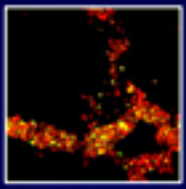
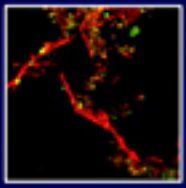
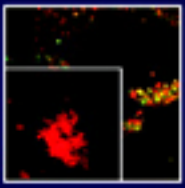
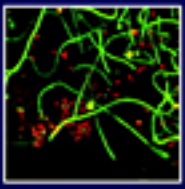
Sulphate-reducing Bacteria

Environmental and Engineered Systems









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Sulphate-reducing Bacteria

The Sulphate-reducing Bacteria (SRB) are a large group of anaerobic organisms that play an important role in many biogeochemical processes. Not only are they of early origins in the development of the biosphere, but their mechanisms of energy metabolism shed light on the limits of life processes in the absence of oxygen. They are widely distributed in nature, and are regular components of engineered systems including, for example, petroleum reservoirs and oil production facilities. SRB are currently subject to extensive genomic studies, which are yielding new understanding of their basic biochemical mechanisms, and aiding in the development of novel techniques for the analyses of their environmental roles. This volume provides a timely update on these important microorganisms, from basic science to applications, and will therefore serve as a valuable resource for researchers and graduate students in the fields of microbial ecology, microbial physiology, bioengineering, biogeochemistry and related areas of environmental science.

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Sulphate-reducing Bacteria

Environmental and Engineered Systems

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Preface

Recognition of the biological nature of sulphate reduction in natural environments, and identification of the bacterial species involved dates to the latter part of the nineteenth century, and the seminal work of such giants of the early days of microbiology as Beijerinck and Winogradsky. The central role of environmental studies in highlighting the issues to be addressed and the problems to be solved, has remained to this day a constant theme in microbiological analyses of the sulphate reducers.

The modern era of such analyses, however, can be said to date from the period around 1960 when the demonstrations by Postgate and Peck, respectively, of the presence of cytochromes and of phosphorylation linked to anaerobic respiration in sulphate-reducing bacteria (SRB), fundamentally altered our view of the biochemical nature of these organisms and, in particular, of their mechanisms of energy conservation.

There then followed a period of intense activity centred on: elucidation of the metabolic pathways of substrate utilisation and the mechanisms of energy generation; cultural techniques and the identification of an ever-increasing number of new species; and the appreciation of their significant role in maintaining, or disrupting, the biological balance of many natural and man-made ecosystems.

These themes of biochemistry and cell physiology, phylogeny, and ecology remain central to the understanding of SRB themselves, and of their interactions with other components of the biosphere. In recent years, however, their study has undergone a further paradigm shift with the introduction of the many powerful experimental techniques and analytical approaches of molecular biology. As a direct consequence of these developments, sulphate-reducing prokaryotes (bacteria and archaea, as we have come to appreciate) are now the chosen organisms of study in many of the

major microbiological laboratories worldwide. Additionally, there is an extensive literature covering their several unique characteristics which, in many cases, may help to shed light on certain issues of fundamental importance to our understanding of the evolution and development of life processes. It is the purpose of this book, therefore, to draw together many of the major players in the field of biological sulphate reduction, and to present a clear and full picture of the current state of our knowledge. We thank our authors who have accepted this challenge, and given so willingly of their time, effort and insight.

It has been a conscious decision to include in the same volume studies of: genomic and proteomic analyses; phylogenetic diversity; molecular characterisation of enzymes and respiratory systems; thermodynamic analyses of metabolic processes, including anaerobic oxidation of hydrocarbons; response to stress, most particularly with regard to oxygen and other alternative electron acceptors; extreme and specialised (micro)environments, including biofilms; environmental impact in, for example, bioremediation and corrosion; and medical microbiology. These apparently disparate subject areas nevertheless form an intellectual continuum, within which it is possible to see the interdependence of the techniques and thought processes of one study area impacting directly on another. Perhaps uniquely, our current knowledge of the SRB is sufficiently extensive for us to be able to recognise and make practical use of this cross-fertilisation, and yet not so extensive and subject to technique-dependency that scientists working in one field have neither knowledge of, nor empathy with, the work in other areas. Thus it may be that this book will be seen in later years to stand at the crossroads before any such parting of the ways, as research into the sulphate-reducers continues at its present exhilarating pace. We would hope so.

W. Allan Hamilton
Larry L. Barton

CHAPTER 1

Energy metabolism and phylogenetic diversity of sulphate-reducing bacteria

Rudolf K. Thauer, Erko Stackebrandt and W. Allan Hamilton

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 $E^{\circ\prime}$ of electron donors thermodynamically capable and not capable of dissimilatory sulphate, thiosulphate or bisulphite reduction*

Redox couple	*n*	$E^{\circ\prime}$ (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: $\Delta G^{\circ\prime} = -n\mathrm{F}\Delta\mathrm{E}$, 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).

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

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 transmembrane 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* (*TpI-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).

1.2.8 Cytoplasmic H_2 oxidation

The genome of *D. vulgaris* indicates that besides the four periplasmic hydrogenases, the SRB contains two energy-conserving membrane-associated hydrogenase complexes, EchABCDEF and CooMKLXUHF, which are phylogenetically closely related (Heidelberg *et al.*, 2004; Rodrigues *et al.*, 2003). These are known to catalyze the reduction of ferredoxin (Fd) with H_2 driven by the electrochemical proton potential (energy-driven reversed electron transport), or the reduction of protons to H_2 by reduced ferredoxin coupled with the generation of an electrochemical proton potential ($\Delta\mu H^+$) (Forzi *et al.*, 2005; Hedderich, 2004; Sapra *et al.*, 2003).

$$H_2 + Fdox + \Delta\mu H^+ = Fdred^{2-} + 2H^+ \quad (1.10)$$

The subunits of the energy-conserving hydrogenases catalyzing H_2 oxidation and ferredoxin reduction have a cytoplasmic orientation within the membrane complex.

For an understanding of the function of these energy-conserving hydrogenases in *D. vulgaris* it has to be appreciated that growth of the SRB on H_2 and sulphate is dependent on the presence of acetate and CO_2 as carbon sources. These are assimilated by the cells via acetyl-phosphate, acetyl-CoA and pyruvate, the last being formed from acetyl-CoA by reductive carboxylation in a reaction catalyzed by pyruvate:ferredoxin oxidoreductase. The redox potential, $E^{\circ\prime}$, of the acetyl-CoA + CO_2/pyruvate couple is −500 mV and thus considerably more negative than that of the H^+/H_2 couple, especially when the H_2 partial pressure is very low (1 to 10 Pa) (–270 to –300 mV). For pyruvate synthesis from acetyl-CoA, CO_2 and H_2 to become exergonic, the electrons from H_2 must be elevated to a more negative potential which is achieved by the energy-driven reversed electron transport from H_2 to ferredoxin, as catalyzed by the energy-conserving hydrogenase complexes EchABCDEF and/or CooMKLXUHF (Fricke *et al.*, 2006; Hedderich, 2004; Meuer *et al.*, 2002). The same arguments hold true for other reductive reactions such as the reductive carboxylation of succinyl-CoA to 2-oxoglutarate (−500 mV) or the reduction of CO_2 to CO (–520 mV).

Energy-driven reversed electron transport is not restricted to the energy-conserving hydrogenases and is of considerable general significance in anaerobic energy metabolism where there is often the need for reducing equivalents at low redox potential/high energy level to drive particular reductive reactions. Further examples to be considered later include lactate oxidation to pyruvate ($E^{\circ\prime} = -190$ mV) with cytochrome c_3 ($E^{\circ\prime} = -400$ mV) (Pankhania *et al.*, 1988), and the oxidation of succinate to

fumarate ($E^{\circ\prime} = +33$ mV) with menaquinone ($E^{\circ\prime} = -60$ mV) as electron acceptor (Paulsen *et al.*, 1986).

When *D. vulgaris* metabolize organic substrates such as pyruvate ($E^{\circ\prime} = -500$ mV) or CO ($E^{\circ\prime} = -520$ mV), the oxidation of which yields reduced ferredoxin, then the two energy-conserving hydrogenases are involved in H_2 formation, as will be outlined in the section ''Lactate oxidation and intraspecies hydrogen transfer''.

These equation schemes are not, however, found in all SRB. *D. fructosovorans*, for example, has been shown to harbour a cytoplasmic NADP-reducing hydrogenase. This enzyme, which is absent from *D. vulgaris*, does not appear to be energy-coupled. Deletion mutants are not lethal (Malki *et al.*, 1997).

1.3 DISSIMILATORY SULPHATE REDUCTION WITH ELECTRON DONORS OTHER THAN H_2

As outlined in the previous section, dissimilatory sulphate reduction with H_2 in *D. vulgaris* involves the oxidation of H_2 in the periplasm, electron transport through the cytoplasmic membrane, and reduction of APS and bisulphite in the cytoplasm. Of the many other electron donors used by SRB for dissimilatory sulphate reduction, probably only formate is also oxidized in the periplasm. Biochemical and genomic data show that the three formate dehydrogenases in *D. vulgaris* are localized in the periplasm and, like the periplasmic hydrogenases, use poly-heme cytochromes *c* as electron acceptors (Heidelberg *et al.*, 2004). The oxidation of all other electron donors appears to occur in the cytoplasm or at the inner aspect of the cytoplasmic membrane. The redox potentials of the intermediates involved are summarized in Table 1.2. The energetic problems involved are illustrated by consideration of: (i) the oxidation of lactate to acetate and CO_2 in *D. vulgaris* (Steger *et al.*, 2002); (ii) the oxidation of acetate (acetyl-CoA) to CO_2 in SRB capable of complete oxidations (Thauer, 1988; Thauer *et al.*, 1989); and (iii) propionate oxidation to acetate and CO_2 in *Desulfobulbus propionicus* (Houwen *et al.*, 1991; Kremer and Hansen, 1988; Widdel and Pfennig, 1982).

1.3.1 Lactate oxidation and intraspecies H_2 transfer

D. vulgaris can grow on sulphate with lactate as the sole energy source. The hydroxy acid is incompletely oxidized to acetate and CO_2,

Table 1.2. *Redox potentials (E°′) of intermediates involved in dissimilatory sulphate reduction*

Redox couple	E°′ (mv)
Acetate/acetaldehyde	−581
CO_2/CO	−520
CO_2 + MFR/formyl-MFR	−520
Succinyl-CoA + CO_2/2-oxogrutarate	−520
Acetyl-CoA + CO_2/pyruvate	−498
CO_2/formate	−432
H^+/H_2	**−414**
$S_2O_3^{2-}/H_2S + HSO_3^-$	−402
Acetyl-CoA/acetaldehyde	−396
2-Oxoglutarate + CO_2/isocitrate	−364
Methenyl-H_4MPT/methylene-H_4MPT	−360
Methenyl-H_4MPT/methylene-H_4MPT	−330
Methenyl-H_4F/methylene-H_4F	−295
Acetoacetyl-CoA/ß-hydroxybutyryl-CoA	−238
Methylene-H_4F/methyl-H_4F	−200
Acetaldehyde/ethanol	−197
Dihydroxyacetone phosphate/ glycerol phosphate	−190
Pyruvate/lactate	−190
$3HSO_3^-/S_3O_6^{2-}$	−173
Oxaloacetate/malate	−172
CoM-S-S-CoB/HS-CoM + HS-CoB	−145
SO_3H^-/HS^-	**−116**
APS/AMP + HSO_3^-	**−60**
Crotonyl-CoA/butyryl-CoA	−10
Fumarate/succinate	+33
Acrylyl-CoA/propionyl-CoA	+69
$S_3O_6^{2-}/S_2O_3^{2-} + HSO_3^-$	+225

Notes: E°′ at pH 7.0 are given for H_2, CO_2 and CO 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 (see legend to Table 1.1).

with the intermediary formation of pyruvate, acetyl-CoA and acetyl phosphate.

$$2\text{Lactate}^- + SO_4^{2-} + H^+ = 2\text{acetate}^- + 2CO_2 + HS^- + 2H_2O$$

$$\Delta G^{\circ\prime} = -196.4\,\text{kJ/mol} \tag{1.11}$$

Equation 1.11 probably involves the following oxidoreduction steps:

$$\text{Lactate}^- + 2\text{cyt}c_3(\text{ox}) + \Delta\mu H^+ = \text{Pyruvate}^- + 2\text{cyt}c_3(\text{red})^{-1} \tag{1.12}$$

$$\text{Pyruvate}^- + \text{CoA} + \text{Fdox} = \text{acetyl-CoA} + CO_2 + \text{Fdred}^{2-} + 2H^+ \tag{1.13}$$

$$\text{Fdred}^{2-} + 2H^+ = \text{Fdox} + H_2 + \Delta\mu H^+ \tag{1.10}$$

$$H_2 + 2\text{cyc}\ c_3(\text{ox}) = 2\text{cyt}c_3(\text{red})^{-1} + 2H^+ \tag{1.14}$$

$$4\text{Cyt}c_3(\text{red})^{-1} + 0.5SO_4^{2-} = 4\text{cyt}c_3(\text{ox}) + 0.5H_2S \tag{1.15}$$

Equation 1.12 is catalyzed by a membrane-associated lactate dehydrogenase complex, with its active site facing the cytoplasm (Hansen, 1994; Ogata *et al.*, 1981; Reed and Hartzell, 1999). From this site, the electrons generated by lactate oxidation ($E^{\circ\prime} = -190$ mV) are transferred through the cytoplasmic membrane, driven by $\Delta\mu H^+$, to one of the periplasmic cytochromes c_3 which are in enzyme-catalyzed equilibrium with the H^+/H_2 couple ($E^{\circ\prime} = -414$ mV) (equation 1.14). The electron flow from lactate to cytochrome c_3 is an example of energy-driven reversed electron transport with the $\Delta\mu H^+$ generated in equation 1.10 used to drive equation 1.12. Equation 1.13 is catalyzed by a cytoplasmic pyruvate:ferredoxin oxidoreductase. The redox potential $E^{\circ\prime}$(–500 mV) of the acetyl-CoA + CO_2/pyruvate couple is well below that of the H^+/H_2 couple. Equation 1.10 is catalyzed by one of the two membrane-associated energy-conserving hydrogenase complexes, EchABCDEF or CooMKLXUHF, which are ferredoxin-specific and face the cytoplasm. Equation 1.14 is catalyzed by one of the four periplasmic cytochrome c_3-specific hydrogenases. The reduced cytochrome c_3 in the periplasm is finally re-oxidized by sulphate, which is reduced in the cytoplasm via transmembrane electron transport (equation 1.15) (see above). The H_2 generated in equation 1.10 has to diffuse into the periplasm in order to react with cytochrome c_3. This formation of H_2 in the cytoplasm and its re-oxidation in the periplasm has been termed intraspecies hydrogen transfer, or hydrogen cycling (Odom and Peck, 1984); Chapter 7, this volume).

Evidence for the proposed sequence of equations comes from the topology and specificity of the enzymes and electron carriers involved, and from the finding that H_2 is formed and re-consumed during growth of *D. vulgaris* on lactate and sulphate. In the absence of sulphate, H_2 is formed without re-consumption (equation 1.16).

$$\text{Lactate}^- + H_2O = \text{acetate}^- + CO_2 + 2H_2$$

$$\Delta G^{\circ\prime} = -8.8\,\text{kJ/mol} \tag{1.16}$$

H_2 formation from lactate was found to be inhibited by protonophores and by arsenate, demonstrating the involvement of an energy-requiring equation (Pankhania *et al.*, 1988). Contrary to the situation with lactate, H_2 formation from pyruvate, which is oxidized to acetate and CO_2, is not inhibited by protonophores and arsenate, showing that this equation does not require energy input (Pankhania *et al.*, 1988). These data demonstrate the validity of the equation scheme proposed above, and in particular show that lactate oxidation to pyruvate is the energy-requiring step.

Intraspecies H_2 transfer is probably also involved in dissimilatory sulphate reduction with CO. The SRB contains a cytoplasmic carbon monoxide dehydrogenase, which catalyzes the reduction of ferredoxin with CO (Soboh *et al.*, 2002; Voordouw, 2002).

$$CO + H_2O + \text{Fdox} = CO_2 + \text{Fdred}^{2-} + 2H^+ \tag{1.17}$$

$$\text{Fdred}^{2-} + 2H^+ = \text{Fdox} + H_2 + \Delta\mu H^+ \tag{1.10}$$

Besides intraspecies hydrogen transfer, formate cycling is another possible mechanism of electron transfer from the cytoplasm to the periplasm. The genome of *D. vulgaris* harbours genes for the expression of an active pyruvate-formate lyase which catalyzes the formation of acetyl-CoA and formate from pyruvate and CoA (Heidelberg *et al.*, 2004).

$$\text{Pyruvate}^- + \text{CoA} = \text{acetyl-CoA} + \text{formate}^- \quad \Delta G^{\circ\prime} = -16.3\,\text{kJ/mol} \tag{1.18}$$

Since pyruvate-formate lyase is a cytoplasmic enzyme and in *D. vulgaris* the three formate dehydrogenases are all localized in the periplasm (Haynes *et al.*, 1995; Heidelberg *et al.*, 2004), the formate generated from pyruvate must pass through the cytoplasmic membrane, most probably catalyzed via a proton symport, before it can be used as electron donor for dissimilatory sulphate reduction, or the reduction of protons to H_2.

This is therefore considered to be a case of intraspecies formate transfer (see Chapter 7).

1.3.2 Acetate (acetyl-CoA) oxidation to CO_2

There are many SRB that can oxidize organic compounds such as acetate, lactate, longer chain fatty acids, alkanes, or benzoic acid (Table 1.1) completely to CO_2 using sulphate as the electron acceptor. In all these cases acetyl-CoA is an intermediate in the pathway to CO_2. Some SRB use the citric acid cycle to oxidize acetyl-CoA to CO_2 with citrate, aconitate, isocitrate, 2-oxoglutarate, succinyl-CoA, succinate, fumarate, malate and oxaloacetate as intermediates. Amongst these is *Desulfobacter postgatei* (Thauer *et al.*, 1977). However, most SRB including *Archaeoglobus fulgidus*, use the oxidative acetyl-CoA synthase (decarbonylase)/carbon monoxide dehydrogenase pathway, either with tetrahydrofolate (H_4F) or tetrahydromethanopterin (H_4MPT) as C_1-carrier. In this pathway acetyl-CoA is oxidized via carbon monoxide, methyl-H_4F (methyl-H_4MPT), methylene-H_4F (methylene-H_4MPT), methenyl-H_4F (methenyl-H_4MPT), N^{10}-formyl-H_4F (N^5-formyl-H_4MPT) and formate (formylmethanofuran) as intermediates (Leaphart *et al.*, 2003; Thauer *et al.*, 1989; Thauer and Kunow, 1995) (for redox potentials see Table 1.2).

With respect to dissimilatory sulphate reduction, the oxidative acetyl-CoA synthase (decarbonylase)/carbon monoxide dehydrogenase pathway has the advantage that all oxidation steps involved proceed at redox potentials more negative than that of the APS/HSO_3^- couple (−60 mV) and that of the HSO_3^-/HS couple (–116 mV). On the contrary, the citric cycle involves one step, the oxidation of succinate to fumarate, with a redox potential of +33 mV. Oxidation of succinate to fumarate with sulphate as terminal electron acceptor is therefore an endergonic reaction which again requires energy-driven reversed electron transport to proceed.

1.3.3 Propionate oxidation

In SRB, propionyl-CoA, generated from propionate or by oxidation of uneven numbered fatty acids, is oxidized via methylmalonyl-CoA, succinyl-CoA, succinate, fumarate, malate, oxaloacetate, pyruvate and acetyl-CoA as intermediates. Biochemical evidence for this pathway has been obtained from *Desulfobulbus propionicus* growing on propionate and sulphate (Houwen *et al.*, 1991; Kremer and Hansen, 1988). The methylmalonyl-CoA pathway appears to be energetically more favourable than the oxidation

of propionyl-CoA via acrylyl-CoA, since the redox potential of the acrylyl-CoA/propionyl-CoA couple is +69 mV and thus more problematical than that of the fumarate/succinate couple (+33 mV), and requiring an even greater input of energy before the reaction can proceed (Sato *et al.*, 1999).

1.4 TROPHIC INTERACTIONS OF SRB WITH OTHER MICROORGANISMS

In natural environments, SRB invariably coexist with a plethora of anaerobic microorganisms which either use other electron donors and acceptors, or compete with SRB through their utilization of the same substrates. The commensals frequently form catabolic end-products which can serve as good electron donors for the SRB. This is particularly important in the case of hydrogen. Oxidation by the SRB keeps the hydrogen partial pressure low such that the primary organisms may use H^+ as an electron acceptor and so be able to produce more oxidized carbon end-products in reactions which would otherwise be endergonic. The SRB and commensals thus derive mutual benefit from living together and generally form stable syntrophic associations in which cellular energetics become community energetics, and the rates of metabolism are controlled by the rates of interspecies transfer of reducing equivalents. In open non-mixed ecosystems, the rates of diffusion of extracellular electron donors and acceptors become dominant.

Specific examples of the metabolic activities associated with sulphate reduction in different parts of a marine sediment will be discussed with reference to two regions of such an ecosystem; (i) the oxic/anoxic interface which are the upper layers, including the overlying water column, where electron acceptors more positive than sulphate/HS^- are also present such as O_2, nitrate, Mn(IV) and Fe(III); and (ii) the deeper anoxic regions where sulphate is the electron acceptor with the most positive potential.

1.4.1 Oxic/anoxic interface

The first benefit SRB derive from the activity of other organisms is essentially physicochemical. As a direct consequence of the oxygenated character of the present day biosphere, anaerobic ecosystems arise from the exclusion or removal of oxygen. In a biologically active environment, this most commonly occurs through the rate of oxygen utilization by the

metabolism of aerobic and facultative species exceeding the rate of its replenishment by diffusion, mixing or bioturbation. Such ecosystems are characterized by an oxic/anoxic interface where oxygen is replaced by a progressively less oxidizing terminal electron acceptor with increasing depth (see Chapters 5 and 6, this volume). Within a water column, the transition from oxic to anoxic conditions may occur over a depth of some centimetres, or even metres, and be subject to diurnal variation with the rise and fall of phototrophic species (Jorgensen, 1982). In sediments, and more particularly within biofilms, the interface may be very sharply defined, and measured in micrometres rather than centimetres or metres (Nielsen *et al.*, 1993); Chapter 12, this volume).

It is generally the case in oxic/anoxic ecosystems that maximal activities of the anaerobic SRB are found close to the interface with the oxic region. This arises from a number of possible causes. In addition to reducing the oxygen within the biosystem as a whole, the activities of aerobic and facultative species in the oxic zone are often responsible for the production of many of the compounds that form the carbon and electron donors utilized by the SRB. The main reservoir of sulphate is also found in oxic environments. Since the end-product of sulphate reduction, sulphide, is highly toxic to all life forms, including the SRB themselves, it is essential for the ongoing maintenance of biological activity that the sulphide be removed, or in some way neutralized. Most commonly, this is achieved by recycling across the interface with re-oxidation, biotic or abiotic, in the oxic region (Jorgensen, 1982).

The presence of other electron acceptors with more positive redox potentials than sulphate can give rise to both cooperative and competitive effects. Oxygen is generally considered to be inhibitory to SRB, although defence mechanisms have been demonstrated and species identified which can use oxygen as the electron acceptor in certain equations (see Chapter 5). However the rates of metabolism of, for example, sugars, alkanes and fatty acids by aerobic organisms greatly outcompete any potential activity found with the SRB using sulphate.

Many sulphate reducers can also use nitrate as terminal electron acceptor (see Chapter 8). This is the basis, for example, of the procedure in which nitrate is introduced to oil production facilities as a means of controlling sulphide generation in the reservoir, which give rise to so-called sour oil. It remains unclear, however, whether the observed reduction in sulphide is due to: nitrate competing as electron acceptor; the resulting nitrite acting as a non-competitive inhibitor of sulphidogenesis; or anaerobic oxidation of produced sulphide with nitrate as electron acceptor.

Recent work on the mechanisms and ecological significance of iron (FeIII) and manganese (MnIV) reduction has shed new light on the relative complexity of anaerobic respiratory processes in nature (Hamilton, 2003; Lovley *et al.*, 2004). Iron and manganese are amongst the most abundant elements found in the biosphere. Their redox potentials are in the biological range (Table 1.1), and many sulphate- and sulphur-reducing species have been shown also to have the capability of using them as terminal electron acceptors. The oxidations of Fe(II) and of Mn(II), and the reductions of Fe(III) and of Mn(IV) are, however, mediated by a wide range of microorganisms (Ehrlich, 1999; Ghiorse, 1984; Lovley *et al.*, 2004). This has been of great significance over geological time periods with the formation of iron and of manganese dioxide deposits. These same processes are now seen to be integral to the overall dynamics of anaerobic respiratory ecosystems, where the redox cycling of Fe(III)/Fe(II) and Mn(IV)/Mn(II) may play significant roles in coupling the re-oxidation of sulphide to oxygen, which thus serves as the ultimate electron acceptor (Thamdrup and Canfield, 1996). By this means, electron flux can be mediated over considerable distances between sulphidogenic and oxic zones in, for example, deepwater column biosystems (Nealson and Saffarini, 1994).

The largely insoluble character of both Fe(III) and Mn(IV) poses particular mechanistic problems for the organisms involved in their reduction. MnO_2, however, is extremely reactive and it has been found that considerable proportions of the reduced sulphide and Fe(II) in anaerobic ecosystems are re-oxidized abiotically in contact with MnO_2, without direct microbial involvement (Schiffers and Jorgensen, 2002; Thamdrup *et al.*, 1994); Chapter 5 this volume). This biotic/chemical redox cycling of Mn(IV)/Mn(II) is directly paralleled by the biotic/electrochemical redox cycling which has been shown to give rise to microbially influenced corrosion of stainless steels (Hamilton, 2003).

1.4.2 Anoxic regions where sulphate is the most positive electron acceptor

In the deeper anoxic regions of sediments, where the only electron acceptors present are sulphate (sulphidogenesis), CO_2 (methanogenesis and acetogenesis) and protons (hydrogen formation), the higher redox potential of the SO_4^{2-}/HS^- couple ensures that sulphidogenesis will be the dominant process, so long as there remains unreacted sulphate. Sulphidogenesis is also favoured by the more extensive range of substrates available to the sulphate reducers.

The major input of energy to anaerobic microbial communities is in the form of carbohydrates derived from the breakdown of plant and animal polysaccharides. Despite genomic evidence, however, for the presence of glycolytic enzymes (Heidelberg *et al.*, 2004), most SRB are not thought to degrade and grow on polysaccharides or their derived sugars in naturally occurring microbial ecosystems.

It appears rather that the many fermentative organisms in nature which are capable of sugar catabolism with high rates of activity outcompete any putative glycolytic activity that might be expressed by the SRB. On the other hand, in the anoxic conditions, sugar breakdown is incomplete and the reduced products derived from fermentative reactions are ideal substrates for sulphate reduction; for example, lactate, acetate formate or H_2. Being only partially degraded, these fermentation products retain redox energy which is available to anaerobic respiratory organisms such as the SRB. By this simple cooperation among species, complex polysaccharide materials can be completely degraded in anaerobic biosystems, with both fermentative and sulphate-respiring organisms each acquiring benefit from the throughput of carbon and energy donors.

The case of hydrogen is particularly significant. The essential character of fermentative mechanisms is that they maintain redox balance, without resource to an external acceptor as electron sink. Where the redox potential of the primary electron donor is more negative than that of the H^+/H_2 couple, however, H^+ can act as an electron acceptor with the formation of molecular hydrogen. Hydrogen is, of course, a highly favoured electron and energy donor for the SRB. Where the hydrogen partial pressure is maintained close to zero by this terminal respiratory mechanism, the primary fermentation is significantly altered with an increased hydrogen production allowing the formation of more oxidized carbon end-products. Where the fermentative and respiratory processes are coupled in this energetically mutually beneficial manner, the term used is ''syntrophism'' and the mechanism is referred to as ''interspecies hydrogen transfer'' (Schink and Stams, 2002). Syntrophism by hydrogen transfer is generally characterized by close physical association of the hydrogen-producing and the hydrogen-utilizing organisms (Conrad *et al.*, 1985). Hydrogen transfer between organisms is thus analogous to hydrogen cycling; or intraspecies hydrogen transfer, between cytoplasm and periplasm within a single organism.

As indicated by the redox potentials listed in Table 1.1, most of the compounds that can be oxidized by sulphate can also be oxidized by protons when the H_2 partial pressure is very low. Thus acetate can be first converted

by a non-sulphate reducer to $2CO_2$ and $4H_2$, and then the hydrogen further oxidized by an SRB (Galouchko and Rozanova, 1996). Alternatively, the acetate can be oxidized directly by an SRB (Thauer *et al.*, 1989). Which of the two possibilities occurs in any one environment is not always evident. Only in the case of methane oxidation with sulphate does interspecies hydrogen transfer appear not to be possible, at least not unless H_2 partial pressures in marine sediments are lower than 1 Pa (Shima and Thauer, 2005). Here the intriguing possibility of extracellular electron transfer via microbial nanowires may have to be considered (Reguera *et al.*, 2005).

1.5 PHYLOGENETIC DIVERSITY

Sulphate-reducing bacteria have been described as early as the turn of the nineteenth century when Beijerinck (1895) reported the formation of hydrogen sulphide from sulphate by a species later reclassified as *Desulfovibrio desulfuricans*. This early period of microbiology saw the dawn of microbial systematics, microbial ecology and microbial physiology when Winogradsky (1890) discovered chemoautotrophy. Over 80 years the number of sulphate-reducing species remained small. The determination of the diversity and phylogenetic incoherence of sulphate- and sulphur-reducing bacteria coincided with the application of 16S rRNA oligonucleotide sequencing in the 1980s, reverse transcriptase sequencing of 16S rRNA genes in the late 1980s, and from 1990, the PCR-mediated amplification and sequencing techniques. Today, more than 120 species of 35 genera, belonging to 3 bacterial phyla and 1 archaeal phylum have been described and their metabolism elucidated. At the turn of the twenty-first century, a sufficient body of information on sequences of 16S rRNA had been accumulated in scientific journals to develop genus-specific oligonucleotide primers used in sequencing protocols on pure cultures and, as dye-labelled DNA fragments, in *in situ* detection of single cells in their natural habitat. It became possible to explore anoxic environments by using cultivation-independent techniques, and the enumeration of sulphate-reducing bacteria demonstrated the crucial role of sulphate reducers in the global sulphur cycle and in the food web of anoxic and mixed oxic/anoxic environments (Venter *et al.*, 2004; Hines *et al.*, 1999; Voordouw *et al.*, 1996). These studies were not exclusively based on the analysis of the 16S rRNA gene but also concentrated on the analysis of key proteins of sulphate reduction. Results of these studies confirm the dominant role of Gram-negative forms. Complete oxidizers were found in eutrophied environments, whereas incomplete oxidizers dominated pristine environments (Castro *et al.*, 2002). Among the

gram-negative organisms members of *Desulfobacteriaceae* thrive on aromatic compounds (Koizumi *et al.*, 2002), while members of *Desulfovibrionaceae* did not use aromatic components of crude oil (Rabus *et al.*, 1996).

The chemical analysis of inorganic molecules of geologically ancient rocks indicated that the 3.8 Ga-old Isua sedimentary rocks did not show discrimination of ^{34}S over the lighter ^{32}S in sulphur compounds (Monster *et al.*, 1979). This finding was interpreted as the lack of biological reduction of sulphate. Enrichment of ^{32}S was, however, detected in rock dated 3.47 Ga indicating the antiquity of microbial sulphate reduction (Shen and Buick, 2004; Johnston *et al.*, 2005). The sulphate present on early Earth (rocks from the moon do not contain sulphate) was probably generated photochemically from volcanic SO_2 and H_2S in the atmosphere (Farquhar and Wing, 2003). All the available evidence indicates that the first SRB evolved before the cyanobacteria, and therefore dissimilatory sulphate reduction arose before oxygenic photosynthesis. New estimates date the origins of oxygenic photosynthesis back to 2.3–2.2 Ga (Kopp *et al.*, 2005).

Plotting the 16S rRNA similarity values against geological time, Stackebrandt (1995) concluded that the main radiation of sulphate-reducing bacteria evolved at the beginning of the formation of marine-banded iron; later than the origin of carbon fixation (photosynthesis, methanogenesis) but significantly earlier than the occurrence of O_2-dependent bacterial species. These data are in contrast to more recent findings on sequence similarities of genes coding for the alpha and beta subunits of the dissimilatory sulphite reductase, a key enzyme in the sulphur metabolism of sulphate reducers (Larsen *et al.*, 1999). These later data point to a common origin of these genes in *Archaeoglobus* (domain Archaea) and *Desulfotomaculum* (domain Bacteria), two groups of organisms that evolved from a common ancestor significantly earlier than the isotope fractionation data seem to indicate. Genes encoding for the subunits of a dissimilatory sulphite reductase-type protein have also been isolated from the hyperthermophilic crenarchaeote *Pyrobaculum islandicum* which shows structural and sequence similarities to the above-mentioned sulphate reducers (Molitor *et al.*, 1998). This indicates that most likely this enzyme-type belonged to the genetic makeup of the progenote, present even before its evolutionary split into archaea and bacteria.

In contrast to the low phylogenetic diversity of archaeal sulphate reducers, restricted to the two species of *Archaeoglobus* (*A. fulgidus*, *A. profundus*), the diversity of bacterial sulphate reducers is found in three phyla. On the other hand, considering that cultivated organisms have been described in more than 40 phyla, the evolution of sulphate reducers must be considered rather restricted. Though present in the ancestors of both

domains, the ability to reduce sulphate was lost in most lineages, even in those descendents which still thrive today in anoxic environments. Only among the mesophilic Gram-negative sulphate reducers, members of the *Deltaproteobacteria*, has a phylogenetic diverse assemblage of forms evolved that have been described as novel taxa.

Members of *Thermodesulfovibrio* can be considered deep branching thermophilic descendants of the early Bacteria. This genus has been described for strictly anaerobic, non-sporeforming, thermophilic (optimum 65–75°C), sulphate-reducing bacteria from hot springs, hot oil reservoirs and hydrothermal vents. Species are either chemoorganotrophic or chemolithoautotrophic, in which sulphate is reduced to sulphide. Hydrogen or C1–C3 acids serve as electron donors. Chemotaxonomically they are unique as they contain non-phytanyl ether-linked lipids. Recently, *Thermodesulfobium* has been described, representing a new phylum, adjacent to candidate division OP9 (Mori *et al.*, 2003) (not shown). This organism, together with *Thermodesulfovibrio*, is placed within the *Desulfovibrio* branch of *Deltaproteobacteria* in the *apsA* genes tree. This indicates that enzymes involved in sulphate respiration may evolve in an independent manner. The finding of horizontal gene transfer among sulphate-reducing bacteria was first reported by Friedrich (2002).

The publications by Widdel and Bak (1992), Widdel and Pfennig (1984) and Rabus *et al.* (2001) summarize recent developments of the taxonomy of Gram-negative sulphate reducers, including a description of families and genera, along with an extensive coverage of metabolic properties. The sulphur- and sulphate-reducing organisms of this phylum are classified in four orders which contain a few genera for which sulphur-dependent pathways have not been detected: (a) the obligately intracellular *Lawsoni intracellularis* (McOrist *et al.*, 1995) and *Bilophila wadsworthia*, isolated from patients with gangrenous and perforated appendicitis (Baron *et al.*, 1989) within the family *Desulfovibrionaceae*; (b) *Malonomonas rubra* (Dehning and Schink, 1989) and *Pelobacter*, which ferment either acetoin or trihydroxybenzenoids to acetate and CO_2, though some species ferment either polyethylene glycol, 2,3-butandiol, or acetylene to acetate and alcohols (Schink, 1992) within the family *Desulfuromonadaceae*. Gram-positive sulphate reducers are located within the phylum Firmicutes in two phylogenetically neighbouring genera within the radiation of the many clostridial lineages.

Taxonomically the combination of phylogenetic position, morphology and physiology facilitate the affiliation of a novel isolate to taxa and decide whether a new taxon can be described (Table 1.3). The relatedness of phyla

Table 1.3. *Main taxonomic differences among genera of sulphate reducers*

Family	Genus	Morphology	Desulfoviridin	Oxidation of organic electron donors	Electron acceptors for growth (other than SO_4^{2-})	mol% G+C of DNA
Desulfovibrionaceae	*Desulfovibrio*	Vibrio	+	incomplete (i)	SO_3^{2-}, $S_2O_3^{2-}$, Fumarate	46–61
Desulfomicrobiaceae	*Desulfomicrobium*	Oval to rod	–	i	SO_3^{2-}, $S_2O_3^{2-}$,	52–60
Desulfohalobiaceae	*Desulfohalobium*	Rod	–	i	SO_3^{2-}, $S_2O_3^{2-}$, S^0	57
	Desulfonatronovibrio	Vibrio	–	nr	SO_3^{2-}, $S_2O_3^{2-}$	49
	Desulfonauticus	Curved rod	–	nr	SO_3^{2-}, $S_2O_3^{2-}$, S^0	34
	Desulfothermus	Rod	–	Complete (c)	$S_2O_3^{2-}$	37
Desulfonatronumaceae	*Desulfonatronum*	Vibrio	–	i	SO_3^{2-}, $S_2O_3^{2-}$	56–57
Desulfobacteraceae	*Desulfobacter*	Oval or vibrio	–	c	SO_3^{2-}, $S_2O_3^{2-}$	45–49
	Desulfobacterium	Oval	–	c	$S_2O_3^{2-}$	45–48
	Desulfobacula	Oval to curved rods	nd	c	$S_2O_3^{2-}$	41–42
	Desulfobotulus	Vibrio	–	i	SO_3^{2-}	53
	Desulfocella	Vibrio	–	i	–	35
	Desulfococcus	Sphere	+	c	SO_3^{2-}, $S_2O_3^{2-}$	56–57
	Desulfofaba (=Desulfomusa)	Rod	–	i	SO_3^{2-}, $S_2O_3^{2-}$	52
	Desulfofrigus	Rod	–	c	SO_3^{2-}, $S_2O_3^{2-}$, Fe(iii)-citrate	52–53

	Desulfonema	Multicellular filaments	v	c	SO_3^{2-}, $S_2O_3^{2-}$,	35–55
	Desulforegula	Rods	+	i	–	nd
	Desulfosarcina	Oval, aggregates	–	c	SO_3^{2-}, $S_2O_3^{2-}$	51–59
	Desulfospira	Vibrio	–	c	SO_3^{2-}, $S_2O_3^{2-}$	50
	Desulfotignum	Curved rods	–	c	SO_3^{2-}, $S_2O_3^{2-}$	62
	Desulfatibacillum	Rods	–	c	SO_3^{2-}, $S_2O_3^{2-}$	41
Desulfobulbaceae	*Desulfobulbus*	Lemon to rod	–	i	SO_3^{2-}, $S_2O_3^{2-}$, NO_3^-	50–60
	Desulfocapsa	Rod	–	i	–	47–51
	Desulfofustis	Rod	–	i	SO_3^{2-}, S^0	56
	Desulforhopalus	Oval	–	i	SO_3^{2-}, $S_2O_3^{2-}$	48–51
	Desulfotalea	Rod	–	i	SO_3^{2-}, $S_2O_3^{2-}$, Fe(iii)-citrate	42–47
	Desulfomonile	Rod	+	nd	$S_2O_3^{2-}$, 3-Cl-benzoate	49
	Desulfarculus	Vibrio	–	c	SO_3^{2-}	66
	Desulfacinum	Oval	–	nd	SO_3^{2-}, $S_2O_3^{2-}$	60–64
	Desulforhabdus	Rod	–	Complete	SO_3^{2-}, $S_2O_3^{2-}$	53

Table 1.3. (*cont.*)

Family	Genus	Morphology	Desulfoviridin	Oxidation of organic electron donors	Electron acceptors for growth (other than SO_4^{2-})	mol% G+C of DNA
	Desulfovirga	Rod	–	Complete, depending on the substrate	SO_3^{2-}, $S_2O_3^{2-}$, S^0	60
	Desulfobacca	Oval	–	c	SO_3^{2-}, $S_2O_3^{2-}$	51
	Desulfospira	Curved rods	–	c	SO_3^{2-}, $S_2O_3^{2-}$, S^0	49
	Thermodesulforhabdus	Rod	–	c	SO_3^{2-}	51
	Thermodesulfobium	Rod	nr	c	$S_2O_3^{2-}$, NO_3^-, NO_2^-	35
	Thermodesulfobacterium	Rod	–	i	$S_2O_3^{2-}$	28–40
Thermodesulfovibrionaceae	*Thermodesulfovibrio*	Vibrio	–	i	SO_3^{2-}, $S_2O_3^{2-}$	38
Firmicutes	*Desulfotomaculum*	Straight or curved rod	Sporulating Gram-positive	i or c	$S_2O_3^{2-}$, fumarate	48–52
	Desulfosporosinus	Straight or curved rod	Sporulating Gram-positive	i	$S_2O_3^{2-}$	45–46
Archaea	*Archaeoglobus*	Sphere	–	c	–	41–46

among each other with an emphasis of anaerobic bacteria has been summarized by Stackebrandt (2004). It should be noted that the order of phyla within the 16S rRNA gene sequences tree is not well resolved and phylogenetic trees, no matter which algorithm used for their generation, have a bush- or fan-like appearance. The hierarchic outline of sulphate reducers has been devised by Garrity *et al.* (2003). The reader interested in the original description of taxa at the genus and species level is referred to the homepage of Jean Euzeby (http://www.bacterio.cict.fr) which provides a nomenclatural database.

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