CHAPTER 1

Energy metabolism and phylogenetic diversity of sulphate-reducing bacteria

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1.1 INTRODUCTION

Sulphate-reducing bacteria (SRB) are those prokaryotic microorganisms, both bacteria and archaea, that can use sulphate as the terminal electron acceptor in their energy metabolism, i.e. that are capable of dissimilatory sulphate reduction. Most of the SRB described to date belong to one of the four following phylogenetic lineages (with some examples of genera): (i) the mesophilic δ-proteobacteria with the genera *Desulfovibrio, Desulfobacterium*, *Desulfobacter*, and *Desulfobulbus*; (ii) the thermophilic Gram-negative bacteria with the genus *Thermodesulfovibrio*; (iii) the Gram-positive bacteria with the genus *Desulfotomaculum*; and (iv) the *Euryarchaeota* with the genus *Archaeoglobus* (Castro *et al.*, 2000). A fifth lineage, the *Thermodesulfobiaceae*, has been described recently (Mori *et al.*, 2003).

Many SRB are versatile in that they can use electron acceptors other than sulphate for anaerobic respiration. These include elemental sulphur (Bottcher *et al.*, 2005; Finster *et al.*, 1998), fumarate (Tomei *et al.*, 1995), nitrate (Krekeler and Cypionka, 1995), dimethylsulfoxide (Jonkers *et al.*, 1996), Mn(IV) (Myers and Nealson, 1988) and Fe(III) (Lovley *et al.*, 1993; 2004). Some SRB are even capable of aerobic respiration (Dannenberg *et al.*, 1992; Lemos *et al.*, 2001) although this process appears not to sustain growth, and probably provides these organisms only with energy for maintenance. Since dissimilatory sulphate reduction is inhibited under oxic conditions, SRB can grow at the expense of sulphate reduction only in the complete absence of molecular oxygen. SRB are thus considered to be strictly anaerobic microorganisms and are mainly found in sulphate-rich anoxic habitats (Cypionka, 2000; Fareleira *et al.*, 2003; Sass *et al.*, 1992). These conditions apply in marine sediments since ocean water is rich in sulphate,

its concentration being as high as 30 mM. SRB are also present, however, in freshwater sediments, where the sulphate concentration is generally well below 1 mM but is continuously maintained at this level by the re-oxidation of the H_2S to sulphate at the oxic/anoxic interface due to the action of chemolithotrophic and photolithotrophic bacteria (Holmer and Storkholm, 2001). Since most SRB may use electron acceptors other than sulphate, they can also be found in anoxic habitats depleted in sulphate such as the human intestinal tract (Chapter 18). SRB are, however, most abundant in habitats where the availability of sulphate is not limiting.

1.1.2 Thermodynamics

The discussion of the energetics of sulphate reduction is introduced with consideration of the thermodynamic parameters that determine the possible interactions between potential electron donors and sulphate as electron acceptor.

The redox potential, $E^{\circ\prime}$, of the sulphate/HS^- couple is −217 mV under standard conditions, which are 1M concentrations of sulphate and HS^- at pH 7.0 and 25°C. Under physiological conditions, however, where the concentrations of sulphate are generally < 30 mM and of HS^- < 1 mM, the redox potential, E', is a little more positive and of the order of −200 mV. Thus at all expected concentrations of sulphate and HS^-, almost any organic compound generated by plants or animals, including carbohydrates, fatty acids, alkanes and aromatic compouds, should, in theory, be able to be completely oxidized to CO_2 since the redox potentials of each of these possible electron donors is significantly more negative than the –200 mV of the sulphate/HS^- couple (Table 1.1). Indeed, each of these bio-organic materials has now been shown to be completely mineralized by individual SRB, either alone or in syntrophic association with other organisms. This is even true for methane with a redox potential $E^{\circ\prime}$ of the CO_2/methane couple of −244 mV and therefore a redox potential difference $\Delta E'$ $\left[E'\left(SO_4^{2-}/HS^-\right) - E^{\circ\prime}(CO_2/CH_4)\right]$ of only +44 mV. Remarkably, it has recently been shown that a $\Delta E'$ of +25 mV, equivalent to a free energy change $\Delta G'$ of –20 kJ/mol ($\Delta G' = -nF\Delta E'$; $n = 8$) is sufficient to sustain growth of SRB (Hoehler *et al.*, 2001).

For the prediction of electron flow in natural environments, in most cases $E^{\circ\prime}$ rather than E' of the reductant can be used since the two generally differ by only around 20 mV. There are, however, important exceptions, e.g. the H^+/H_2 couple and the S°/HS^- couple. The redox potential at pH 7.0 (H^+ concentration constant at 10^{-7} M) of the H^+/H_2 couple increases

Table 1.1. *Redox potentials E°′ of electron donors thermodynamically capable and not capable of dissimilatory sulphate, thiosulphate or bisulphite reduction*

Redox couple	*n*	E°′ (Mv)
$2CO_2$ + 2acetate /hexose	8	−670
CO_2 + acetate /pyruvate	2	−660
$FeS_2/FeS + H_2S$[d]	2	−613
SO_4^{2-}/HSO_3^-	**2**	**−516**
CO_2/CO	2	−520
$Fe^{2+}/Fe°$	2	−447
CO_2 + acetate/lactate	4	−430
CO_2 /formate	2	−432
$2H^+/H_2$	2	−414
		(−270 to −300)[a]
$6CO_2$ /hexose	24	−410
$S_2O_3^{2-}/HS^- + HSO_3^-$	**2**	**−402**
CO_2 + acetate + NH_3/alanine	4	−400
Acetate/ethanol	4	−390
CO_2/methanol	6	−370
$4CO_2$ /succinate	12	−312
$7CO_2$ /benzoate[e]	30	−300
2Acetate/butyrate	4	−290
CO_2 + acetate /glycerol	6	−290
$2CO_2$ /acetate	8	−290
$4CO_2$/butyrate	20	−280
$3CO_2$ /propionate	7	−280
N_2/NH_3[f]	6	−276
$S°/H_2S$*	**2**	**−270 (−120)**[b]
$6CO_2$/hexane[g]	38	−250
CO_2/CH_4	8	−244
SO_4^{2-}/HS^-	**8**	**−217 (−200)**[c]
SO_3H^-/HS^-	**6**	**−116**
Glycine/acetate + NH_3	2	−10
Fumarate/succinate	2	+33
Trimethylamine N-oxide/trimethylamine	2	+130
Dimethylsulfoxide/dimethylsulphide	2	+160
$Fe(OH)_3 + HCO_3^-/FeCO_3$[h]	1	+200
NO_2^-/NH_3	6	+330
NO_3^-/NH_3	8	+360

Mn^{4+}/Mn^{2+}	2	+407
NO_3^-/NO_2^-	2	+430
$2NO_3^-/N_2$	10	+760
$O_2/2H_2O$	4	+818
$2NO/N_2O$	2	+1175
$H_2O_2/2H_2O$	2	+1350
N_2O/N_2	2	+1.360

[a] Calculated for a H_2 partial pressure of 1 Pa and 10 Pa, respectively.
[b] Calculated for a $[HS^-] = 0.1$ mM.
[c] Calculated for [sulphate] = 30 mM and $[HS^-] = 0.1$ mM.
[d] (Wächtershäuser, 1992)
[e] Calculated from the free energy of formation. $\Delta G°f$ of benzoate was estimated from $\Delta G^{\circ}f$ for benzoic acid (crystalline solid state) (−245 kJ/mol), from the solubility of benzoic acid at 25 °C (27.8 mM) and from the pK of benzoic acid (4.2) to be –212.3 kJ/mol (Thauer and Morris, 1984).
[f] N_2 cannot be used as electron acceptor for energy conservation because of the too high energy of activation required for its reduction.
[g] (Zengler *et al.*, 1999)
[h] (Ehrenreich and Widdel, 1994)
Notes: $E^{\circ\prime}$ at pH 7.0 are given for H_2, CO_2, CO, CH_4 and O_2 in the gaseous state at 10^5 Pa, for S° in the solid state and for all other compounds in aqueous solution at 1M concentration. The values in brackets are E′ values calculated for physiological substrate and product concentrations. $E^{\circ\prime}$ values were calculated from $\Delta G^{\circ\prime}$ values: $\Delta G^{\circ\prime} = -nF\Delta E$, where n is the number of electrons and $F = 96\,487$ J/mol/volt. Except were indicated, $\Delta G^{\circ\prime}$ values were taken from (Thauer *et al.*, 1977).

from −414 mV at an H_2 partial pressure of 10^5 Pa (standard condition) to values between –270 mV and –300 mV at H_2 partial pressures between 1 and 10 Pa, which is the concentration range of H_2 prevailing in sediments. Thus the oxidation of acetate to CO_2 ($E^{\circ\prime} = -290$ mV) with H^+ as electron acceptor ($E' = -270$ mV at 1 Pa:H_2) becomes thermodynamically feasible and there are organisms that appear to live at the expense of this equation (Galouchko and Rozanova, 1996; Lee and Zinder, 1988; Shigematsu *et al.*, 2004).

$$CH_3COO^- + H^+ + 2H_2O = 2CO_2 + 4H_2 \quad \Delta G^{\circ\prime} = +95\,\text{kJ/mol} \qquad (1.1)$$

The redox potential of the $S°/HS^-$ couple (S° in the solid state and therefore constant) increases from –270 mV under standard conditions

to −120 mV under physiological conditions where the HS^- concentrations can be 0.1 mM or even lower. As a consequence, under *in situ* conditions the reduction of sulphate with H_2S to S° is endergonic. In agreement with this prediction, SRB have been found that can grow at the expense of S° disproportionation to sulphate and HS^- (Finster *et al.*, 1998):

$$4S^0 + 4H_2O = SO_4^{2-} + 3HS^- + 5H^+ \qquad \Delta G^{\circ\prime} = +41\,kJ/mol \qquad (1.2)$$

1.1.3 Energy coupling

Growth of SRB with dissimilatory sulphate reduction indicates that substrate oxidation is coupled with adenosine triphosphate (ATP) synthesis from adenosine diphosphate (ADP) and inorganic phosphate. It leaves open, however, whether coupling is by substrate level phosphorylation and/or electron transport-linked phosphorylation (Thauer *et al.*, 1977). In substrate level phosphorylation an "energy-rich" intermediate is formed from organic substrates during exergonic oxidation reactions. The "energy-rich" intermediate is generally an acyl phosphate or an acyl thioester, which have group transfer potentials equivalent to that of ATP, and are in an enzyme-catalyzed equilibrium with the ADP/ATP system. In electron transport-linked phosphorylation the redox potential difference between electron carriers and the terminal electron acceptor is conserved in a transmembrane electrochemical proton or sodium ion gradient, which drives the phosphorylation of ADP via a membrane-bound ATP synthase.

For many years it was thought that it was only possible to grow SRB on organic substrates as electron donors for dissimilatory sulphate reduction, and this led to the belief that in these organisms energy is conserved mainly or exclusively via substrate level phosphorylation. However, in 1978 it was unambiguously shown that *Desulfovibrio vulgaris* can grow with H_2 and sulphate as the sole energy source (Badziong and Thauer, 1978).

$$4H_2 + SO_4^{2-} + H^+ = HS^- + 4H_2O \quad \Delta G^{\circ\prime} = -151.8\,kJ/mol. \qquad (1.3)$$

During growth on H_2 and sulphate, energy must be conserved by electron transport-linked phosphorylation since substrate level phosphorylation is only possible when the substrate oxidized is organic. There is, however, an exception to this general rule. The oxidation of bisulphite to sulphate can be coupled by substrate level phosphorylation via an energy-rich adenosine phosphosulphate (APS) intermediate. Using this reaction some SRB can grow at the expense of bisulphite disproportionation to sulphate and

hydrogen sulphide (Bak and Cypionka, 1987; Frederiksen and Finster, 2003; Kramer and Cypionka, 1989).

$$4HSO_3^- + 4H^+ = 3SO_4^{2-} + HS^- \quad \Delta G^{\circ\prime} = -235.6\,kJ/mol. \tag{1.4}$$

This chapter first summarizes what is presently known about the biochemistry of dissimilatory sulphate reduction with H_2. Thermodynamic problems associated with the sulphate-dependent oxidations of substrates other than H_2 are then outlined. Subsequently, also under energetic aspects, the trophic interactions of SRB with other microorganisms in their habitats are described. Finally, the phylogenetic diversity of SRB is discussed.

Some of the arguments that will be put forward are based on genome sequence information. Until now, only the genome sequences of three SRB, of *Archaeoglobus fulgidus* (Klenk *et al.*, 1997), of *Desulfovibrio vulgaris* (Hildenborough) (Heidelberg *et al.*, 2004; Hemme and Wall, 2004) and *Desulfotalea psychrophila* (Rabus *et al.*, 2004), have been published. The genomes of many other SRB with different metabolic capacities and from different phylogenetic origins are presently being sequenced. Only when we have these data will we have a complete picture of the energy metabolism of SRB (Chapter 3).

For a previous review on the bioenergetic strategies of sulphate-reducing bacteria see Peck (1993).

1.2 DISSIMILATORY SULPHATE REDUCTION WITH H_2

The equations and proteins involved in dissimilatory sulphate reduction with H_2 will be described for *Desulfovibrio vulgaris* (Hansen, 1994; Matias *et al.*, 2005). The biochemistry of this δ-proteobacterium and of closely related species has been studied in detail and the genome sequence of the Hildenborough strain has recently been published (Heidelberg *et al.*, 2004). Most of the results can probably be generalized to other SRB capable of growth on H_2 as sole energy source such as *D. desulfuricans*, *Thermodesulphobacterium commune*, *Desulfobacterium autotrophicus*, *Desulfotomaculum orientis* and *Archaeoglobus profundus*.

1.2.1 Sulphate activation

Dissimilatory sulphate reduction with H_2 to H_2S in *D. vulgaris* proceeds via HSO_3^- as intermediate. The redox potential, $E^{\circ\prime}$, of the SO_4^{2-}/HSO_3^- couple is −516 mV and thus more than 100 mV more negative that of

the H^+/H_2 couple (Table 1.1). Reduction of sulphate to bisulphite with H_2 can therefore only proceed after some input of energy. It has been shown that sulphate is first activated with ATP to adenosine phosphosulphate (= adenylylsulphate) (APS), and that the redox potential of the resulting APS/HSO_3^- couple is −60 mV. The reaction is catalyzed by ATP sulphurylase (Sperling *et al.*, 1998; Taguchi *et al.*, 2004) and consumes the energy of up to two enegy-rich bonds.

$$SO_4^{2-} + ATP + 2H^+ = APS + PPi \quad \Delta G^{\circ\prime} = -46\,kJ/mol \tag{1.5}$$

$$PPi + H_2O = 2Pi \quad \Delta G^{\circ\prime} = -21.9\,kJ/mol. \tag{1.6}$$

Desulfovibrio contain an active cytoplasmic inorganic pyrophosphatase that probably catalyzes the hydrolysis of most of the inorganic pyrophosphate generated in reaction 1.5 (Kobayashi *et al.*, 1975; Liu and Legall, 1990; Ware and Postgate, 1971; see also Weinberg *et al.*, 2004). Based on the genome sequence of *D. vulgaris*, there is no evidence of a membrane-associated inorganic pyrophosphatase which would allow conservation of part of the energy released during pyrophosphate hydrolysis in the form of a trans-membrane electrochemical proton potential, thus reducing somewhat the energy cost of sulphate activation.

1.2.2 Cytoplasmic APS reduction

The sulphate activation to APS increases the redox potential of the first step in dissimilatory sulphate reduction from −516 mV to −60 mV. This is well above the redox potential of the H^+/H_2 couple and allows the reduction with H_2 to proceed even at low H_2 concentrations. *Desulfovibrio* contain a cytoplasmic APS reductase (= adenylylsulphate reductase) whose direct electron donor is not yet known (Fritz *et al.*, 2002; Kremer and Hansen, 1988; Lopez-Cortes *et al.*, 2005; Yagi and Ogata, 1996).

$$APS + 2e^- + 2H^+ = HSO_3^- + AMP \quad E^{\circ\prime} = -60\,mV \tag{1.7}$$

1.2.3 Cytoplasmic bisulphite reduction

The reduction of APS to HSO_3^- is followed by the reduction of HSO_3^- to HS^-, a reaction catalyzed by a cytoplasmic bisulphite reductase (Crane *et al.*, 1997; Friedrich, 2002; Kremer and Hansen, 1988; Larsen *et al.*, 1999; Steger *et al.*, 2002; Zverlov *et al.*, 2005), whose direct electron donor has

also still to be identified. For assay of enzyme activity reduced viologen dyes are generally used.

$$HSO_3^- + 6e^- + 6H^+ = HS^- + 3H_2O \quad E^{\circ\prime} = -116\,mV \tag{1.8a}$$

With respect to the mechanism of this equation, there has been considerable controversy over the last 30 years, which is not yet completely resolved. One of the reasons for this is that bisulphite reductase also catalyzes reactions 1.8b and 1.8c when the HSO_3^- concentration is high and the reductant concentration is limiting (Akagi, 1995). SRB contain a thiosulphate reductase which catalyzes equation 1.9.

$$3HSO_3^- + 2e^- + 3H^+ = S_3O_6^{2-} + 3H_2O \quad E^{\circ\prime} = -173\,mV \tag{1.8b}$$

$$S_3O_6^{2-} + 2e^- + H^+ = S_2O_3^{2-} + HSO_3^- \quad E^{\circ\prime} = +225\,mV \tag{1.8c}$$

$$S_2O_3^{2-} + 2e^- + H^+ = HS^- + HSO_3^- \quad E^{\circ\prime} = -402\,mV \tag{1.9}$$

Thus bisulphite reduction could proceed in three two-electron steps rather than in one six-electron step, especially when SRB are grown with bisulphite or thiosulphate rather than sulphate as terminal electron acceptor (Fitz and Cypionka, 1990; Sass *et al.*, 1992). However, recently it has been shown that when *D. vulgaris* is genetically impaired in thiosulphate reduction, this does not affect its ability to grow on sulphate and H_2 (Broco *et al.*, 2005). This is interpreted as indicating that, at least under these growth conditions, bisulphite is reduced in a single step. In the following, it is therefore assumed that APS and HSO_3^- are the only intermediary electron acceptors involved in dissimilatory sulphate reduction with H_2.

1.2.4 Periplasmic H_2 oxidation

D. vulgaris contains four periplasmic hydrogenases, three [NiFe]-hydrogenases and one [FeFe]-hydrogenase: of these, three hydrogenases couple with the major periplasmic poly-heme cytochrome *c* (*Tp1-c3*), and one, the [NiFe]-hydrogenases 2, most probably with a second poly-heme cytochrome *c* (*TpII-c3*) (Heidelberg *et al.*, 2004; Matias *et al.*, 2005). When the organism is grown on H_2 and sulphate in medium depleted in nickel, only the [FeFe]-hydrogenase is synthesized without this having a noticeable effect on the growth rate (R. K. Thauer, unpublished results). Growth of *D. vulgaris* is also not impaired when the genes for the [FeFe]-hydrogenase (Haveman *et al.*, 2003; Pohorelic *et al.*, 2002) or one of the NiFe hydrogenases (Goenka *et al.*, 2005) are deleted. These findings indicate that the four hydrogenases

can fully functionally replace each other, at least under the growth conditions employed in the laboratory where the H_2 concentration in the fermenters is kept high.

1.2.5 Transmembrane electron transport

Coupling of periplasmic hydrogen oxidation with cytoplasmic APS and HSO_3^- reduction must involve electron transport through the cytoplasmic membrane. The electron transport is most probably catalyzed by the Hmc complex, which is associated on the periplasmic side with a poly-heme cytochrome *c*, and on the cytoplasmic side with an iron-sulphur protein with sequence similarity to heterodisulphide reductase (Keon and Voordouw, 1996; Matias *et al.*, 2005; Rossi *et al.*, 1993). *D. vulgaris* deleted in the *hmc* genes grew normally on lactate and sulphate, but growth on H_2 and sulphate was hampered (Dolla *et al.*, 2000; Haveman *et al.*, 2003; Keon and Voordouw, 1996). The genome of *D. vulgaris* harbours two other polycistronic transcription units predicted to encode for transmembrane protein complexes (TpII-c_3 and Hme), associated on the periplasmic side with a cytochrome *c* and on the cytoplasmic side with an iron-sulphur protein, again with sequence similarity to heterodisulphide reductase (Heidelberg *et al.*, 2004; Matias *et al.*, 2005). The *Hme* genes are part of a locus that includes the genes for bisulphite reductase. The three transmembrane complexes Hmc, Hme and TpII-c_3 could have overlapping functions, in a like manner to the four periplasmic hydrogenases. In the genome, a further gene cluster for a transmembrane protein complex is found which lacks the periplasmic cytochrome *c* (Qmo complex) (Pires *et al.*, 2003). There is indirect evidence that the Qmo complex is involved in APS reduction (Matias *et al.*, 2005).

Heterodisulphide reductase from methanogens catalyzes the reduction of the heterodisulphide CoM-S-S-CoB to coenzyme M (HS-CoM) and coenzyme B (HS-CoB) (Hedderich *et al.*, 1998). Both coenzymes are absent from SRB, and cell extracts of SRB neither catalyze the reduction of CoM-S-S-CoB nor the oxidation of CoM-SH plus CoB-SH (Mander *et al.*, 2002; 2004). The iron-sulphur proteins in SRB with sequence similarity to heterodisulphide reductase must therefore have a different substrate specificity and/or a different function. However, since in methanogenic archaea heterodisulfide reduction links H_2 oxidation with methyl-coenzyme M reduction to methane, it is tempting to speculate that in SRB a disulphide/–SH couple might also be involved in the electron transport from H_2 to HSO_3^-.

1.2.6 ATP synthesis and sulphate transport

As indicated above, up to two ATP equivalents are required to activate sulphate before it can be reduced to HSO_3^-. The reduction of HSO_3^- to HS^- must therefore be coupled with the phosphorylation of at least 2 mol ADP in order that the SRB can grow on H_2 and sulphate. Growth yield data, extrapolated to infinite growth rates, for *D. vulgaris* on H_2 and sulphate were 12.2 g/mol sulphate, and on H_2 and thiosulphate 33.5 g/mol, indicating that HSO_3^- reduction with H_2 to HS^- is coupled with the net synthesis of approximately three ATP (Badziong and Thauer, 1978). In the interpretation of these growth yield data it has to be considered that sulphate must be transported into the cells before it can be reduced and that this transport also requires energy. Available evidence indicates that sulphate is symported with three protons (Cypionka, 1987; Kreke and Cypionka, 1992), or three sodium ions (Kreke and Cypionka, 1994) which is probably equivalent to the consumption of one third or one fourth of an ATP, as discussed below.

1.2.7 Proton stoichiometries

D. vulgaris contains a F_0F_1-type proton-translocating ATPase/ATP synthase (Heidelberg *et al.*, 2004; Hemme and Wall, 2004) of as yet unknown H^+ to ATP stoichiometry. Recent structural analyses of F_0F_1 ATPases from different organisms indicates that the H^+ to ATP stoichiometry may differ from organism to organism, and may be as high as five or as low as three in some organisms (Dimroth and Cook, 2004; Meier *et al.*, 2005; Mueller, 2004; Murata *et al.*, 2005). If the enzyme in *D. vulgaris* has a stoichiometry of three protons per ATP, then at least nine electrogenic protons are required for the synthesis of the three ATP predicted from growth yields to be formed during bisulphite reduction to HS^-. If the stoichiometry is five protons per ATP, then fifteen protons are required. Of these, six are generated from H_2 in the periplasm in a scalar reaction catalyzed by the periplasmic hydrogenases. The other protons required for ATP synthesis must therefore be generated during bisulphite reduction with H_2 by electrogenic proton translocation from the cytoplasm to the periplasm. Although menaquinone is the major quinone found in the cytoplasmic membrane of all SRB, its involvement in this proton translocation is unlikely since the redox potential, $E^{\circ\prime}$, of the menaquinone ox/red couple is −74 mV and thus more positive than that of the HSO_3^-/HS^- couple (−116 mV).