Chapter 5
Ecophysiology and Application of Acidophilic Sulfur-Reducing Microorganisms

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1 Sulfur Compounds in Nature

Sulfur is an important element in the Earth crust, representing about 0.05 % of the lithosphere weight (Steudel and Eckert 2003). However, it is highly concentrated in various continental rocks, such as metal sulfide ore deposits [e.g. pyrite (FeS_2), chalcopyrite ($CuFeS_2$), pyrrhotite (FeS)] or sulfate deposits [e.g. gypsum ($CaSO_4{\cdot}2H_2O$), barite ($BaSO_4$)]. Sulfur exhibits nine different oxidation states, however the most abundant in nature are −2 (sulfide and reduced organic sulfur), 0 (elemental sulfur) and +6 (sulfate) (Steudel 2000; Tang et al. 2009).

Electronic supplementary material: The online version of this chapter (doi:10.1007/978-3-319-13521-2_5) contains supplementary material, which is available to authorized users.

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P.H. Rampelotto (ed.), *Biotechnology of Extremophiles*, Grand Challenges in Biology and Biotechnology 1, DOI 10.1007/978-3-319-13521-2_5

The oxidation reaction of sulfide to sulfate implies the transference/loss of eight electrons and can be performed in different steps, in which elemental sulfur, thiosulfate, sulfite, and polysulfide (Hedderich et al. 1999) can appear as intermediates. The importance and stability of these intermediates in solution depends on pH, temperature, presence of chemical oxidizing and reducing agents, catalysts, and the species involved (Knickerbocker et al. 2000).

Transformations of sulfur forms in the environment are highly dependent on microbial activities (Steudel 2000). Transformation of organic and inorganic sulfur compounds performed by microorganisms greatly affects chemical, physical and biological properties of the biosphere.

The sulfur cycle can be analyzed through two points of view (Canfield and Farquhar 2012). From a geological perspective, the generation of oceanic crust is associated with the transfer of sulfur from the earth mantle to the earth surface and to the oceans (Canfield 2004), which occurs via volcanic outgassing of SO_2 and H_2S, release of H_2S during hydrothermal circulation, and the erosion of igneous sulfide minerals (Canfield and Farquhar 2012). From the biological perspective, sulfate and/or sulfur reduction may be either assimilatory, when the sulfide produced is used for anabolic reactions, or dissimilatory, when used for energy conservation and growth (Tang et al. 2009; Canfield and Farquhar 2012).

Sulfide is used as electron donor by several anoxygenic phototrophic bacteria which perform photosynthesis. They form elemental sulfur, sulfate (Ghosh and Dam 2009) or, sometimes, thiosulfate (Pfennig 1975) as products (Eqs. 5.1–5.3). Sulfide may be also oxidized by chemotrophic prokaryotes coupled to O_2, nitrate, manganese or iron reduction (Hedderich et al. 1999; Ohmura et al. 2002).

$$H_2S + ½O_2 \rightarrow S^0 + H_2O \tag{5.1}$$

$$H_2S + 2O_2 \rightarrow H_2SO_4 \tag{5.2}$$

$$4H_2S + 5O_2 \rightarrow 2S_2O_3 + 4H_2O \tag{5.3}$$

Elemental sulfur (S^0), thiosulfate ($S_2O_3^{2-}$) and sulfite (SO_3^{2-}), as products of sulfide oxidation, can be oxidized, reduced, or disproportionated to sulfate and sulfide by microorganisms. The disproportionation of elemental sulfur seems to be of great significance in the environment (Steudel 2000; Tang et al. 2009; Canfield and Farquhar 2012).

Biological reactions described in this section are summarized in Fig. 5.1. Chemical reactions are described in the next section.

2 Chemistry of Elemental Sulfur

Elemental sulfur (S_8^0) is the molecule with the largest number of solid structural forms that can be divided into ambient pressure and high-pressure allotropes. Although there exist over 180 different allotropes and polymorphs (Box 5.1), the only steady form of elemental sulfur at standard temperature and pressure conditions (273.15 K and 1 bar) is the orthorhombic α-S_8^0 modification (Steudel and Eckert 2003).

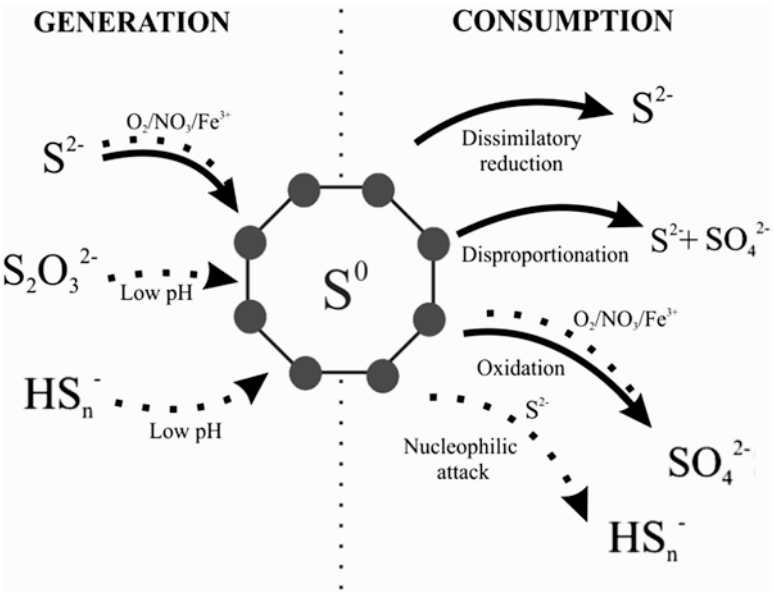

Fig. 5.1 Possible reactions with elemental sulfur as product or reagent. On the left side, reactions that lead to sulfur (by oxidation processes or acidification of the medium) are shown. On the right side, consuming reactions (sulfur reduction, disproportionation, oxidation and nucleophilic attack by sulfide) shown. Biological reactions are represented as *full lines* and chemical reactions as *dashed lines*

Box 5.1
Allotropy: ability of a material to have more than one structure under different conditions of temperature and pressure and to regain these structures when conditions are reversed. Hence, allotropy is a reversible polymorphism.

Polymorphism: ability of solid material to exist in more than one form or crystal structure. If there is change in temperature and pressure, and it is not accompanied by melting or vaporization of the solid, it will cause the solid to change its internal structure of atoms.

Oxo-compounds: compounds containing an oxygen atom doubly bound to carbon or another element (=O).

Sulfur is hardly soluble in water; the solubility of the α-S_8^0 at 20 °C is only 5 μg L^{-1} (Boulegue 1978). In general, the higher the molecular size of the sulfur allotropes, the lower is the solubility in organic solvents. Carbon disulfide, toluene and dichloromethane are the best sulfur solvents, while *cyclo*-alkanes are worthy only at ambient temperatures, dissolving smaller ring molecules (Steudel and Eckert 2003). At higher temperatures (65–140 °C), elemental sulfur is also soluble in compressed gases like nitrogen, methane, carbon dioxide, and hydrogen sulfide, which is of importance for the gas industry since many natural gas reservoirs also contain H_2S

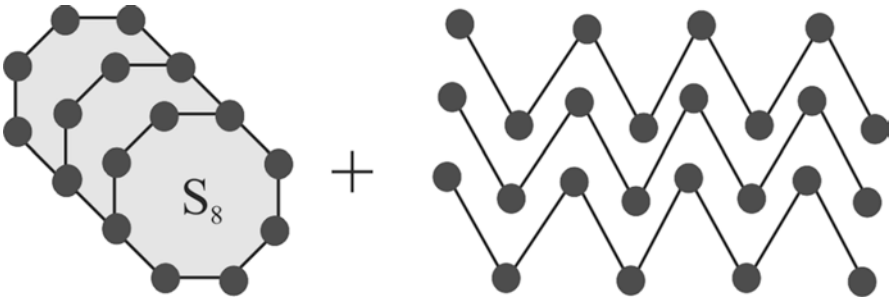

Fig. 5.2 Rings and chain-like macromolecules of polymeric sulfur that compose the commercialized sulfur flower

and elemental sulfur. For example, in a range of pressure from 10 to 30 MPa, solubility of elemental sulfur in hydrogen sulfide increases from 38.6 mg L^{-1} at 65 °C (Roof 1971) to 65.7 at 90 °C (Gu et al. 1993), 68.1 at 100 °C, 91.2 at 110 °C (Roof 1971) and 110.8 mg L^{-1} at 140 °C (Brunner and Woll 1980).

The customary form in which elemental sulfur is typically traded, also called sulfur flower, mainly consists of S_8^0-rings and some polymeric sulfur composed by chain-like macromolecules (Steudel and Eckert 2003) (Fig. 5.2). The heat of reaction from S_8^0 (ring) to S (chain) is 115.14 kJ mol^{-1} per sulfur atom, which is 2.3 kJ mol^{-1} stronger than the bond strength between S–S bonds in polymeric sulfur (Franz et al. 2007). Therefore, polymeric sulfur might be easier to access by sulfur-reducing or sulfur-oxidizing microorganisms.

When sulfide (S^{2-}) is present in the same environment as elemental sulfur, normally at high pH values, a nucleophilic attack of HS^- anion cleaves the S_8^0-ring of elemental sulfur, generating polysulfide (Blumentals et al. 1990). Polysulfide is considered to be preferred over elemental sulfur as electron acceptor by microbes at high temperature and neutral-high pH values due to its higher solubility at these conditions (Schauder and Müller 1993). The most important polysulfide species are tetrasulfide S_4^{2-} and pentasulfide S_5^{2-} (Rabus et al. 2006) which can interconvert rapidly at neutral environments, supporting the growth of neutrophilic sulfur-reducing microorganisms (Schauder and Müller 1993).

The equilibrium concentration of polysulfide (S_n^{2-}) in sulfide solution depends on pH, temperature and sulfide concentration. When the pH decreases, the pH equilibrium concentration of polysulfide immensely shrinks, due to the instability of S_n^{2-} at low pH, and the reaction goes towards elemental sulfur and sulfide, as represented in Eq. (5.4) (Schauder and Müller 1993).

$$2S_5^{2-} + 4H^+ \leftrightarrow \alpha S_8 + 2H_2S \tag{5.4}$$

However, the equilibrium concentration increases with increasing temperatures. Thus, 0.1 mM will dissolve at pH 6.7 and at 30 °C, while at pH 5.5, the same amount will only dissolve at 90 °C. Due to the dissociation constant, the maximum amount of S_8^0 that can be converted into polysulfide in a sulfide solution at pH 8.0

and 37 °C is roughly comparable to the sulfide concentration (Klimmek et al. 1991). However, at pH values below the pK_a1 of H_2S, which is 7.0 at 25 °C, polysulfide is formed in much lower concentration (Hedderich et al. 1999).

Also thiosulfate is unstable under acidic pH conditions and decomposes into sulfur oxides, sulfide and colloidal/dissolved sulfur as nanocrystals (Eq. 5.5) (Wang et al. 1998), which turn the solutions into a milky suspension. In natural environments, an adhesion of organic polymers to colloidal sulfur particles occurs, which alters the surface properties of elemental sulfur and increases its hydrophilicity (Breher 2004). As sulfur particles are generated together with sulfide, they can react, producing an aqueous solution of polysulfide ions, which affects the mobility of sulfur in the environment, its availability for bio-oxidation, and the formation kinetics of polysulfide and sulfide (Breher 2004). However, colloidal sulfur is thermodynamically unstable and eventually precipitates as small settleable crystals (Kleinjan et al. 2005).

$$3S_2O_3^{2-} + 4H^+ \leftrightarrow 2H_2S + SO_3^{2-} + 3SO_2 \tag{5.5}$$

Another form of elemental sulfur, more hydrophilic than the orthorhombic form, is the so-called bio-sulfur (Steudel and Eckert 2003), which is formed when sulfide is biologically oxidized and can be stored inter- or extra-cellularly as sulfur globules (Kleinjan et al. 2005). It has been suggested that adsorbed organic polymers, such as proteins, or organic end groups are the responsible for the more hydrophilic nature of the bio-sulfur, and so, its structure may differ between species of sulfur bacteria (Steudel et al. 2003). Organic groups also stabilize the long sulfur chains that are produced by phototrophic bacteria. The chemotrophic bacteria, however, mainly form rings composed by eight sulfur atoms (Kleinjan et al. 2005).

3 Sulfur-Reducing Microorganisms

Many prokaryotes are able to colonize environments without any presence of oxygen, evolving not only fermentation pathways, but also respiration, conserving energy for anaerobic growth by coupling the oxidation of hydrogen or organic substrates with the reduction of organic or inorganic compounds (Hedderich et al. 1999; Rabus et al. 2006). Nitrate, manganese (IV), ferric iron, carbon dioxide, protons, selenite, uranium (VI), chromate (VI), arsenate, trymethylamine-N-oxide (TMAO), and sulfur compounds, such as sulfate, elemental sulfur, sulfite, thiosulfate, sulfoxides, dimethylsulfoxides (DMSO), and organic disulfides can be used as electron acceptors by prokaryotes under anoxic conditions (Rabus et al. 2006).

Dissimilatory reduction of Fe (III) and sulfur compounds are significant geobiochemical reactions that occur in soils, aquatic and subsurface environments (Lovley et al. 1995). Reduction of iron has a pronounced influence on the dispersal of iron and trace metals and nutrients. Additionally, it is involved in the degradation of organic matter and can be a promising agent for bioremediation of organic and

metals contaminated environments (Lovley et al. 2004). Reduction of Fe (III) can be performed by several microorganisms in the presence of sulfur compounds as energy source.

Reduction of sulfur compounds by it turn attracts attention as it generates hydrogen sulfide as the main end product. Sulfide is known by its remarkable impact on the chemistry of the environment and, furthermore, can serve as electron donor for a considerable high diversity of microorganisms (Rabus et al. 2006). Due to the abundancy and thermodynamic stability, sulfate is the sulfur compound most studied as electron acceptor for anaerobic respiration.

Elemental sulfur reduction, however, is of great importance especially in deep-sea vents, hot springs and other extreme environments, from where microorganisms have most frequently been isolated and their diversity is equivalent to that of sulfate reducers (Stetter 1996).

3.1 Ecophysiology of Sulfur Reducers

Currently known sulfur reducers are spread over about 69 genera within 9 phyla in the *Bacteria* domain (Fig. 5.3a, b) and 37 genera within 2 phyla in the *Archaea* domain (Fig. 5.4). They use elemental sulfur as the main electron acceptor for the oxidation of organic compounds or H_2.

Although microbial sulfur reduction was already reported in early studies as for example by Beijerink (1895), Pelsh (1936) reported the first evidence of elemental sulfur reduction as the only source of energy for microbial growth in enrichments of a vibrioid bacterium from mud, with sulfur and H_2 as electron acceptor and donor, respectively. The first pure culture growing by sulfur reduction was *Desulfuromonas acetoxidans*, an obligatory anaerobic acetate-degrading mesophile and obligate sulfur reducer, not able to use sulfate (SO_4^{2-}) (Pfennig and Biebl 1976).

Afterwards, many sulfur reducers were isolated and showed the ability to reduce other compounds such as thiosulfate, iron (III), nitrate and even oxygen, though anoxic environments are more favorable (Rabus et al. 2006). The capability for sulfur reduction was also observed for microorganisms isolated with other electron acceptors, such as sulfate (Biebl and Pfennig 1977), iron (III) (Caccavo et al. 1994) and manganese (IV) (Myers and Nealson 1988). Only a few species of sulfate reducers are able to grow by sulfur reduction, and sometimes the growth can even be inhibited by elemental sulfur (Bak and Pfennig 1987; Burggraf et al. 1990).

Sulfur-reducing prokaryotes are able to grow at a broad range of temperature (from −2 to 110 °C) and pH (from 1 to 10.5) (Supplemental material—Table 1). Most of the sulfur reducers identified thrive at neutral environments. However, some hyperthermophilic *Archaea* isolated from solfatara fields are reported to grow at pH as low as 1, such as *Acidianus ambivalens*, *Acidianus brierleyi*, *Styogiolobus azoricus*, *Thermoplasma volcanium* and *Thermoplasma acidophilum* (Segerer et al. 1986, 1988, 1991). The lowest pH reported so far for sulfur-reducing bacteria growth is 1.3 for *Acidithiobacillus ferrooxidans* (Ohmura et al. 2002), but several

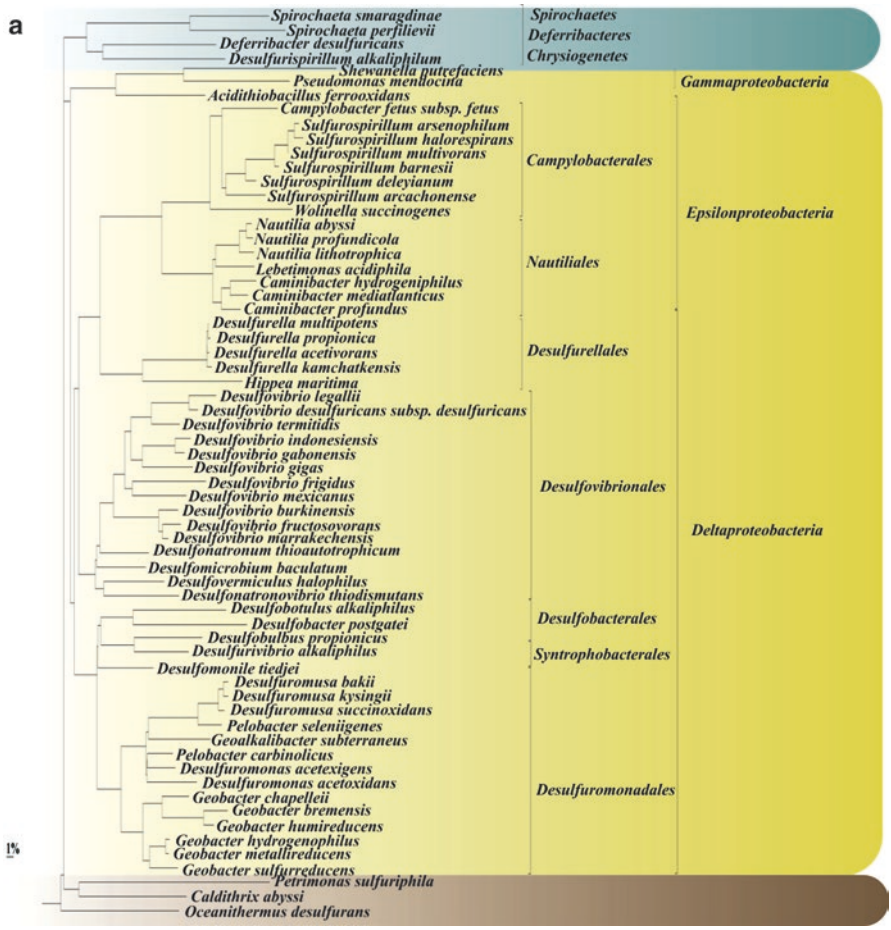

Fig. 5.3 Phylogenetic affiliation of 16S rRNA gene sequences of sulfur-reducing bacteria in The All-Species Living Tree Project (Yarza et al. 2008). In **a**, the sequences belonging to the phyla *Proteobacteria*, *Spirochaetes*, *Deferribacteres* and *Chrysiogenetes* are represented, and in **b** sequences belonging to the phyla *Firmicutes*, *Aquificae*, *Thermodesulfobacteria*, *Synergistetes* and *Thermotogae* are represented. 1 % estimated sequence divergence

acidophilic and acidotolerant species have been described within the bacterial domain, such as *Desulfosporosinus acididurans* (pH 3.8), *Desulfurobacterium thermolithotrophum*, *Marinitoga hydrogenitolerans* and *Thermanaerovibrio velox* (pH 4.5) (L'Haridon et al. 1998; Zavarzina et al. 2000; Postec et al. 2005; Sánchez-Andrea et al. 2015).

Even though several mesophilic microorganisms able to reduce elemental sulfur have been described such as *Desulfuromonas*, *Beggiatoa,* or *Sulfurospirillum* (Pfennig and Biebl 1976), sulfur respiration seems to be more widespread at higher temperature. Slightly thermophilic bacteria (T_{opt} = 40–60 °C) such as *Desulfurella*

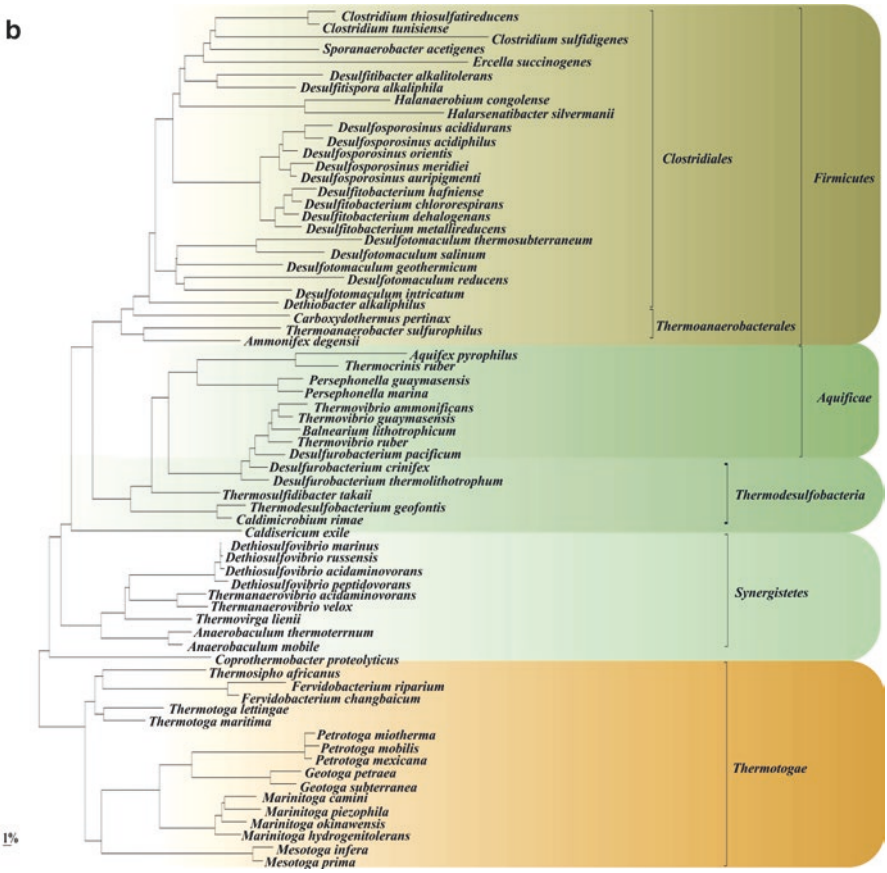

Fig. 5.3 (continued)

and *Thermoanaerobacter* (Bonch-Osmolovskaya et al. 1990b; Bonch-Osmolovskaya et al. 1997) and moderately thermophilic bacteria (T_{opt} = 60–80 °C), such as *Ammonifex* (Huber et al. 1996) and *Desulfurobacterium* (L'Haridon et al. 1998) have been described as well as some hyperthermophilic sulfur reducers, such as *Aquifex* (Huber et al. 1992).

Extreme habitats, such as hot water pools in solfataric fields, acidic hot springs, hydrothermal systems in shallow and deep sea, hypersaline lakes and anoxic mud sediments harbor sulfur reducers that grow at high temperature and low pH (Stetter 1996; Rabus et al. 2006). Due to their abundance and specialized metabolic activities sulfur-reducing prokaryotes are thought to play an important role in the sulfur biogeochemical cycle in deep-sea vents, hot springs and other extreme environments (Bonch-Osmolovskaya et al. 1990a; Alain et al. 2009; Birrien et al. 2011).

In anoxic mud sediment environments, sulfur-reducing microorganisms often form associations with sulfide oxidizers, which provide them with elemental sulfur. The sulfur reducers by their turn reduce the elemental sulfur back to sulfide that is

used as electron donor by the sulfide oxidizers (Pfennig 1975). In hydrothermal vents, some sulfur reducers can be found as free-living organisms on vent chimneys or plumes, or as endosymbionts of animals such as tube worms and shrimps, in which they play the same role as their counterparts in the vents by reducing and oxidizing sulfur compounds (Alain et al. 2009).

Described sulfur-reducing bacteria are widespread within the phylogenetic tree of life. They belong to the phyla *Proteobacteria (Delta, Epsilon-* and *Gammaproteobacteria* classes), *Thermodesulfobacteria, Spirochaetes, Deferribacteres, Chrysiogenetes, Firmicutes, Aquificiae, Synergistetes and Thermotogae* (Fig. 5.3a, b). In the order *Clostridiales* and *Thermoanaerobacterales*, sulfur reduction seems to be a quite widespread metabolic trait (Hernandez-Eugenio et al. 2002; Sallam and Steinbüchel 2009). Within the *Archaea*, sulfur reduction occurs in the phyla *Euryarchaeota* (Fiala and Stetter 1986; Burggraf et al. 1990) and *Crenarchaeota* (Fig. 5.4) (Itoh et al. 1998; Prokofeva et al. 2000; Itoh et al. 2003).

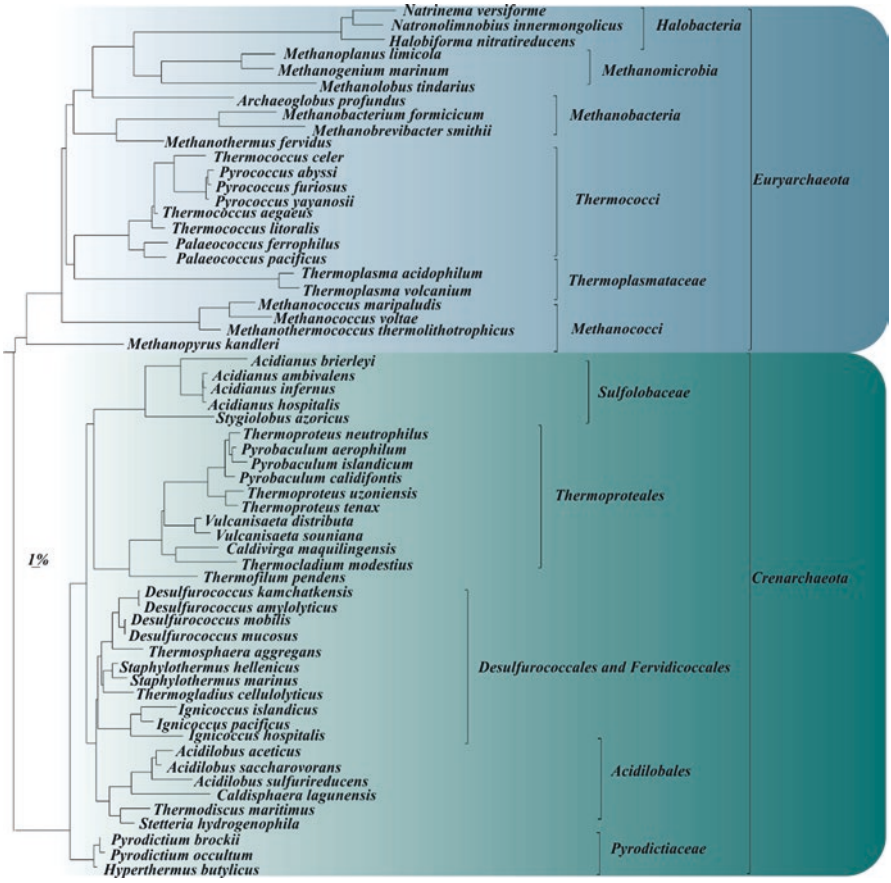

Fig. 5.4 Phylogenetic affiliation of 16S rRNA gene sequences of sulfur-reducing archaea in The All-Species Living Tree Project (Yarza et al. 2008). 1 % estimated sequence divergence

The metabolism of sulfur reducers has been poorly studied, with the exception of few microorganisms, such as the bacterium *Wolinella succinogenes* and the archaeon *Pyrococcus furiosus*. Besides to the biochemistry and bioenergetics of sulfur respiration, little attention has been paid to the conversion of the electron donors in sulfur reducers. Most of the literature related to metabolic pathways and energy conservation is focused on lithotrophic growth on hydrogen or formate as electron donors. Heterotrophic growth on acetate has been investigated only in a few bacteria (Schröder et al. 1988; Klimmek et al. 1991; Kreis-Kleinschmidt et al. 1995). For instance, oxidation of acetate with sulfur as electron acceptor was studied in *Desulfurella* and *Desulfuromonas* species, which occurs via the citric acid cycle. The electron transport is carried out by ferredoxin that might accept electrons from the 2-oxoglutarate via NADP in a 2-oxoglutarate dehydrogenase reaction and menaquinone mediates electron flow to sulfur reductase (Schmitz et al. 1990; Rosenberg et al. 2013). Acetate activation and succinate formation, however, are performed by different mechanisms in those bacteria. In *D. acetoxidans,* it is most likely that only one enzyme (succinyl-CoA:acetate CoA transferase) is involved in the formation of acetyl-CoA and succinate from acetate and succinyl-CoA. In *D. acetivorans,* however, acetate forms acetyl-CoA via acetyl phosphate, which involves the enzymes acetate kinase and phosphate acetyltransferase, and succinyl-CoA forms succinate via succinyl-CoA synthetase (Schmitz et al. 1990).

Other substrates, including alcohols, such as methanol and ethanol; organic acids, like propionate, butyrate, and lactate; sugars, such as glucose, fructose, cellobiose, cellulose, lactose, arabinose, rhamnose, maltose; starch and molasses have also been described as organic substrates for sulfur reducers (Bonch-Osmolovskaya et al. 1990b; Finster et al. 1997; Dirmeier et al. 1998; Boyd et al. 2007).

The oxidation of carbon substrates by sulfur reducers can be complete or incomplete. In the first case, it leads to the solely production of CO_2 (*Desulfuromonas* and *Desulfurella*) (Pfennig and Biebl 1976; Rainey and Hollen 2005) while in the second, acetate and CO_2 are produced as final products (*Wolinella* and *Shewanella*) (Macy et al. 1986).

3.2 Sulfur Metabolism

The poor solubility of α-S_8^0 is a bottleneck for fast growth of sulfur reducers (Bonch-Osmolovskaya et al. 1990b; Schauder and Müller 1993; Miroshnichenko et al. 1998; Prokofeva et al. 2000). Two possible mechanisms to overcome the low solubility of elemental sulfur have been reported (Cammack et al. 1984; Zöphel et al. 1991; Schauder and Müller 1993). One possibility is that sulfur is converted to a more hydrophilic and/or soluble form, such as polysulfide, that can support faster growth (Blumentals et al. 1990; Schauder and Müller 1993). It is likely that the increasing solubility of sulfur and the formation of polysulfide at higher temperatures and pH is beneficial for growth of thermophilic and hyperthermophilic microorganisms (Belkin et al. 1985).

However, as polysulfides are unstable at low pH, it can be that the binding proteins synthesized by sulfur reducers, such as polysulfide sulfur transferases, allow fast polysulfide respiration at low polysulfide concentration (Klimmek 2005), and thus polysulfide is still the substrate. Alternatively, it could be that acidophiles use nanocrystalline that is formed from polysulfide decomposition as electron acceptor. So far, there is still no agreement if polysulfides or nanocrystalline can serve as electron acceptor for acidophilic/acidotolerant microorganisms (Boyd and Druschel 2013). Besides polysulfide, hydrophilic sulfur formed by the association of elemental sulfur with small portions of oxo-compounds (Box 5.1), such as aldehydes, carboxylic acids, ketones, amides, and esters (Steudel et al. 1989) can serve as electron acceptor for microorganisms.

It is remarkable, however, that some bacteria are reported to grow with elemental sulfur when there is no possibility of solubilization in the form of polysulfide (Thamdrup et al. 1993; Finster et al. 1998). As an alternative mechanism, a direct conversion of sulfur into sulfide is suggested to occur due to a physical attachment of the microorganisms to the elemental sulfur.

Even though it is still not clear which mechanism of sulfur reduction is used by the different sulfur reducers, it is likely that hyperthermophilic chemolithoautotrophic archaea reduce elemental sulfur to sulfide via physical attachment (Pihl et al. 1989; Stetter et al. 1993). Moreover, since polysulfides are unstable at low pH and rapidly dissociate into sulfur and sulfide, it is reasonable to hypothesize that elemental sulfur can be the real substrate for the sulfur reductase identified in *A. ambivalens*, an extreme acidophile (Laska et al. 2003).

The reductases that mediate sulfur reduction (either via attachment or via polysulfide) have been purified and characterized from a few sulfur reducers (Schröder et al. 1988; Childers and Noll 1994; Ng et al. 2000; Laska et al. 2003), but sulfur reduction via polysulfide has only been confirmed in *W. succinogenes* (Klimmek et al. 1991), *P. furiosus* (Blumentals et al. 1990) and some *Clostridium* species (Takahashi et al. 2010).

3.3 Enzymes Involved in Sulfur Reduction

In general, the nomenclature of the enzymes involved in sulfur reduction is not well standardized in the published literature. Sometimes the enzymes receive one name related to specific characteristics when they are first isolated and, afterwards, due to more general properties, the name is changed. That was the case for the enzyme sulfhydrogenase. The two hydrogenases isolated from *P. furiosus* were formerly called sulfhydrogenases (Shy). However, as these enzymes seem to be regulated by metabolites other than sulfur, the name sulfhydrogenase became confusing and out of date; so, it was proposed to rename as hydrogenase from hyperthermophiles (Hyh) (Vignais et al. 2001). However, sulfhydrogenase is still present in the database as the main name of the enzyme and is therefore used in this manuscript.

In the genomes database, it is common to find enzymes in reported sulfur reducers named only as sulfur reductase, without specificity about the groups to which they are related. It is also possible to find the mentioned names as synonyms, when they actually refer to different enzymes. In some searches on the available databases, for example MetaCyc (http://metacyc.org/), sulfide dehydrogenase can be referred as sulfhydrogenase and vice-versa.

So far, three enzymes involved in reduction of elemental sulfur and polysulfide to hydrogen sulfide are characterized and described in literature: polysulfide reductase, isolated from *Wolinella succinogenes* (Hedderich et al. 1999), and sulfide dehydrogenase and sulfhydrogenase, both isolated from *P. furiosus* (Ma and Adams 1994).

3.3.1 Polysulfide Reductase

The membrane-bound enzyme is a molybdopterin-containing protein that consists of three subunits predicted by the operon *psrABC* (Krafft et al. 1995). The molybdopterein cofactor is located at the catalytic subunit PsrA, which has an [4Fe-4S] iron-sulfur center. The purified enzyme contains 20 mol of free iron and sulfide per mol of enzyme. Since the subunit PsrB contains four [4Fe-4S] iron-sulfur centers, the mentioned amount is consistent with the whole enzyme (Hedderich et al. 1999).

The hydrophobic subunit of polysulfide reductase (PsrC) anchors the enzyme in the membrane. The transference of electron from the membrane anchor and the catalytic subunit of the enzyme is most likely mediated by the subunit PsrB. The PsrB is probably bound to the other subunits at the periplasmic side of the membrane (Dietrich and Klimmek 2002). The purified enzyme contains menaquinone as cofactor. Due to its lipophilic nature, it is likely that the menaquinone is bound to the subunit PsrC of the enzyme.

The hypothetical mechanism of polysulfide reduction at the catalytic subunit PsrA indicates that the polysulfide chain is cleaved at the last sulfur atom, which is released and bound to the molybdenum cofactor that is further oxidized. The molybdenum cofactor in the PsrA is most likely coordinated by two molybdopterin guanine nucleotide molecules. Thus, after the uptake of a proton, probably via sulfide dehydrogenase, and two electrons, HS^- is released and the molybdenum returns to its reduced stage (Fig. 5.5) (Klimmek et al. 1991).

Sequences of the gene subunits deposited in the JGI genome database are available under accession numbers: PsrA: NP906381; PsrB: NP906382; PsrC: NP906383.

3.3.2 Sulfide Dehydrogenase

Sulfide dehydrogenase, also called flavocytochrome c sulfide dehydrogenase, is a bifunctional cytoplasmic enzyme that catalyzes the reduction of polysulfides to sulfide using NADPH as electron donor (Ma and Adams 1994), but it can also function as a ferredoxin:$NADP^+$ oxidoreductase (Ma and Adams 1994). Reduction of $NADP^+$ is thought to be a required step in the disposal of reducing equivalents as H_2.

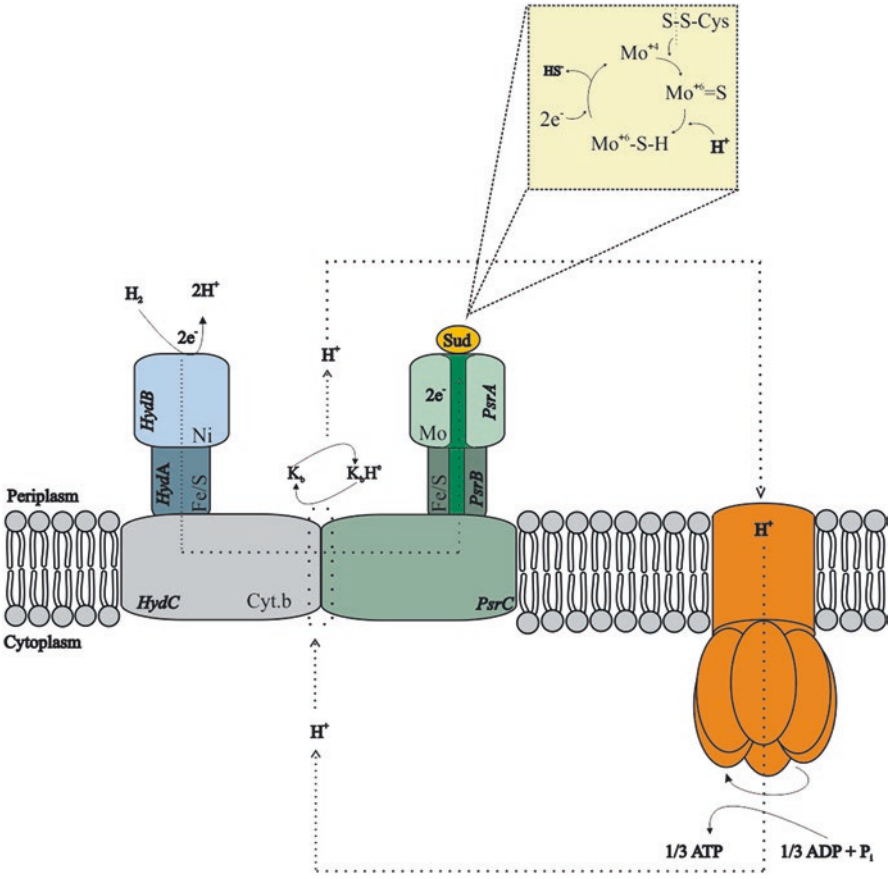

Fig. 5.5 Hypothetical view of elemental sulfur reduction (via polysulfide) and anaerobic electron transport chain in *W. succinogenes*. For the electron transfer to happen between the enzymes, collision of the enzymes is assumed to be required and menaquinone seems to be bound to the subunit C of the polysulfide reductase. Protons are also assumed to be translocated to the periplasm via menaquinone. Subunits of the hydrogenase are labeled HydA, HydB and HydC and subunits of the polysulfide reductase are labeled PsrA, PsrB and PsrC. K stands for quinone and Sud stands for a sulfur/polysulfide transferase. Model adapted from Hedderich et al. (1999) and Rosenberg et al. (2013)

The protein contains two flavins and three different [Fe-S] centers: a putative [2Fe-2S] cluster coordinated by a motif of an aspartate and three cysteine ($Asp(Cys)_3$) that combines physic-chemical properties known as exclusive from protein clusters coordinated by hystidine (Rieske-type), a regular [3Fe-4S] cluster with high reduction potential, and a [4Fe-4S] cluster also with unusual reduction properties (Hagen et al. 2000). The role of the high reduction potentials for the last two clusters is not yet clear, but the redox potential of the flavins is consistent with the function of sulfide dehydrogenase and ferredoxin: $NADP^+$ oxidoreductase.

As the properties of the iron-sulfur clusters in the subunits of the sulfide dehydrogenase are not yet completely understood, the mechanism of action is not clear.

Sequences of the gene subunits deposited in the JGI genome database are available under accession numbers: SudHA: AAL81451/AAL82034; SudHB: AAL81452/AAL82035.

3.3.3 Sulfhydrogenase

Two different cytoplasmic hydrogen-metabolizing enzymes were purified from *P. furiosus* and showed sulfur reductase activity. Both are referred as sulfhydrogenases, I and II, also called NAD(P)H:sulfur oxidoreductase, or coenzyme A (CoA)-dependent NADP(H) sulfur oxidoreductase (Bryant and Adams 1989; Ma et al. 1993, 2000).

Both, sulfhydrogenases I (Bryant and Adams 1989) and II (Ma et al. 2000) can reduce $S_8^0$and polysulfide to H_2S using H_2 as electron donor. Both proteins have four subunits, with nickel, iron-sulfur centers and flavin adenine dinucleotide, but their subunits differ in catalytic activities and arrangements; sulfhydrogenase I is a heterotetramer ($\alpha\beta\gamma\delta$) and sulfhydrogenase II is suggested to be a dimer of heterotetramer $(\alpha\beta\gamma\delta)_2$ (Bryant and Adams 1989). In both cases β and γ subunits play a sulfur reductase role, while α and δ function as hydrogenases.

There are three main differences between the enzymes: (1) sulfhydrogenase II was shown to be less active for hydrogen production, uptake and sulfur reduction assays developed by Ma et al. (2000). (2) The authors also showed that sulfhydrogenase II has higher affinity for elemental sulfur and polysulfide, suggesting a physiological relevance of this enzyme when the concentration of sulfur is low. (3) Sulfhydrogenase II shows greater affinity for NAD(H) and NADP(H) the sulfhydrogenase I, and potentially uses both nucleotides with equivalent efficiency.

Sequences of the gene subunits of the two complexes deposited in the JGI genome database are available under accession numbers: shyA: AAL81018/AAL81456; shyB: AAL81015/AAL81453; shyC: AAL81016/AAL81454; shyD: AAL81017/AAL81455.

A possible novel enzyme involved in elemental sulfur reduction was purified from the acidophilic archaeon *A. ambivalens* (Laska et al. 2003), which reduces elemental sulfur with H_2 or $NADPH_2$ as electron donors. The sulfur reductase is shown to be a membrane-bound protein related to the one from *W. succinogenes,* with subunits sharing similar structure and properties. At least three proteins likely compose the main structure of the core enzyme: a catalytic subunit, probably a molybdopterin (SreA), an iron-sulfur protein (SreB) and a membrane anchor (SreC). The membrane anchor, however, was shown to be phylogenetically unrelated to the analogous protein in *W. succinogenes*. As the enzyme was isolated in the absence of sulfide, it is most likely that it reduces elemental sulfur itself, instead of polysulfide. Deeper investigations on the sulfur reductase were not possible, as the enzyme could not be purified in the absence of hydrogenase (Laska et al. 2003). A complete characterization of the enzyme is still necessary to reveal if it is a true

novel enzyme in sulfur-reducing microorganisms which will help in the elucidation of the mechanisms.

A similar enzyme is present in several microorganisms within *Archaea* and *Bacteria* domains, such as *Deferribacter desulfuricans, Desulfitobacterium dehalogenans, Pelobacter carbinolicus Desulfovibrio frigidus, Acidilobus sulfurireducens, Desulfurella acetivorans, Thermanaerovibrio acidaminovorans, Thermodesulfobacterium geofontis, Acidilobus sulfurireducens, Caldisphaera lagunensis, Vulcanisaeta distributa, Pyrobaculum islandicum, Methanococcus maripaludis* and *Natronolimnobius innermongolicus*.

A general overview of the enzymes present in reported sulfur reducers is given as supplemental material (Supplemental material—Table 2). A search on the online Joint Genome Institute database (http://img.jgi.doe.gov/) shows that the aforementioned enzymes are present in the genome of many microorganisms not reported so far as sulfur reducers. These potential sulfur-reducing prokaryotes are spread over the tree of life, including some phyla without reported species of sulfur-reducing bacteria, such as *Chloroflexi, Actinobacteria, Nitrospira, Chlorobi* or *Rikenellaceae* (Supplemental material—Figure 1). In *Archaea*, the potential sulfur reducers are spread only over the phyla *Crenarchaeota* and *Euryarchaeota* (Supplemental material—Figure 2), where the reported sulfur reducers are also distributed. Even though some of these microorganisms have been tested and did not show sulfur reduction activity, it is not known whether the conditions applied were optimal for growth and/or sulfur reduction. In some cases, e.g. *Desulfonatronovibrio thiodismutans, Desulfonatronum thioautotrophicum* and *Desulfobotulus alkaliphilus* elemental sulfur reduction occurred in resting cells, but sulfur did not support growth. It is suggested that the reaction between the sulfide produced and elemental sulfur generates polysulfide. Due to its toxicity, the polysulfide produced inhibits growth of some of those microorganisms (Sorokin et al. 2011).

3.4 Reduction of Sulfur via Polysulfide

Analyzing *Sulfurospirillum deleyianum*, formerly called *Spirillum* 5175, Zöphel et al. (1991) showed that the addition of thiols, such as glutathione and sulfide, to the medium facilitated elemental sulfur reduction by the membrane fractions of cell extract; and cleaving of S–S bonds by nucleophilic attack was enhanced, which increased the activity. It has also been suggested that polysulfide chains formed from sulfide and sulfur are intermediates in the reduction of sulfur by cytochrome c_3 of *Desulfovibrio desulfuricans* (Cammack et al. 1984). The sulfide (S^{2-}) formed by reduction of the polysulfide cleaves the S_8^0-ring by nucleophilic attack leading to the generation of new polysulfide molecules, which are quickly reduced to S^{2-} by cytochrome c_3 (Cammack et al. 1984).

Sulfur reduction via polysulfide has been extensively studied in *W. succinogenes*. Macy et al. (1986) reported growth of *W. succinogenes* on formate and elemental sulfur, with H_2S and CO_2 as products. Later, Klimmek et al. (1991) reported growth of *W. succinogenes* with formate and polysulfide.

Ringel et al. (1996) questioned the involvement of polysulfide as intermediate for sulfur respiration in *W. succinogenes* and added Fe^{2+} to the medium to precipitate all the sulfide produced by the bacterium as FeS. In that case, polysulfide formation was prevented. Under the mentioned conditions, anaerobic growth of *W. succinogenes* was observed with formate and elemental sulfur and it was concluded that elemental sulfur was the terminal electron acceptor for sulfur reduction in *W. succinogenes*. Three years later, Hedderich et al. (1999) isolated a soluble sulfur-containing fraction and a periplasmic sulfide dehydrogenase, so-called Sud protein, from the cultures to which Fe^{2+} was added. When they treated the isolated protein with CN^- and thiosulfate, no reaction was observed; but when polysulfide was added to the medium, thiocyanate was formed (Eq. 5.6).

$$S_n^{2-} + CN^- \rightarrow SCN^- + S_{n-1}^{2-} \tag{5.6}$$

The Sud protein was found to be involved in the transfer of sulfur from polysulfide in solution to the catalytic site of the polysulfide reductase (Psr) (Klimmek et al. (1991). The menaquinone present in the Psr is thought to serve as electron acceptor of the hydrogenase in polysulfide/sulfur reduction (Rosenberg et al. 2013). The electron transport chain of polysulfide reduction with hydrogen or formate is composed by polysulfide reductase (Psr) and hydrogenases or formate dehydrogenase. Hydrogenases and polysulfide reductase are assumed to be randomly dispersed in the membrane of *W. succinogenes* (Jankielewicz et al. 1995).

Later studies indicated that 8-methyl-menaquinone is essential for sulfur reduction in *W. succinogenes* (Jankielewicz et al. 1995; Hedderich et al. 1999). As most of the menaquinones are assumed to be dissolved in the lipid bilayer phase of the membrane and to play a role in the transference of electrons by diffusion, this was the first hypothesis for its involvement in the mechanisms of sulfur/polysulfide reduction by *W. succinogenes*. However, the redox potential of the menaquinone dissolved in the membrane is much more positive than that of polysulfide, which makes the electron transfer from formate dehydrogenase to polysulfide reductase mediated by diffusion improbable (Hedderich et al. 1999). Alternatively, the menaquinone is likely bound to polysulfide reductase and is the primary electron acceptor for the cytochrome b subunit of the hydrogenase (Hedderich et al. 1999). Therefore, it is possible that the electron transfer from hydrogenase to polysulfide reductase requires collision or aggregation of the two enzymes within the membrane (Fig. 5.5). As the menaquinone is intramembrane, it is assumed that its reduction is coupled to the uptake of protons from the cytoplasm by the hydrogenase and the oxidation is coupled to proton release at the periplasm, by the polysulfide reductase (Dietrich and Klimmek 2002).

Several genes were subcloned from genomic libraries of *W. succinogenes*, such as *frh* genes, encoding for formate dehydrogenase (Bokranz et al. 1991), psr genes encoding for polysulfide reductase (Krafft et al. 1995), and sud genes encoding for sulfide dehydrogenase (Kreis-Kleinschmidt et al. 1995).

Blumentals et al. (1990) investigated the mechanism of sulfur reduction in the archaeon *P. furiosus.* The authors observed sulfide and polysulfide formation in cultures in which elemental sulfur was physically separated from the microorganism, indicating that contact between the archaeon and elemental sulfur is not necessary

for the metabolism and that soluble polysulfides serve as substrates for sulfur reduction. It is not yet clear whether sulfur reduction in *P. furiosus* is coupled to energy conservation. Sulfur can serve merely as electron sink allowing a more effective fermentation of organic compounds (Rosenberg et al. 2013).

P. furiosus can use protons as terminal electron acceptors, coupling directly the production of H_2 to the synthesis of ATP. The multiprotein membrane bound hydrogenase complex and the ferredoxin, which functions as an electron donor of low-potential, couple the electron transfer to proton reduction and proton translocation (Sapra et al. 2003).

3.5 Reduction of Sulfur via Physical Attachment to Solid Phase

Due to the low solubility of elemental sulfur in water, some microorganisms reduce it at the surface of the outer membrane. The mechanisms adopted by these microorganisms are poorly studied. As some prokaryotes are also able to reduce insoluble mineral-oxides outside the membrane (Lovley 1991; Lovley et al. 2004; Hartshorne et al. 2009), different strategies for electron transfer have been proposed, which can be related to sulfur reducers.

For example, in species of the iron-reducing genera Shewanella and Geobacter, in which some sulfur reducer members can be found, external insoluble iron oxides reduction is reported to happen by four different mechanisms: (1) cytochrome c extends the respiratory chain to the cell surface (Richardson 2000; Lovley et al. 2004; Richter et al. 2012); (2) extracellular redox mediators, such as humic acids, quinones, phenazines and cysteine, can shuttle electrons between the terminal electron donor of the electron transport chain and the insoluble acceptor (Lovley et al. 1998; Scott et al. 1998; Newman and Kolter 2000; Hernandez and Newman 2001); (3) in the absence of cytochrome c, microorganisms can produce modified pili, so-called nanowires, that can serve as an electrical connection between the cell and the surface of the oxides (Reguera et al. 2005); and, some strains can construct electrically conductive networks with nanoparticles of crystalline, conductive or semiconductive minerals, such as iron oxides (Kato et al. 2010).

Some microorganisms are reported to reduce elemental sulfur directly to sulfide, such as *A. ambivalens, A. ferrooxidans, Pyrodictium abyssi* and *Pyrodictium brockii*, from which several studies were performed and are here summarized.

Hydrogenase, quinone and cytochrome c were detected in membranes of *P. brockii* (Pihl and Maier 1991; Pihl et al. 1992). The purified hydrogenase is of the Ni-Fe type, with two subunits (Pihl and Maier 1991). Even though the quinone in this microorganism shows chromatographic properties of migration like ubiquinone-6, nuclear magnetic resonance analysis performed by Pihl et al. (1992) evidentiated a quinone different from all the compared quinones. When the quinone was inactivated by exposition to UV light, the electron transport activity was inactivated. The addition of quinone reactivated the process, implying that the electron transfer sequence is: hydrogenase → quinone → cytochrome c. With this, cytochrome c is supposed to be the electron donor for the not yet identified sulfur reductase.

Dirmeier et al. (1998) isolated a sulfur-oxidoreductase complex from the membrane fraction of *P. abyssi* isolate TAG11 and showed that the electron transport chain that catalyzes sulfur reduction by hydrogen is different from *P. brockii* in composition and organization of its constituents. The complete respiratory chain of the organism is suggested to be represented by an enzyme multi-complex, in which the components of the electron transport, the hydrogenase and the sulfur reductase are consistently arranged. The reductase is composed by at least nine subunits, with two b-type cytochromes and one c-type. No quinone has been detected in the membrane fraction complex enzyme of *P. abyssi.* The presence of nickel in the sulfur-oxidoreductase indicates that its hydrogenase is of the Ni-Fe type (Rosenberg et al. 2013), as for *P. brockii*.

A sulfur reductase purified from *A. ambivalens* was shown to reduce elemental sulfur with hydrogen as electron donor in the presence of a co-purified hydrogenase, with a quinone as electron carrier (Laska et al. 2003). The hydrogenase has similar main subunits as the hydrogenase purified from *W. succinogenes*, one homologous Ni-containing catalytic subunits (HynL/HydB), one homologous Fe-S containing electron transfer subunit (HynS/HydB) and one non-homologous membrane anchor (IspI/HydC) (Laska et al. 2003). Thus, the electron transport chain in this microorganism is most likely composed of the two enzymes connected by quinones (Fig. 5.6).

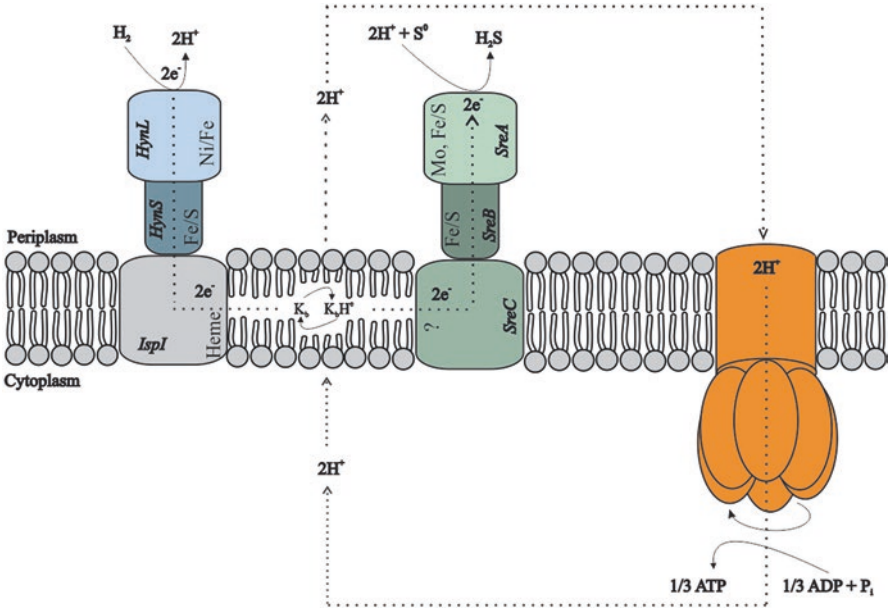

Fig. 5.6 Hypothetical view of elemental sulfur reduction and anaerobic electron transport chain in *A. ambivalens.* Protons are assumed to be translocated to the periplasm via quinone. Only major structural subunits are represented. Subunits of the hydrogenase are labeled HynL, HynS and IspI and subunits of the sulfur reductase are labeled SreA, SreB and SreC. K stands for quinone. Model adapted from Laska et al. (2003)

As the net balance of protons from the periplasmic reactions is zero, an electrochemical gradient is most likely generated with protons taken up by quinone from the cytoplasm and released at the periplasm.

4 Mechanisms of Adaptation to Acidic Conditions

Many sulfur-reducing microorganisms prefer neutral pH to grow. Nonetheless, several species that are capable to thrive in acidic environments have been identified (Stetter 1996; Hedderich et al. 1999; Yoneda et al. 2012) (Supplemental material—Table 1). Those species of acidophiles or acidotolerants tolerate larger pH gradients across the cytoplasmic membrane than neutrophilic organisms. These microorganisms normally face a proton motive force beyond the cell membrane which can drive energy dependent processes to promote pH homeostasis (Baker-Austin and Dopson 2007). To maintain a physiological pH despite the external acidic conditions, microorganisms adopt several strategies. Baker-Austin and Dopson (2007) presented an extremely valuable review on the pathways and mechanisms proposed that enable microorganisms to thrive at low pH, which are summarized in this section, such as utilization of specific transporters and enzymes for proton export, adoption of particular permeability properties, increment of buffer capacity and enhancement of positive surface charges.

In general, acidophiles and acidotolerants have a highly impermeable cell membrane or low membrane fluidity to restrict proton influx to the cytoplasm (Benjamin and Datta 1995; Dilworth and Glenn 1999; Konings et al. 2002). The membranes of some acidophilic archaea are composed of tetraether lipids which make them rather impermeable to protons. Additionally, ether linkages are less sensitive to acid hydrolysis than ester linkages, commonly found in bacterial and eukaryotic cell membranes (Macalady and Banfield 2003; Golyshina and Timmis 2005). Moreover, the lipids from the membranes are also characterized by a substantially higher content of glycolipids, in which one or more sugar units are exposed at the outer surface of the cell (De Rosa et al. 1983; Chong 2010). Although there is still a lack of direct evidence, it was suggested that the abundant modifications of sugar on the cell surface of archaea can provide a protection against proton influx (Shimada et al. 2008; Wang et al. 2012)

Reduction of the size and permeability of the membrane channels is another mechanism for pH homeostasis in acidophiles. The membrane pore reduces its size and the selection of ions to enter the porin occurs based on their charge and size (Amaro et al. 1991).

Another mechanism adopted by acidophiles to reduce the influx of protons is the maintenance of a difference in electrical potential between the intra and extracellular environment without current coursing through the membrane, developing an inside positive $\Delta\Psi$ against the inside negative $\Delta\Psi$ in neutrophiles, the so-called Donnan potential. This Donnan potential is probably generated by a greater influx of potassium ions. The importance of this mechanism is suggested by a very high

number of putative cation transporters identified in the genomes of several acidophiles, including some related to sulfur cycle, such as *Acidithiobacillus thiooxidans* (Suzuki et al. 1999), *Acidithiobacillus caldus* (Dopson et al. 2002), *A. ferrooxidans* (Cox et al. 1979) and *Acidiphilium acidophilum* (Goulbourne et al. 1986).

Proton efflux pump systems, such as proton ATPases, antiporters and symporters (Box 5.2), are also used by some acidophiles to maintain the pH homeostasis (Tyson et al. 2004; Golyshina and Timmis 2005; Baker-Austin and Dopson 2007). Protons that enter the cell must be balanced by extrusion during electron transport and reduction of terminal electron acceptors.

Box 5.2

Antiporters: integral membrane proteins that actively transport a substance through the membrane, while transporting ions in the opposite direction. The ions, typically hydrogen (H^+) or sodium (Na^+) ions, flow down their concentration gradient, and in doing so provide the energy for the transport of another substance in the other direction.

Symporters: integral membrane proteins that simultaneously transports two substances across membrane in the same direction. Often, one molecule can move up an electrochemical gradient because the movement of the other molecule is more favourable.

The cytoplasm of all microbes presents a buffering capacity (Box 5.3) to sequester or release protons, according to the shifts in pH. Amino acids or other small organic molecules and ionizable groups in proteins and inorganic polymers, such as polyphosphates, have this buffering capacity (Slonczewski et al. 1982; Zychlinsky and Matin 1983; Krulwich et al. 1985; Leone et al. 2007). Zychlinsky and Matin (1983) compared the buffering capacity of *Acidiphilium acidophilum* and *Escherichia coli* and the result showed a slightly higher capacity for the acidophile, 97 and 85 mmol H^+ per pH unit, respectively. It was also found by Krulwich et al. (1985) that *Bacillus acidocaldarius* has a higher buffering capacity (around 600 mmol H^+ per pH unit) than other bacilli in neutrophilic conditions (around 400–550 mmol H^+ per pH unit). However, the results obtained in both studies show that the buffering capacity of the acidophiles is not necessarily higher than their counterpart of neutrophiles. This suggests that the buffering capacity can contribute to pH homeostasis only together with other mechanisms.

Box 5.3

Buffering capacity: It is the ability of a solution to resist to changes in pH by either absorbing or desorbing H^+ and OH^- ions. It is represented by the moles of an acid or base needed to change the pH of a solution by 1, divided by the pH change and the volume of buffer.

The low pH of the environments can damage biomolecules in the cell, which requires repair mechanisms. This can explain the great number of DNA and protein repair genes present in the genomes of several acidophiles (Crossman et al. 2004). At low pH, chaperones involved in protein refolding are highly expressed in a wide range of acidophiles, suggesting that they can play a role in the survival of microorganisms under acidic conditions.

Investigation on *Ferroplasma acidiphilum*, an obligate acidophile with an intracellular predicted pH of 5.6 during active growth, showed that several enzymes were functional at pH values in a range of 1.7–4.0, suggesting that they need to be functional to get the metabolism started when the cells grow at extreme low pH values. It has been detected a higher amount of iron proteins in the proteoms of many acidophiles which contributes to the pH stability of enzymes at low pH (Ferrer et al. 2007). The removal of iron from purified proteins of these acidophiles makes them to lose the secondary structure of the proteins and, therefore, their activity. Iron is then thought to play an important role on the maintenance of three dimensional structures of the proteins and then serves as an iron rivet—an early property that has a role in stabilizing proteins in acidic condition (Ferrer et al. 2007).

Most of the organic acids, such as acetic and lactic acid facilitate transfer of protons across the membrane at low pH. In this condition, there is a diffusion of acids in protonated form into the cell and consequently the protons dissociate in the cytoplasm, where the pH is higher (Baker-Austin and Dopson 2007). Therefore, the organic acid degradation ability in some acidophiles can play a detoxifying role.

5 Biotechnological Application

5.1 Industrial Wastes and Acid Mine Drainage

The biological oxidation of sulfidic minerals and formation of acidic metal-rich mine drainage waters have been described in several studies (Hoffert 1947; Johnson 1995, 2003). Briefly, due to their exposure to oxidants (O_2 or Fe^{3+}), the geobiochemical oxidation of metal sulfides such as pyrite is the root cause of acid mine drainage (AMD) (Johnson and Hallberg 2005). In most situations, ferric iron is the primary oxidant which chemically oxidize the ores (Eqs. 5.7 and 5.8) and its biological regeneration (Eq. 5.9) maintains the open-ended oxidation of the mineral (Schippers and Sand 1999; Johnson and Hallberg 2005; Vera et al. 2013) and the acidic environment formation, in which metals are commonly dissolved.

$$FeS_2 + 6Fe^{3+} + 3H_2O \rightarrow S_2O_3^{2-} + 7Fe^{2+} + 6H^+ \tag{5.7}$$

$$S_2O_3^{2-} + 8Fe^{3+} + 5H_2O \rightarrow 2SO_4^{2-} + 8Fe^{2+} + 10H^+ \tag{5.8}$$

$$4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O \tag{5.9}$$

Copper, zinc, cadmium, arsenic, manganese, aluminium, lead, nickel, silver, mercury, chromium and iron are metals of remarkable interest in acid mine drainage and industrial wastewaters, as they can be present in a wide range of concentration, from 10^{-6} to 10^{2} g L^{-1} (Huisman et al. 2006). As examples, in Tinto River, a natural acidic rock drainage, iron can be detected up to 20.2 g L^{-1}, copper up to 0.7 g L^{-1}, and zinc up to 0.56 g L^{-1} (Lopez-Archilla et al. 2001); while in the effluent of a textile industry iron was detected up to 0.11 g L^{-1}, and copper and zinc up to 0.01 g L^{-1} (Joshi and Santani 2012).

5.2 State of the Art Methods for Metal Removal and Recovery

5.2.1 Chemical/Physical Methods

Many chemical/physical methods have been applied to remove heavy metals from contaminated wastewaters, such as absorption, ion exchange, complex formation and precipitation by addition of chemicals, which is the most widely applied chemical/physical approach for the treatment of acid mine drainage (AMD) and other metal-contaminated streams (Johnson and Hallberg 2005).

To raise the pH and consequently precipitate metals in a mitigation process, some neutralizing agents are added to the medium, such as calcium carbonate, calcium oxide, calcium hydroxide or sodium hydroxide (Weijma et al. 2002). Despite effective treatments, these methods are relatively expensive and produce large volumes of residual metal-contaminated sludge with no or low metal reuse potential (Gallegos-Garcia et al. 2009; Tekerlekopoulou et al. 2010).

5.2.2 Microbiological Methods

Microbial processes, such as methanogenesis, denitrification, and reduction of iron and manganese, generate alkalinity, which may result in metal precipitation as hydroxides (Johnson and Hallberg 2005). Even though hydroxides can be removed from the effluent, as all the metals precipitate together, the generated waste needs to be disposed, which results in extra costs of the process. Metals may also be recovered bioelectrochemical systems, where an organic substrate is biologically oxidized at the anode, thereby generating electrons which are used to reduce metals like Cu^{2+} at the cathode (Heijne et al. 2010). Much research in the past used the concept of metal biosorption, i.e. the adsorption of metal ions to the surface of biological matter such as bacterial cells and plants. This method is not widely applied, presumably due to the low metal loading capacity and the production of a residue from which metal recovery is hardly feasible.

Bioreactors systems to precipitate metals based on sulfidogenesis are as effective as the physical methods while operating at substantially lower costs and producing lower amounts of residual sludge (Johnson and Hallberg 2005). Sulfidogenesis is

based on the oxidation of simple organic compounds or hydrogen by microorganisms under anaerobic conditions, generating sulfide from the reduction of sulfur compounds, such as sulfate, sulfite, thiosulfate, organic sulfoxides, elemental sulfur, polysulfide, and organic disulfides. The versatility of sulfidogenic microorganisms allows for many combinations of electron donor and sulfur sources, and also for a wide range of operational conditions for the process (temperature, salinity, pH).

5.3 Sulfidogenesis for Metal Removal and Recovery

In sulfidogenic processes for metal removal and recovery, the biologically produced sulfide binds to dissolved heavy metals, such as Cu^{2+}, Zn^{2+}, and Ni^{2+} precipitating as insoluble metal sulfides (Hulshof et al. 2006; Neculita et al. 2007). The theoretical solubility of most metal sulfides at neutral to alkaline pH is extremely low, much lower than that of the corresponding metal hydroxides. Thus, better effluent qualities can be reached and more metal can be recovered. Also the reactions rates are higher and the acid-stable metal sulfides, such as Co, Ni and Cu, present good settling properties and high potential for re-use (Tsukamoto et al. 2004; Gallegos-Garcia et al. 2009; Lewis 2010; Sánchez-Andrea et al. 2014). Smelter facilities for base metal production use ore concentrates that often contain the metal in their sulfidic mineral form, such as sphalerite in the case of ZnS. This facilitates the use of biologically precipitated metals sulfides as feedstock for smelters. For ZnS, this is practised at the zinc refinery of Nyrstar in The Netherlands (Weijma et al. 2002).

Sulfate reduction is the most used biological process for the treatment of mining and metallurgical streams. However, there are only a few described species of moderate acidophilic sulfate-reducing bacteria: *Thermodesulfobium narugense*, which can grow at pH 4 (Mori et al. 2003), *Desulfosporosinus acidiphilus,* which can grow at pH 3 (Jameson et al. 2010), and *Desulfosporosinus acididurans*, which can grow at pH 3.8 (Sánchez-Andrea et al. 2015). The use of biogenic sulfur is of particular interest for the treatment of acid mine and acid rock drainage (Hoffert 1947; Johnson 1995, 2003).

For treatment of metal-contaminated streams such as acid mine/rock drainage, two designs of sulfidogenic bioreactors have been proposed. One is based on a biological and a chemical compartment operating independently (Tabak et al. 2003). In the biological compartment, hydrogen sulfide is produced and transferred via a gas circulation to the chemical circuit, which receives the raw influent (Fig. 5.7a). Thus, the biological production of sulfide and the precipitation of metals are separated by stripping hydrogen sulfide from the biological solution with a carrier gas (nitrogen) and then the hydrogen sulfide gas dissolves in the metal-contaminated (waste)water. In this device, there is no contact between the sulfidogenic biomass and the metal-contaminated stream. This is the major advantage of this design because it prevents possible biomass toxicity effects due to high acidity and metal concentrations (Johnson and Hallberg 2005). The drawback is that the carrier gas recycle requires a high energy input. This technique has been studied with metals like Cu and Zn

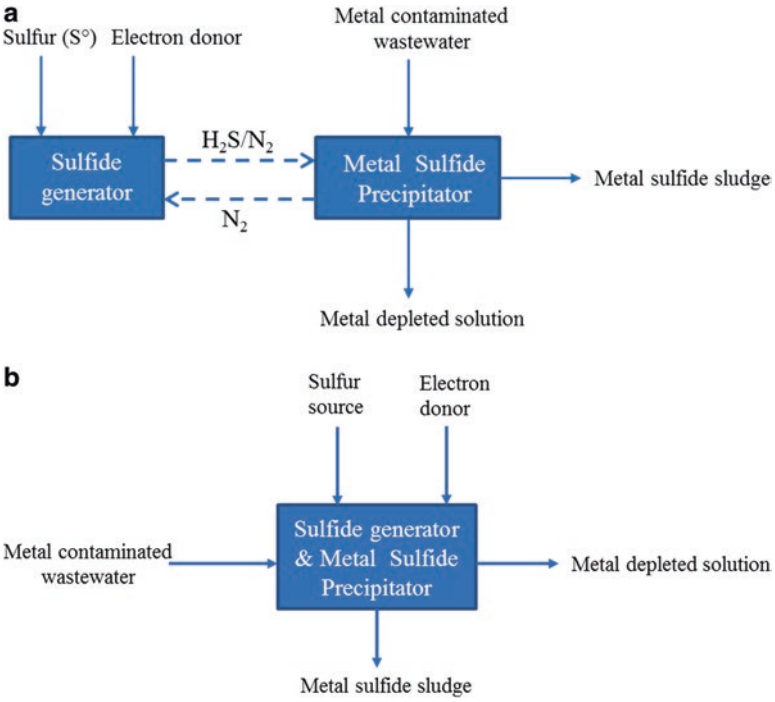

Fig. 5.7 Flowsheet for two-stage biological metal removal with no direct contact between the sulfidogenic microorganisms and the metal-contaminated wastewater (**a**). One-stage biological metal removal with direct contact between the sulfidogenic microorganisms and the metal-contaminated wastewater (**b**)

(Foucher et al. 2001; Al-Tarazi et al. 2005; Gramp et al. 2009). Because of the separate sulfide production and metal sulfide precipitation, both process parts can be controlled at their optimal conditions. For example, selective precipitation of individual heavy metals can be achieved by carefully controlling the pH and the pS ($-\log[S^{2-}]$) in the precipitator (Veeken and Rulkens 2003; König et al. 2006; Sampaio et al. 2009). These results in relatively pure precipitates of metal sulfides that have a higher value as supplement to ore concentrate feedstock in the metallurgical industry (Grootscholten et al. 2008).

The other designed system has only one compartment, in which biological sulfide production and metal precipitation occur simultaneously (Fig. 5.7b).

In this configuration, since the sulfidogenic culture comes into contact with the dissolved metals from the influent, metal toxicity is a design and operation concern. By keeping some excess of hydrogen sulfide relative to the metals, a 'sulfide buffer' is created that can accommodate fluctuations in metal loading and biological activity. The advantage of this configuration is that sulfide generation and metal sulfide precipitation take place in a single unit, thereby eliminating the need for energy-intensive recirculation of a carrier gas. This flow scheme has been studied by amongst others (Labrenz et al. 2000; Steed et al. 2000; Kaksonen et al. 2003; Johnson and Hallberg 2005;

Sierra-Alvarez et al. 2006; Gallegos-Garcia et al. 2009; Sánchez-Andrea et al. 2012). Full-scale operations for biogenic sulfide production are described in Weijma et al. (2002) and Möbius et al. (2015).

5.4 Comparative Analysis of Cost Between Sulfate and Sulfur Reduction Processes

Wastewater from mining or metals industries contains, normally, low organic matter content. To completely reduce the sulfur compounds to sulfide, electron donors need to be added (Liamleam and Annachhatre 2007). Based on the stoichiometry of the reactions, elemental sulfur is more attractive as electron acceptor than sulfate, since only two electrons per mol of sulfide produced are needed in the process (Eq. 5.10), instead of eight needed for sulfate (Eq. 5.11). The sulfide produced determines the amount of metals to be recovered (Eq. 5.12), therefore with the same amount of metal precipitated, the process needs four times less of electron donor for sulfur reduction in comparison with sulfate reduction.

Hedrich and Johnson (2014) performed analysis of costs for modular reactors to oxidize iron and reduce sulfate to precipitate metals operating at low pH. The 42 m^3 sulfidogenic reactor needed to treat 1 m^3 mine water operated with glycerol as electron donor, would produce 3.96 mol of sulfide. As the stoichiometric reaction of glycerol with sulfate is 4–7 (Eq. 5.12), 2.26 mol (208.52 g) of glycerol would be required in the reactor. Assuming the market price of glycerol as 2400 $/ton, the cost of this reagent in the process result on 0.5 $, as described in the article.

If instead of sulfate, sulfur is applied as electron acceptor, to reach the same amount of sulfide in a 42 m^3 reactor, an input of 0.126 kg of sulfur is required. As an estimated market price of sulfur of 61 $/ton, an additional cost of 0.008 $ is needed in the process. However, as sulfur reduction requires four times less electron donors (Eq. 5.13), the same amount of sulfide is reached with only 52.13 g of glycerol, implying a global reduction in costs of $ 0.37 per m^3 of mine water treated.

In accordance with Eq. (5.11) and as expressed in Hedrich and Johnson (2014), with the amount of sulfide produced via sulfur or sulfate reduction, 0.46 kg can be recovered, which represents about 0.80 $ of return per m^3 of mine water treated. Considering copper, which is common in acid mine drainage, 0.46 kg Cu recovered would imply 2.71 $ of return per m^3 of mine treated, taking 5900 $/ton as an average market price of copper.

Another advantage of implementing elemental sulfur reduction for remediation of AMD streams is that sulfur reducers can generally reduce elemental sulfur at pH values lower than the so far described sulfate reducers. Sulfur reduction is reported in extremely acidophilic microorganisms, such as *A. ferrooxidans* (pH 1.8) (Osorio et al. 2013), *Acidilobus sulfurireducens* (pH 2) (Boyd et al. 2007), *Acidianus infernus* (pH 1.5) (Stetter 1996), *Stygiolobus azoricus* (pH 1) (Svetlichnyi et al. 1987; Stetter 1996), *Thermoplasma acidophilum* and *volcanicum* (pH 1) (Segerer et al. 1988). The lowest reported pH for sulfate reduction by isolates is 3.6–3.8 by members of *Desulfosporosinus* genus (Alazard et al. 2010;

Sánchez-Andrea et al. 2015) and Ňancucheo and Johnson (2012) reported activity at a pH as low as 2.5 in bioreactors.

$$\text{Half reaction}: H_2 \rightarrow 2e^- + 2H^+$$

$$\text{Half reaction}: S + 2e^- \rightarrow S^{2-}$$

$$\textit{Global reaction}: S + H_2 \rightarrow S^{2-} + 2H^+ \tag{5.10}$$

$$\text{Half reaction}: 4H_2 \rightarrow 8e^- + 8H^+$$

$$\text{Half reaction}: SO_4^{2-} + 8e^- + 8H^+ \rightarrow S^{2-} + 4H_2O$$

$$\textit{Global reaction}: SO_4^{2-} + 4H_2 \rightarrow S^{2-} + 4H_2O \tag{5.11}$$

$$H_2S + Me^{2+} \rightarrow MeS_{(s)} + 2H^+ \tag{5.12}$$

where Me^{2+} = metal, such as Zn^{2+}, Cu^{2+}, Pb^{2+} and Ni^{2+}

$$4C_3H_8O_3 + 7SO_4^{2-} \rightarrow 7H_2S + 12CO_2 + 16H_2O \tag{5.13}$$

$$C_3H_8O_3 + 7S + 3H_2O \rightarrow 7H_2S + 3CO_2 \tag{5.14}$$

Additionally, many sulfate reducers are incomplete oxidizers (e.g.: *Desulfotomaculum* sp., *Desulfobulbus* sp., *Archaeoglobus* sp. (Castro et al. 2002), *Desulfovibrio* sp., *Thermodesulfobacterium* sp. (Widdel and Pfennig 1981; Widdel 1988; Widdel and Pfennig 1991), *Desulfosporosinus* sp. (Sánchez-Andrea et al. 2015) which means that they contribute to the accumulation of acetic acid in the medium, with the consequent possible inhibition of the process. This is not the case for most of the sulfur reducers, especially the ones belonging to the *Deltaproteobacteria* class, which are able to oxidize organic substrates to CO_2, such as *Desulfuromonas* sp., *Geobacter* sp., *Pelobacter* sp. *and Desulfurella* sp. the latter ones are usually found in acid environments (Bonch-Osmolovskaya et al. 1990b; Miroshnichenko et al. 1998).

Sulfur reduction looks more promising for treatment of metal-laden streams in metallurgical processes, which are free of sulfate, often acidic and sometimes hot. However, for obvious reasons such as the natural presence of sulfate in AMD water, sulfate reduction might be still the easiest option for *in situ* systems such as permeable reactive barriers.

6 Concluding Remarks and Future Perspectives

Microorganisms involved in the sulfur cycle are of great importance from the industrial and environmental point of view, especially the ones that perform sulfidogenesis. Sulfur-reducing prokaryotes are ubiquitously distributed in marine and terrestrial

environments and able to grow in a broad range of temperature and pH. Species able to thrive in acidic environments are of interest for selective metals precipitation and bioremediation processes.

Several acidophilic sulfur reducers were described but their physiology and specific mechanisms adopted to face extreme conditions are still poorly understood. Ongoing and future research on these microorganisms will provide more insight into the real substrate used by sulfur reducers, physiology and ecology of those microorganisms and their behavior in engineered ecosystems such as reactors for the selective precipitation and recovery of heavy metals from mining and metallurgical industries.

Acknowledgements The doctoral study program of A.P. Florentino is supported by the organization of the Brazilian Government for the development of Science and Technology CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico). Research of I. Sánchez-Andrea and A.J.M. Stams is financed by ERC grant project 323009 and by Gravitation grant project 024.002.002 from the Netherlands Ministry of Education, Culture and Science.

Conflict of Interest Anna P. Florentino, Jan Weijma, Alfons J. M. Stams, and Irene Sánchez-Andrea declare that they have no conflict of interest.

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