}$), as expected from the chemical reactions shown in equations (2), (3) and (5) (Table 1). Thiosulfate was reduced incompletely and only after the addition of sulfate. By contrast, thiosulfate-grown cells reduced thiosulfate without the addition of sulfate [Table 1, equation (4)]. Sulfate reduction was completely inhibited by the uncoupler CCCP, whereas sulfite reduction was not affected.

In the absence of electron donors the cells dismutated sulfite, thiosulfate and sulfur to sulfate and sulfide. Disproportionation of sulfite was accompanied by alkalization, whereas disproportionation of thiosulfate and sulfur was accompanied by acidification [Table 1, equations (6)–(8)].

Reduction of sulfate and sulfite depended on the presence of sodium ions. If the cells were incubated in a salt solution without sodium ions added no formation of sulfide after pulses of sulfate or sulfite occurred. After adding NaCl (200 mM), sulfide formation started (Fig. 2). Elemental sulfur was reduced independently of sodium ions. Cells grown with thiosulfate dismutated this compound also in the absence of sodium ions. The cells were also able to oxidize sulfide with oxygen, as described in detail by Fuseler *et al.* (1996).

Dependency of ATP synthesis on protons or sodium ions

To investigate which ion was translocated by the ATPase, proton or sodium-ion pulses were added (Kreke & Cypionka, 1994). Pulses of Na^+ (0.52 M NaCl) to resting cells did not result in ATP formation, whereas the ATP level of the cells increased sixfold upon HCl pulses, shifting the pH from 9.2 to 7.0 . ATP production

Fig. 3. ATP synthesis driven by proton or sodium-ion pulses. Resting cells of *D. hydrogenovorans* ($0.725 \text{ mg protein ml}^{-1}$) were incubated in a N_2 -saturated salt solution ($\text{pH } 9.2$). ATP synthesis was induced by the addition (indicated by an arrow) of HCl (■) to give a pH of 7.0 , but not by the addition of 520 mM NaCl (●). If the cells were de-energized with TCS no ATP was synthesized (▲).

after proton pulses was inhibited by the protonophore TCS (Fig. 3).

Demonstration of a $\text{Na}^+ \text{--} \text{H}^+$ antiporter

To prove the existence of a $\text{Na}^+ \text{--} \text{H}^+$ antiporter, cells were preincubated with 200 mM KSCN to destroy the membrane potential. Na^+ pulses (0.1 mM NaCl) were then added, which caused acidification of the bulk medium and thus indicated the presence of a $\text{Na}^+ \text{--} \text{H}^+$ antiporter. To test whether the antiport mechanism was electroneutral or electrogenic, light scattering due to plasmolysis after adding various salts was studied. The addition of NaCl (350 mM) to cell suspensions resulted in an increase in optical density due to plasmolysis of the cells. Uptake of this salt requires transport of both sodium and chloride ions, which obviously did not occur (Table 2). In contrast, ammonium acetate was taken up and led to a slight decrease in the optical density. This salt is easily transformed into the uncharged permeable form of NH_3 plus CH_3COOH , which dissociate again once inside the cell. The addition of sodium acetate resulted in plasmolysis, indicated by a stable increase in the optical density (Fig. 4, A). Obviously, there was no electroneutral $\text{Na}^+ \text{--} \text{H}^+$ antiporter, which would have exported protons that could re-enter the cells together with acetate as CH_3COOH . Sodium acetate was taken up after electroneutral $\text{Na}^+ \text{--} \text{H}^+$ antiport was made possible by adding monensin (Table 2). Furthermore, after the addition of TCS, which dissipates the membrane potential and thus the electrogenic effect of an antiporter, uptake of sodium acetate was observed (Fig. 4, B). Finally, the addition of propylbenzylcholine mustard.HCl (PrBCM), a covalently binding inhibitor of $\text{Na}^+ \text{--} \text{H}^+$ antiporters (Glasmacher & Schönheit, 1992; Kreke & Cypionka, 1994),

Table 2. Uptake of various salts by *D. hydrogenovorans* determined by following the changes in light scattering

Cells were suspended in 10 mM MOPS (pH 7.0) with 175 mM KCl (A–H) or 175 mM NaCl (I). The ΔOD_{436} values were read about 10 min after addition of the salts, when the changes had ended almost completely.

Addition of 350 mM:	Observed effect (ΔOD_{436})	Interpretation
(A) NaCl	+0.090	No uptake of sodium chloride
(B) NH_4CH_3COO	−0.190	Uptake, because salt can enter as NH_3 plus CH_3COOH
(C) $NaCH_3COOH$	+0.055	Little uptake, because Na^+ cannot enter electroneutrally
(D) Monensin (50 μM) + $NaCH_3COOH$	0.000	Complete uptake, because monensin allows electroneutral Na^+-H^+ antiport
(E) $NaCH_3COOH$ + TCS (100 μM)	−0.030	Complete uptake, Na^+ entered the cells after dissipation the membrane potential by TCS
(F) $NaCH_3COOH$ + TCS (100 μM) + PrBCM (50 μM)	+0.010	Partial uptake, because PrBCM inhibited the Na^+-H^+ antiporter
(G) $(NH_4)_2SO_4$	+0.037	No uptake
(H) $(NH_4)_2SO_4$ (214 mM) + TCS (100 μM)	+0.032	The dissipation of membrane potential did not cause uptake
(I) Na_2SO_4 (175 mM)	−0.020	Electroneutral sodium–sulfate symport

Fig. 4. Changes in OD_{436} after the addition of various substances to a cell suspension of *D. hydrogenovorans*. Cells were incubated in air-saturated 10 mM MOPS buffer (pH 7.0) containing 175 mM KCl. In all cases, arrows indicate when each of the substances was added to the cell suspension. (A) Addition of 320 mM sodium acetate (NaAc). (B) Addition of 320 mM NaAc followed by the addition of 100 μM TCS. (C) Addition of 320 mM NaAc followed by the addition of 100 μM TCS and later by the addition of 50 μM PrBCM.

stopped reswelling of the cells (Fig. 4, C). Thus, the existence of an electrogenic Na^+-H^+ antiport system could be clearly demonstrated.

Sulfate transport

Obviously, sulfate was taken up in symport with sodium ions, as shown in the plasmolysis experiments. Addition of 200 mM ammonium sulfate to cells suspended in 175 mM KCl with 10 mM MOPS buffer (pH 7.0) resulted in plasmolysis, causing increased light scattering. Since ammonia could enter the cells, as described above, there must have been a lack of sulfate entering the cells. TCS, added to dissipate the membrane potential, did not change this result. However, if KCl

Fig. 5. Transient proton uptake upon the reduction of small pulses of O_2 . Washed cells (0.42 mg protein ml^{-1}) were incubated in a H_2 -saturated salt solution, pH 8.0, at 30 °C. Oxygen (50 nmol) was added as a O_2 -saturated salt solution. For calibration of the pH effects, 50 μl of 1 mM N_2 -saturated HCl (50 nmol) was added.

was replaced by NaCl reswelling of the cells occurred, even in the absence of TCS. This indicated an electro-neutral sodium sulfate symport.

Proton translocation coupled to the reduction of oxygen

A transient alkalinization of the bulk medium was observed when small oxygen pulses (5–100 nmol O_2 , which were reduced within a few seconds) were added to cells incubated in weakly buffered H_2 -saturated salt solution (Fig. 5). The curves obtained were very similar to those observed during electron-transport-coupled proton translocation with neutrophilic sulfate reducers (Fitz & Cypionka, 1989; Cypionka, 1995, 2000), except

that an alkalization instead of an acidification was observed. The $H^+ : O_2$ ratios observed with different cell preparations were 0.2–1.0, independent of the pH of the assay (pH 7.5–9). The effect was hardly sensitive to the presence of the uncoupler CCCP (by contrast to neutrophilic sulfate reducers). Highest $H^+ : O_2$ ratios (2.1–3.4) were obtained in the presence of valinomycin, an electrogenic K^+ transporter added to dissipate the membrane potential, and monensin, an electroneutral $Na^+ - H^+$ antiporter, whereas either of these reagents alone had little effect. These results indicated that the pH effects might be due to electron-transport-driven sodium ion translocation coupled to $Na^+ - H^+$ antiport as discussed below.

DISCUSSION

Growth parameters

The electron donor H_2 , used in this study, allows only chemiosmotic energy conservation, but no substrate-level phosphorylation. Whereas the free energy of sulfate or thiosulfate reduction with H_2 is similar in alkaline and in neutral environments (Table 1), the growth yields in the chemostat were lower than those of neutrophilic sulfate reducers (Nethe-Jaenchen & Thauer, 1984; Cypionka & Pfennig, 1986). Yields extrapolated to infinite growth rates were about half of those found in neutrophilic *Desulfovibrio* species. This was to be expected, since the inverse ΔpH results in a decreased proton-motive force (Dimroth, 1992). However, the maintenance energy coefficient was not higher than in neutrophilic sulfate reducers. Obviously, the inverse ΔpH leads to an increased energy requirement for ATP conservation. However, the cells do not lose much energy by outleaking protons.

Sulfur metabolism

Although the spectrum of electron donors utilized by *D. hydrogenovorans* is very restricted (Zhilina *et al.*, 1997) the bacterium reveals a versatile sulfur metabolism (Table 1). Growth yields with thiosulfate were higher than those with sulfate. This indicates that sulfate activation requires energy, as is known from neutrophilic bacteria. Correspondingly, sulfate, but not sulfite, reduction was inhibited by uncouplers. In the absence of electron donors, disproportionation of sulfite, thiosulfate and sulfur was catalysed, but did not support growth, as found in many neutrophilic sulfate reducers (Krämer & Cypionka, 1989). With oxygen as electron acceptor even aerobic respiration (not coupled to growth) and oxidation of sulfide were observed. The mechanism of sulfur compound disproportionation and oxidation appears to follow the same pathway in neutrophilic sulfate reducers (Fuseler *et al.*, 1996).

Energy coupling

It was clearly demonstrated that ATP is conserved by a proton-translocating ATPase. HCl pulses to the bulk medium resulted in ATP formation, whereas NaCl or

Fig. 6. Suggested model of chemiosmotic energy conservation by *D. hydrogenovorans*. Primary sodium translocation is transformed to a proton gradient by electrogenic $Na^+ - H^+$ antiport, and also used for sulfate uptake. ATP is conserved by a H^+ -translocating ATP synthase. The stoichiometry of the $Na^+ - H^+$ antiport system (n, x) is not known, but it is assumed that more protons than sodium ions are translocated.

KCl pulses showed no effect. Furthermore, ATP formation was sensitive to protonophores. The mechanism of chemiosmotic ATP synthesis in *D. hydrogenovorans* thus resembles that in neutrophilic sulfate reducers (Fitz & Cypionka, 1989), but clearly differs from that in the second species of alkaliphilic sulfate reducer *Desulfonatronum lacustre*, which was shown to couple ATP conservation to Na^+ translocation (Pusheva *et al.*, 2000).

Although ATP is conserved by proton translocation, three important functions of sodium ions were found. First, sulfate is taken up by electroneutral symport with (two) sodium ions. In this regard, *D. hydrogenovorans* resembles marine sulfate reducers. The ability to induce an electrogenic mechanism under sulfate limitation as observed in neutrophilic sulfate reducers (Stahlmann *et al.*, 1991) was not tested and can not be excluded. Second, as demonstrated in plasmolysis experiments, sodium ions were used for electrogenic antiport of protons. As in other alkaliphiles (Krulwich *et al.*, 1997), sodium ions are important not only for substrate uptake, but also for pH homeostasis of the cytoplasm. Furthermore, this transport system must play an essential role in the generation of the membrane potential required for H^+ -dependent ATP synthesis. In *Desulfovibrio salexigens* it was found that the electrogenic $Na^+ - H^+$ antiporter transports more protons than sodium ions (Kreke & Cypionka, 1994). The same stoichiometry would help *Desulfonatronovibrio hydrogenovorans* to increase the membrane potential as required for proton-dependent ATP synthesis at an inverse ΔpH . The third and most important role of Na^+

was demonstrated indirectly and can be derived from our proton translocation experiments with oxygen. Although the mechanism of energy coupling of oxygen reduction in sulfate-reducing bacteria is not fully understood (Cypionka 2000), a transient vectorial proton extrusion coupled to ATP conservation has been shown repeatedly (Fitz & Cypionka, 1989; Dilling & Cypionka, 1990). However, in *D. hydrogenovorans* we observed a transient vectorial proton uptake instead. This certainly cannot be explained by electron-transport-driven proton uptake and an ATP synthase coupled to proton extrusion instead of uptake. First, it was shown that ATP synthesis is coupled to proton uptake. Second, electron-transport-coupled proton uptake would lower the membrane potential, which is inside-negative in all bacteria studied so far, and which must be the main driving force for ATP synthesis. Third, the transient proton uptake was not sensitive to uncouplers, although ATP synthesis was. In contrast, we observed the most pronounced effects when both valinomycin, which in the presence of K^+ should destroy the membrane potential, and the Na^+-H^+ antiporter monensin were added. Our observations are, however, in accordance with energy conservation by electron-transport-driven Na^+ translocation and electrogenic Na^+-H^+ antiport as suggested in Fig. 6. Our data on the $H^+:O_2$ ratios are not yet robust enough to calculate stoichiometries. However, the finding that CCCP did not stimulate alkalization of the medium is in accordance with the electrogenic antiport mechanism (more H^+ than Na^+ ions translocated) discussed above. Of course, the intracellular concentrations of the ions involved and the sodium- and proton-motive forces remain to be determined.

Our study has shown that the energy metabolism of *D. hydrogenovorans* is well adapted to life in an alkaline environment. The chemiosmotic mechanisms include primary sodium and proton transport, mediated by electrogenic Na^+-H^+ antiport.

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