The ecology and biotechnology of sulphate-reducing bacteria

Gerard Muyzer and Alfons J. M. Stams‡*

Abstract | Sulphate-reducing bacteria (SRB) are anaerobic microorganisms that use sulphate as a terminal electron acceptor in, for example, the degradation of organic compounds. They are ubiquitous in anoxic habitats, where they have an important role in both the sulphur and carbon cycles. SRB can cause a serious problem for industries, such as the offshore oil industry, because of the production of sulphide, which is highly reactive, corrosive and toxic. However, these organisms can also be beneficial by removing sulphate and heavy metals from waste streams. Although SRB have been studied for more than a century, it is only with the recent emergence of new molecular biological and genomic techniques that we have begun to obtain detailed information on their way of life.

Chemolithotropic
Metabolism of an organism that obtains energy from inorganic compounds and carbon from carbon dioxide.

**Department of Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands.*
‡Laboratory of Microbiology, Wageningen University, Dreijenplein 10, 6703 HB, Wageningen, The Netherlands.
Correspondence to G.M.
e-mail: g.muyzer@tudelft.nl
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Sulphur is among the most abundant elements on the Earth. It is mainly present as pyrite (FeS_2) or gypsum ($CaSO_4$) in rocks and sediments and as sulphate in seawater. The sulphur cycle (FIG. 1) is complex, because sulphur has a broad range of oxidation states, from –2 (completely reduced) to +6 (completely oxidized), and can be transformed both chemically and biologically. In addition, the sulphur cycle is closely linked to other element cycles, such as the carbon and nitrogen cycles.

Microorganisms play an important part in sulphur transformations (FIG. 2). Sulphate is taken up as a nutrient and reduced to sulphide, which is then incorporated into sulphur-containing amino acids and enzymes. Oxidation and reduction reactions for the generation of metabolic energy are also important, such as sulphide oxidation by chemolithotropic sulphur bacteria and dissimilatory sulphate reduction by sulphate-reducing bacteria (SRB). Because members of the Bacteria and Archaea can use sulphate as a terminal electron acceptor, some researchers use the term sulphate-reducing prokaryotes or sulphate-reducing microorganisms. In this Review, however, we use the term SRB to refer to members of both domains.

SRB are anaerobic microorganisms that are widespread in anoxic habitats, where they use sulphate as a terminal electron acceptor for the degradation of organic compounds, resulting in the production of sulphide. Subsequently, the sulphide can be oxidized under oxic conditions by chemolithotrophic sulphur bacteria or under anoxic conditions by phototrophic sulphur bacteria. It has been estimated that sulphate reduction can account for more than 50% of the organic carbon mineralization in marine sediments[1], which indicates the importance of sulphate reducers in both the sulphur and carbon cycles and, consequently, why SRB have been studied extensively[2]. In this Review, we provide an overview of the diversity, physiology and distribution of SRB and their applications in environmental biotechnology for the removal of heavy metals and sulphur compounds from waste water and flue gas.

Physiology of SRB

Electron-donor metabolism. Until the early 1980s, it was thought that sulphate reducers played only a minor part in the carbon cycle. The *Desulfovibrio* and *Desulfotomaculum* species that were known at that time used hydrogen and a number of organic compounds, such as ethanol, formate, lactate, pyruvate, malate and succinate, for growth. Typically, carbon compounds are incompletely oxidized to acetate by these SRB. However, through the research of Fritz Widdel at the University of Göttingen, Germany, it became clear that, particularly in marine sediments, SRB are the main players in anaerobic carbon cycling. Widdel[3] isolated and characterized a large number of novel sulphate reducers that had the ability to grow on short-chain fatty acids (including acetate), long-chain fatty acids and aromatic compounds, such as benzoate and phenol. Currently, sulphate reducers can be divided into two main groups: those that degrade organic compounds incompletely to acetate and those that degrade organic compounds completely to carbon dioxide. Sulphate reducers that degrade organic

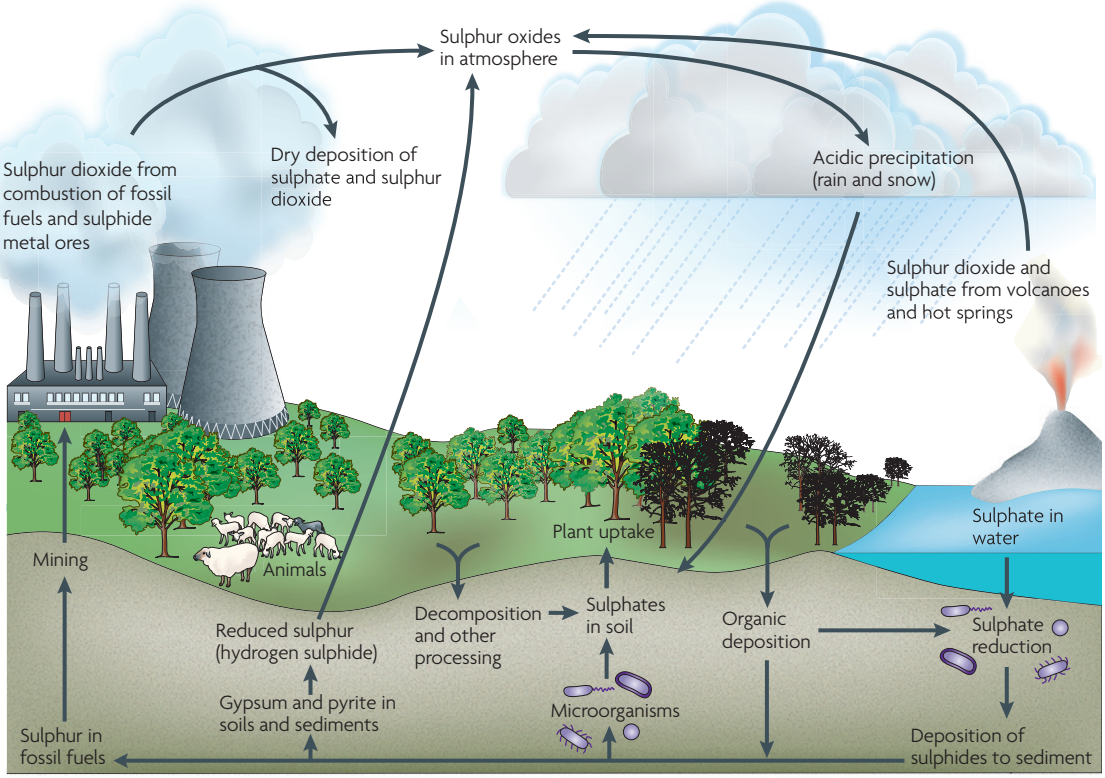


Figure 1 | **The sulphur cycle.** The largest sulphur reservoirs on the Earth are iron sulphides (pyrite; FeS_2) and gypsum ($CaSO_4$) in sediments and rocks (7,800 x 10^{18} g sulphur) and sulphate in seawater (1,280 x 10^{18} g sulphur). Sulphur, which is a necessary element for life, is taken up as sulphate by microorganisms and plants, and subsequently by animals. Decomposition of dead organisms in the absence of oxygen releases the sulphur again as hydrogen sulphide. The combustion of fossil fuels and emission of volcanic fumes releases sulphur dioxide into the atmosphere, where it reacts with water, thereby forming sulphuric acid and resulting in acid rain. Microorganisms play an important part in the recycling of these sulphur compounds.

compounds completely to carbon dioxide commonly also use acetate as a growth substrate and two different pathways for acetate oxidation are employed, a modified citric acid cycle, as used by *Desulfobacter postgatei*[4], and the acetyl-CoA pathway, as used by, for example, *Desulfobacterium*, *Desulfotomaculum* and *Desulfococcus* species[5] and *Desulfobacca acetoxidans*[6].

A huge range of novel sulphate reducers have been described over the past 25 years that have the ability to grow on various different substrates, including sugars[7,8], amino acids[9,10] and one-carbon compounds, such as methanol[11,12], carbon monoxide[13,14] and methanethiol[15]. SRB can also grow by the dismutation of thiosulphate, sulphite and sulphur, which results in the formation of sulphate and sulphide[16,17]. In addition to benzoate and phenol, aromatic hydrocarbons (for example, toluene and ethylbenzene) are also degraded by a number of SRB[18–20]. Recently, SRB that can grow on long-chain alkanes[21–24], alkenes[25] and short-chain alkanes[26] have also been described. Typically, polymeric organic compounds, such as starch, cellulose, proteins, nucleic acids (DNA and RNA) and fats are not direct substrates for SRB. Therefore, in nature, SRB are dependent on other microorganisms that degrade these polymeric substrates and ferment them to products that are substrates for SRB (FIG. 3a).

The anaerobic oxidation of methane can be coupled to sulphate reduction, as proposed by Reeburgh[27] in 1976. Much research has been done to unravel the microbiology of sulphate-dependent methane oxidation. There is solid evidence that this process is carried out by syntrophic communities of archaea, which perform reverse methanogenesis, and SRB that oxidize the intermediates formed by the archaea[28–32], the identities of which are still unknown. Initially, one intermediate was thought to be hydrogen[28]; however, research by Nauhaus *et al.*[33] excluded hydrogen, formate, methanol and acetate as intermediates. The option of methyl sulphide as an intermediate has also been proposed[34]. Typically, archaea are phylogenetically most closely related to the *Methanosarcina* genus and the sulphate reducers to the *Desulfosarcina–Desulfococcus*, *Desulfobulbus* or *Desulfobacter* genera[29,35–37]. However, successful attempts to enrich these SRB from methane-oxidizing sediments have not yet been reported.

Electron-acceptor metabolism. Sulphate reducers use sulphate as the terminal electron acceptor for growth. However, from a chemical viewpoint, sulphate is an unfavourable electron acceptor for microorganisms. The $E^{0\prime}$ of the redox couple sulphate–sulphite is –516 mV,

Citric acid cycle
A cyclic series of reactions that result in the conversion of acetate to carbon dioxide and NADH.

Acetyl-CoA pathway
A pathway of autotrophic carbon dioxide fixation and acetate oxidation in obligate anaerobes.

Dismutation
The splitting of a chemical compound into two new compounds, one that is more oxidized and one that is more reduced than the original compound.

Syntrophic
Growth of two or more organisms that depend on each other for their growth.

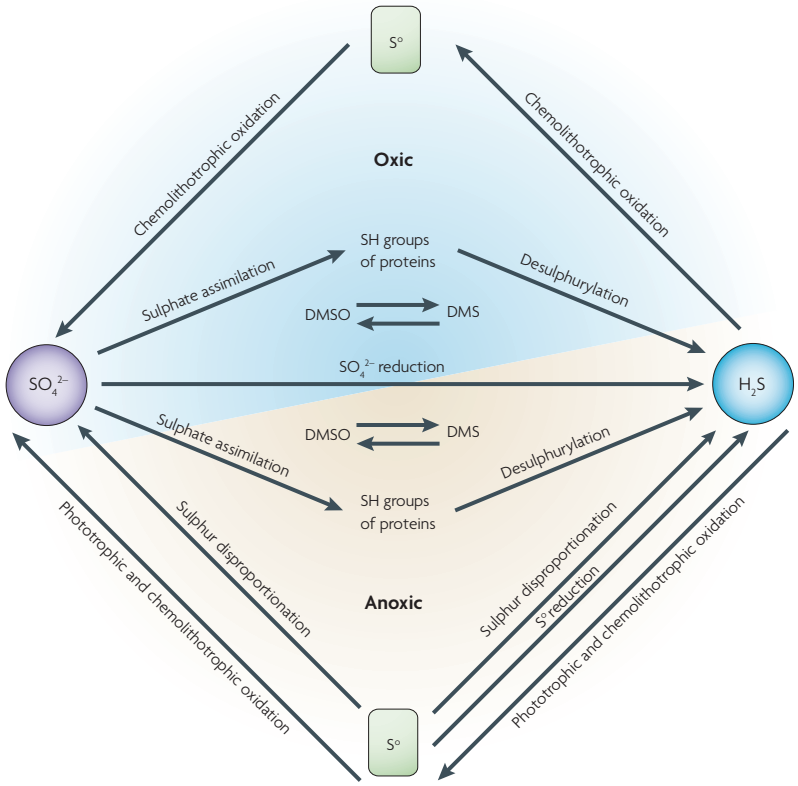


Figure 2 | **Sulphur transformations.** Sulphate-reducing bacteria have a key role in the sulphur cycle. They use sulphate (SO_4^{2-}) as a terminal electron acceptor in the degradation of organic matter, which results in the production of hydrogen sulphide (H_2S). Subsequently, the sulphide can be oxidized aerobically by chemolithotrophic sulphur-oxidizing bacteria (for example, *Thiobacillus* or *Beggiatoa* spp.) or anaerobically by phototrophic sulphur bacteria (for example, *Chlorobium* spp.) to elemental sulphur (S°) and SO_4^{2-}. Other transformations, which are carried out by specialized groups of microorganisms, result in sulphur reduction (for example, *Desulfuromonas* spp.) and sulphur disproportionation (*Desulfovibrio sulfodismutans*). Organic sulphur compounds, such as dimethylsulphoxide (DMSO) can be transformed into dimethylsulphide (DMS) and vice versa by several groups of microorganisms. SH, sulfhydryl. Figure modified, with permission, from REF. 148 (2006) Pearson Education.

which is too negative to allow reduction by the intracellular electron mediators ferredoxin or NADH ($E^{0\prime}$ of −398 mV and −314 mV, respectively) that are present in sulphate reducers. Therefore, before reduction, sulphate is activated by an ATP sulphurylase, resulting in the formation of adenosine-phosphosulphate (APS) and pyrophosphate, which is hydrolysed by pyrophosphatase to 2-phosphate. The $E^{0\prime}$ of the redox couple APS–sulphite plus AMP is −60 mV, which allows the reduction of APS with reduced ferredoxin or NADH. AMP, which is formed by the reduction of APS, is converted by ATP-dependent adenylate kinase into two molecules of ADP. Thus, the activation of sulphate occurs at the expense of two ATP molecules. Sulphite is further reduced to sulphide; the $E^{0\prime}$ of the redox couple sulphite–sulphide is −116 mV, but how sulphite is reduced to sulphide is not yet clear. A pathway through trithionate and thiosulphate would allow a reduction in three two-electron reduction steps, but a reduction in one six-electron reduction step still cannot be excluded[38,39].

Substrate-level phosphorylation
Synthesis of high-energy phosphate bonds through the reaction of inorganic phosphate with an activated organic substrate.

As many SRB are able to grow on hydrogen and sulphate as sole energy substrates, it is clear that sulphate reduction results in electron-transport phosphorylation. More than two ATP molecules need to be synthesized by electron-transport phosphorylation to compensate for the loss of ATP that is necessary for sulphate activation. By comparing yields of a *Desulfovibrio* strain grown with hydrogen and sulphate or hydrogen and thiosulphate, a net yield of one ATP molecule per sulphate reduced was proposed by Badziong and Thauer[40]. Taking into account the energy costs for the uptake of sulphate, the net yield would therefore be one-third of an ATP molecule or one-quarter of an ATP molecule per sulphate reduced[41]. When a *Desulfovibrio* strain is growing on lactate, substrate-level phosphorylation also occurs. The observation that hydrogen is formed when SRB are growing on lactate plus sulphate led Odom and Peck[42] to propose the hydrogen-cycling model. In this model, lactate is first converted to acetate, carbon dioxide and hydrogen; the hydrogen diffuses out of the cell and is used as an electron donor for sulphate reduction. This model, even today, is controversial, but has never been refuted or confirmed convincingly. Hydrogen formation during growth on lactate might reflect the high investment of ATP to transport sulphate across the cytoplasmic membrane and to activate sulphate to APS. After a period of starvation, the ATP levels in the cell are probably low. Sulphate-independent lactate degradation might be a way to produce the ATP that is needed to initiate sulphate metabolism.

Although named after their ability to use sulphate as a terminal electron acceptor, sulphate reducers can use many other electron acceptors for growth and can ferment substrates in the absence of inorganic electron acceptors. Therefore, the occurrence of high numbers of SRB in an environment does not necessarily reflect the occurrence of sulphate reduction in that environment, but in many recent publications this link is made too easily. Sulphate reducers can reduce other sulphur compounds (thiosulphate, sulphite and sulphur) to sulphide or can reduce nitrate and nitrite to ammonium[43–46]. Even oxygen respiration is performed by sulphate reducers (BOX 1). Other compounds that are electron acceptors for some SRB include iron (Fe(III))[47,48], uranyl (U(VI))[49], pertechnetate (Tc(VII))[50], selenate (Se(VI))[51], chromate (Cr(VI))[52] and arsenate (As(VI))[53]. However, not all of these reduction processes are coupled to growth.

Organic compounds can also be used as terminal electron acceptors for growth. Fumarate is used as an electron acceptor by many SRB. Some marine SRB use dimethylsulphoxide as an electron acceptor[54]. Additionally, sulphonates can act as electron acceptors for SRB[55]. *Desulfomonile tiedjei* has been isolated from a methanogenic microbial community that mineralizes 3-chlorobenzoate. In this community, *D. tiedjei* grows by the reductive conversion of monochlorobenzoate to benzoate, with hydrogen formed by benzoate-degrading bacteria[56]. Interestingly, *D. tiedjei* was only identified as being a member of the SRB after it had been isolated[57].

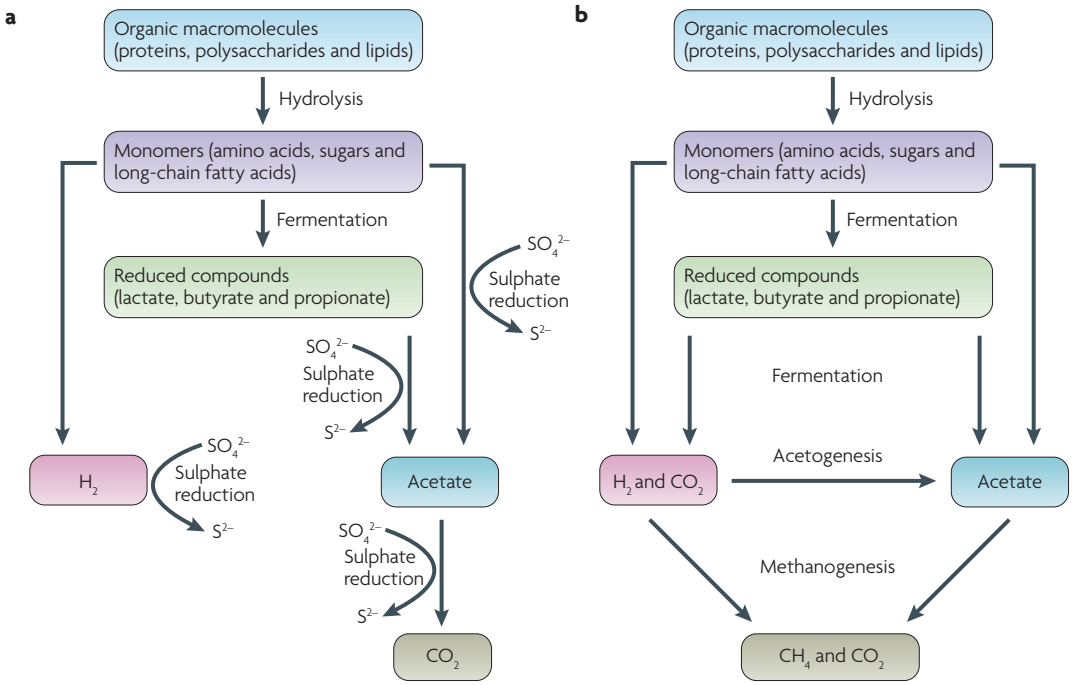


Figure 3 | **The sequential pattern of microbial degradation of complex organic matter in anoxic environments in the presence and absence of sulphate.** Macromolecules, such as proteins, polysaccharides and lipids are hydrolysed by hydrolytic bacteria. Subsequently, the monomers — amino acids, sugars and fatty acids — are fermented by fermentative bacteria into a range of fermentation products, such as acetate, propionate, butyrate, lactate and hydrogen. In the presence of sulphate (**a**), sulphate-reducing bacteria consume these fermentation products. However, in the absence of sulphate (**b**), hydrogen and acetate — the acetate having been produced directly by fermentation or indirectly by acetogenesis — are consumed by the methanogens.

In freshwater environments, which are low in sulphate, SRB have an important role in the fermentation and anaerobic oxidation of organic compounds. Many *Desulfovibrio* and *Desulfomicrobium* species grow by fermenting pyruvate to form acetate, carbon dioxide and hydrogen as products. They are also able to oxidize lactate and ethanol to acetate, but only when hydrogen is efficiently removed by hydrogen-consuming methanogens. This syntrophic growth of sulphate reducers with methanogens was first demonstrated by Bryant and colleagues[58]. Furthermore, sulphate reducers were the dominant acetogenic bacteria in a methanogenic reactor that was used to treat whey[59].

Syntrophobacter species are a special group of sulphate reducers[60]. They can grow on propionate and sulphate, but were isolated as bacteria that grow by converting propionate to acetate, carbon dioxide and hydrogen in a co-culture with hydrogen-utilizing methanogens. Similarly, sulphate-dependent or syntrophic growth was found for *Desulfotomaculum thermobenzoicum* subsp. *thermosyntrophicum*[61]. *Syntrophobacter wolinii* was obtained in a defined co-culture with a *Desulfovibrio* species[62]. *S. wolinii* is a sulphate reducer that, in the presence of a hydrogen-utilizing sulphate reducer, suppresses sulphate reduction and grows as an acetogen[63,64]. One can speculate if this is not a more common property among SRB. An alternative way to interpret the hydrogen-cycling model of Odom and Peck[42] is that in mixed cultures with SRB syntrophic degradation takes place, in which one sulphate reducer oxidizes the lactate and another uses the hydrogen for sulphate reduction.

Desulfobulbus species grow on propionate and sulphate, but unlike *Syntrophobacter* species they cannot oxidize propionate to acetate in co-culture with methanogens. However, in the absence of sulphate, they can ferment lactate and ethanol (plus carbon dioxide) to acetate and propionate.

Fermentative and acetogenic growth of SRB might not only explain why they are present in high numbers in anaerobic environments that are low in sulphate, but also why the addition of sulphate to sulphate-depleted sediments results in instantaneous sulphate reduction.

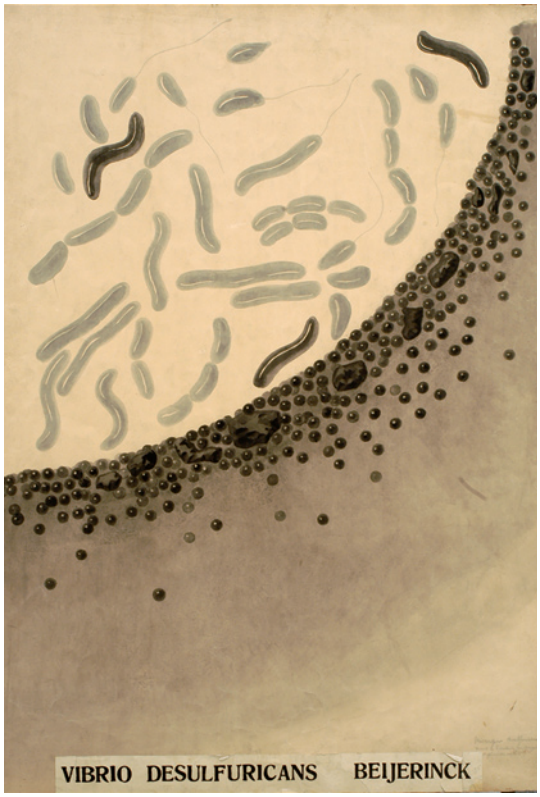
Competition with methanogens and acetogens

In anaerobic environments that have a low redox potential, SRB compete with other anaerobes, including fermentative bacteria, proton-reducing acetogenic bacteria, homoacetogens and methanogens, for the available common substrates. Some important conversions are listed in TABLE 1. The presence of sulphate is crucial in this competition. The degradation of organic matter in sulphate-reducing environments (FIG. 3a) is different from the degradation in methanogenic environments[60] (FIG. 3b). In contrast to sulphate reducers, methanogens use a limited number of substrates for growth. Quantitatively, hydrogen, carbon dioxide and acetate are the most important and best-known substrates for methanogens. Thus far, no methanogens

Homoacetogen
A bacterium that produces acetate as the sole product from sugar fermentation or from hydrogen and carbon dioxide.

Box 1 | **Sulphate reducers and oxygen**

In mixed microbial communities, sulphate-reducing bacteria (SRB) are present in the vicinity of microorganisms that consume oxygen, which creates conditions in which strict anaerobic bacteria can thrive. This finding was made more than 100 years ago by the Dutch microbiologist and founder of the Delft School of Microbiology Martinus Beijerinck, when he described *Spirillum desulfuricans*[137], which was later reclassified as *Desulfovibrio desulfuricans* (see the figure). At that time, the environmental conditions and optimal medium composition for SRB were unknown, but by adding aerobic bacteria Beijerinck obtained better and more reproducible growth of the SRB. The view that sulphate reducers are strictly anaerobic, which can still be found in recent publications, started to change with the demonstration of the occurrence of sulphate reduction in oxic environments[138]. Much research has been done since then to obtain some insights into the oxygen response of SRB[139]. Some strains of sulphate reducers are irreversibly inactivated by low oxygen concentrations, whereas others survive aeration, even though sulphate reduction is suppressed by oxygen[140,141]. Dilling and Cypionka[142] described SRB that respire using oxygen and can even couple aerobic respiration to ATP formation. *Desulfovibrio oxyclinae*, which was isolated from the upper layer of a hypersaline microbial mat, showed oxygen-dependent growth, as indicated by higher growth yields after exposure to oxygen[143]. The figure is a reproduction of *Vibrio desulfuricans*, which was painted by Henriëtte Beijerinck, the sister of Martinus Beijerinck, and is reproduced courtesy of the Beijerinck Museum.



have been described that grow on organic acids, such as lactate, propionate and butyrate, which are common substrates for sulphate reducers. Consequently, these compounds are degraded by bacteria to form products that are the substrates for methanogens (FIG. 3b). Typically, these conversions are carried out by syntrophic communities of acetogenic bacteria and methanogenic archaea.

In the presence of an excess of sulphate, sulphate reducers compete with methanogens for the common substrates hydrogen and acetate and with syntrophic methanogenic communities[65]. Owing to the higher affinity and lower threshold values for hydrogen, hydrogen-utilizing methanogens and homoacetogens are easily and rapidly out-competed by hydrogen-utilizing SRB. However, many SRB have a requirement for acetate as a carbon source and, therefore, when acetate is not provided, sulphate reducers will coexist with homoacetogens[66,67]. Acetate-utilizing sulphate reducers also out-compete acetoclastic methanogens[68,69]. However, this competition is not so clear-cut as for hydrogen. In experiments in which sulphate was added to a fully methanogenic anaerobic bioreactor, it took years before the acetotrophic *Methanosaeta* species were out-competed by sulphate reducers[70]. The sulphate reducer that became dominant was *Desulfobacca acetoxidans*, a bacterium that is specialized in growth on acetate[6] and has only slightly better growth kinetics than *Methanosaeta* spp. Propionate and butyrate-degrading sulphate reducers grow much faster than syntrophic propionate- and butyrate-degrading methanogenic or sulphate-reducing communities, which gives these sulphate reducers a competitive advantage.

From an ecological viewpoint, it is interesting to understand how sulphate reducers interact with methanogenic communities when the sulphate that is available is insufficient for complete oxidation of organic compounds. Under these conditions, SRB will compete with each other for the available sulphate. Unfortunately, only a few studies have addressed the competition between sulphate reducers for sulphate. Laanbroek *et al.*[71] found that *Desulfovibrio* spp. had the highest affinity for sulphate followed by *Desulfobulbus* spp. and *Desulfobacter* spp. This suggests that under sulphate limitation sulphate reducers use hydrogen, lactate and ethanol as substrates, but not propionate and acetate. It is likely that under sulphate-limited conditions, syntrophic communities have a role in the degradation of organic acids, whereby the hydrogen-utilizing methanogens are replaced by hydrogen-utilizing sulphate reducers.

Diversity and activity of SRB

Different techniques have been used to detect SRB and study their diversity and activity. One of the oldest techniques that has been used in this context is cultivation. Although successful, this technique has limitations, as only a small percentage of bacteria

Acetoclastic methanogen
A methanogen that uses acetate as a substrate to produce methane and carbon dioxide.

Table 1 | **Sulphate-reducing, methanogenic and acetogenic reactions**

Equation	$\Delta G^{o\prime}$ (kJ/reaction)*
Sulphate-reducing reactions	
$4\,H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4\,H_2O$	−151.9
$Acetate^- + SO_4^{2-} \rightarrow 2\,HCO_3^- + HS^-$	−47.6
$Propionate^- + 0.75\,SO_4^{2-} \rightarrow Acetate^- + HCO_3^- + 0.75\,HS^- + 0.25\,H^+$	−37.7
$Butyrate^- + 0.5\,SO_4^{2-} \rightarrow 2\,Acetate^- + 0.5\,HS^- + 0.5\,H^+$	−27.8
$Lactate^- + 0.5\,SO_4^{2-} \rightarrow Acetate^- + HCO_3^- + 0.5\,HS^-$	−80.2
Acetogenic reactions	
$Propionate^- + 3\,H_2O \rightarrow Acetate^- + HCO_3^- + H^+ + 3\,H_2$	+76.1
$Butyrate^- + 2\,H_2O \rightarrow 2\,Acetate^- + H^+ + 2\,H_2$	+48.3
$Lactate^- + 2\,H_2O \rightarrow Acetate^- + HCO_3^- + H^+ + 2\,H_2$	−4.2
Methanogenic reactions	
$4\,H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3\,H_2O$	−135.6
$Acetate^- + H_2O \rightarrow CH_4 + HCO_3^-$	−31.0
Homoacetogenic reactions	
$4\,H_2 + 2\,HCO_3^- + H^+ \rightarrow Acetate^- + 4\,H_2O$	−104.6
$Lactate^- \rightarrow 1.5\,Acetate^- + 0.5\,H^+$	−56.5

*Data from REF. 151.

in nature (less than 1%) can be cultured. Another classical technique used to determine the presence of SRB in natural samples is the analysis of phospholipid fatty acids[72]. This technique has been used to detect groups of SRB, but the taxonomic resolution that can be obtained is limited. Most of the information on the diversity of SRB in both natural and engineered ecosystems has therefore been obtained by the use of marker genes. The most commonly used marker gene is the gene that encodes 16S ribosomal RNA (rRNA).

Based on comparative analysis of 16S rRNA sequences, the known SRB can be grouped into seven phylogenetic lineages, five within the Bacteria and two within the Archaea (FIG. 4). Most of the sulphate reducers belong to the ~23 genera within the Deltaproteobacteria, followed by the Gram-positive SRB within the Clostridia (*Desulfotomaculum*, *Desulfosporosinus* and *Desulfosporomusa* genera). Three lineages, Nitrospirae (*Thermodesulfovibrio* genus), Thermodesulfobacteria (*Thermodesulfobacterium* genus) and Thermodesulfobiaceae (*Thermodesulfobium* genus)[73], only contain thermophilic sulphate reducers. Within the Archaea, SRB belong to the genus *Archaeoglobus* in the Euryarchaeota, and to the genera *Thermocladium*[74] and *Caldirvirga*[75] in the Crenarchaeota.

Different primer sets have been described[76] for the specific amplification of 16S rRNA gene fragments from different groups of SRB, such as the *Desulfotomaculum*, *Desulfobulbus*, *Desulfobacterium*, *Desulfobacter*, *Desulfonema–Desulfosarcina–Desulfococcus* and *Desulfovibrio* genera. A more powerful approach for the detection of SRB is the use of so-called functional genes which encode enzymes that play an important part in the sulphate-reduction pathway, such as *dsrAB*, which encodes the dissimilatory sulphite reductase[77], or *aprBA*, which encodes the dissimilatory adenosine-5′-phosphosulphate reductase[78]. Cloning or denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA[79,80], *dsr*[81–83] or *aprA*[84] gene fragments has been used to determine the diversity of SRB in many different habitats. Recently, a DNA microarray, the SRP-PhyloChip[85], has been used to detect SRB in natural samples, such as acidic fen soils[86]. However, these methods have the disadvantage that they provide little or no information on the number of SRB cells that are present.

Quantitative real-time PCR is a highly sensitive technique that can be used to quantify the number of SRB, and has been used, for example, to determine the number of SRB in rice field soils[87,88], soda lakes[89] and industrial waste water[90]. Moreover, this technique can also be used to study the expression of functional genes, such as *dsrAB*[91]. Another technique that can be used to quantify the number of SRB is fluorescence *in situ* hybridization (FISH), which also allows their spatial distribution to be visualized[81,92]. Many different probes have been developed to target the rRNA of different taxonomic groups of SRB[93]. Mussmann *et al.*[94] used a combination of FISH with catalysed reporter deposition (CARD–FISH) to study the vertical distribution of SRB in intertidal mud-flat samples. They found that up to 11% of all cells were SRB and that organisms related to the genera *Desulfosarcina* and *Desulfobulbaceae* dominated the surface layer of the sediment.

In combination with the use of radioactively labelled substrates, the activity of specific populations can be visualized. Ito and co-workers[95] used a microautoradiography–FISH (MAR–FISH) approach to determine the relative abundance of SRB in sewer biofilms and their substrate-uptake patterns in the presence of different electron acceptors. They found that *Desulfobulbus* was the most dominant SRB genus in the biofilms, preferentially taking up ^{14}C-propionate and ^{3}H-acetate with sulphate as an electron acceptor, whereas *Desulfovibrio* spp. showed a positive uptake of ^{14}C-bicarbonate in the presence of hydrogen and sulphate.

Instead of radioisotopes, stable isotope probing (SIP) can be used to determine the compositions of active populations. By phospholipid fatty acid analysis of samples from estuarine sediments that were incubated with ^{13}C-acetate, Boschker *et al.*[96] found that this substrate was mainly consumed by relatives of the Gram-positive *Desulfotomaculum acetoxidans* and not by the Gram-negative *Desulfobacter* spp., as was expected. Webster and co-workers[97] compared SIP of DNA and phospholipid fatty acids to identify the active community constituents in sulphate-reducing sediment enrichments. After short incubations with different ^{13}C-labelled substrates, they found that bacteria related to the acetate-utilizing genus *Desulfobacter*, as well as a member of the new candidate division JS1, which comprises only uncultured members, had taken up ^{13}C-acetate. Unfortunately, this result could not be substantiated by phospholipid fatty acid analysis.

Phospholipid fatty acid
A key component of the cellular membrane of living cells that can be used to identify specific groups of microorganisms and to monitor their physiological state.

CARD-FISH
Fluorescence *in situ* hybridization with horseradish peroxidase-labelled oligonucleotide probes and fluorochrome-labelled tyramides. The tyramides are deposited at the hybridization site, resulting in enhanced fluorescence intensity.

Microautoradiography
A photographic technique to visualize the uptake of radioactive substrates by single cells.

Stable isotope probing
A technique to identify microorganisms in environmental samples that have taken up a stable isotope-labelled substrate.

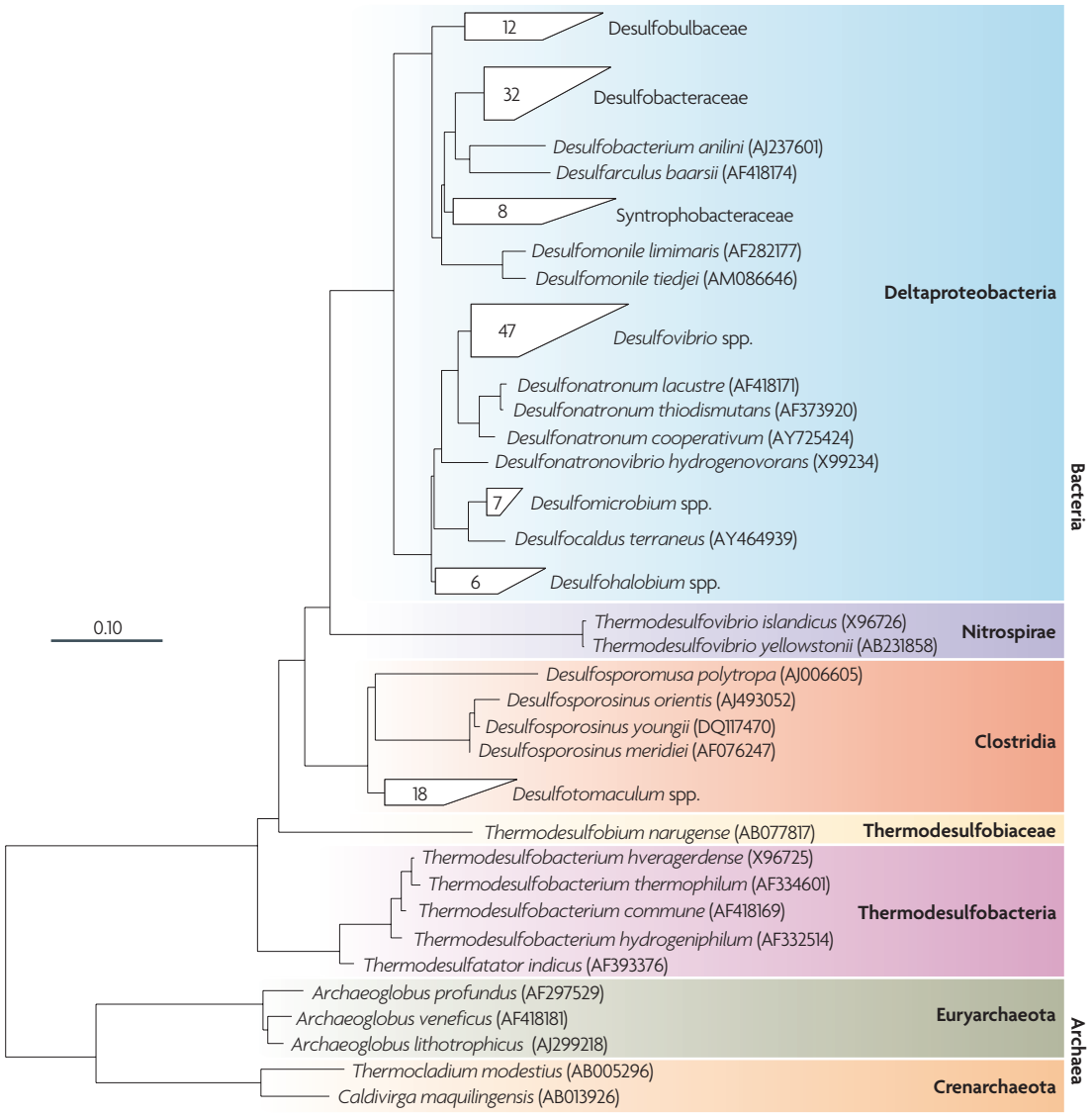


Figure 4 | **Phylogenetic tree based on nearly complete 16S ribosomal RNA (rRNA) sequences of described sulphate-reducing bacterial species.** The sequences were obtained from the SILVA small subunit (SSU) rRNA database (version 03 08 22)[149] and the tree was created using ARB software[150] (see Further information). Note the seven phylogenetic lineages of sulphate-reducing bacteria, two in the Archaea and five in the Bacteria. The number within the collapsed clusters indicates the number of different species within a particular group. The scale bar indicates 10% sequence difference.

Other tools to study the activity of SRB are the use of microelectrodes for sulphide measurements[98] and the use of radiolabelled sulphate to determine sulphate-reduction rates. Recently, gene-expression studies — for example, the detection of mRNA of genes that encode key enzymes in the sulphate-reduction pathway — were carried out to infer the activity of SRB in natural samples. Wawer and co-workers[99] studied the expression of the NiFe hydrogenase gene to infer the niche differentiation of coexisting *Desulfovibrio* spp., and Dar *et al.*[81] studied the expression of *dsrB* genes to infer the activity of all SRB. However, all these methods have their advantages and disadvantages, and so to obtain a comprehensive understanding of the diversity and activity of SRB in their natural habitat an integrated approach using different traditional and molecular methods should be used[81].

Distribution of SRB

SRB are not only versatile in their use of various electron acceptors and electron donors, they can also thrive in a range of different environmental conditions. They are ubiquitous and can be found in many natural and engineered environments where sulphate is present. SRB have been detected or isolated from marine sediments[94,96,97,100], hydrothermal vents[101], hydrocarbon seeps[26,102] and mud volcanoes[103], and are abundantly present in hypersaline microbial mats, even at saturating oxygen concentrations[83,104]. They have been detected in habitats with extreme pH values, such as acid-mine drainage sites, where the pH can be as low as 2 (REF. 105) and in soda lakes, where the pH can be as high as 10 (REF. 82). SRB have been detected and isolated from oil fields[106], as well as from

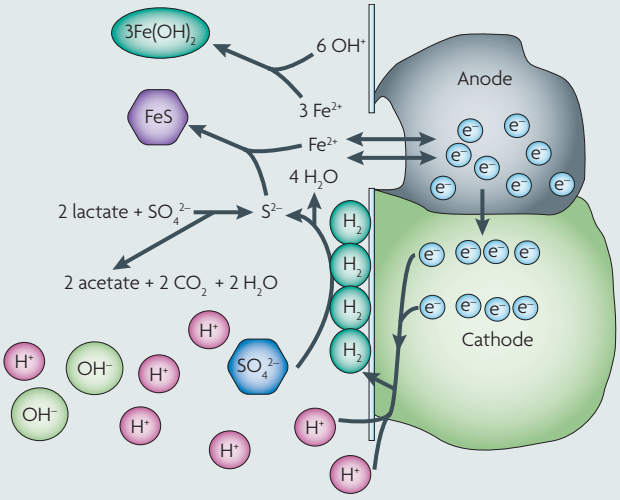
Niche differentiation
The tendency for coexisting species to differ in their use of resources.

Acid-mine drainage site
Acid water that contains H_2SO_4 derived from microbial oxidation of sulphidic minerals.

Box 2 | **Sulphate reducers and corrosion**

Microbially induced corrosion or biocorrosion of steel results in huge financial losses that amount to US$100 million per annum in the United States alone[144]. Both aerobic and anaerobic corrosion occurs. Steel and iron surfaces act as a substratum for microbial communities to form biofilms. Owing to oxygen consumption by aerobic microorganisms, biofilms are largely anaerobic at the metal surface, which creates a niche for anaerobic bacteria. Fermentation of decaying biomass also takes place. Fermentation products, such as lactate, are used as electron donors for sulphate-reducing bacteria (SRB). At the steel and iron surface, electrochemical corrosion occurs (see the figure). Chemical dissolution of iron then results in the formation of hydrogen according to Equation 9.

$$Fe + 2H^+ \rightarrow Fe^{2+} + H \qquad (9)$$



SRB consume hydrogen and influence the equilibrium of the chemical dissolution. Iron sulphide is formed as a product of chemical dissolution and sulphate reduction. Some sulphate reducers have the ability to enhance hydrogen formation from steel (F. Widdel, personal communication). Iron and steel corrosion is most severe in marine water that contains a high concentration of sulphate. However, SRB also have an important role in freshwater owing to an active sulphur cycle. It is therefore important that sulphate reduction can occur at freshwater sulphate concentrations[145] because *Desulfovibrio* species have a high affinity for sulphate[71,146]. Figure modified, with permission, from REF. 147 © (2005) Taylor & Francis.

the deep sub-surface[107]. They are also present in freshwater sediments[108], in the rhizosphere of plants[109,110], in aquifers and in engineered systems, such as anaerobic waste-water treatment plants[69,80,81,90,98,99]. Most SRB are free-living, but some are present in consortia with other microorganisms, such as methanotrophic archaea[29], or even in a more intimate relationship, for example, together with sulphur-oxidizing Gammaproteobacteria as endosymbionts in the marine worm *Olavius algarvensis*[111], thereby providing the host with nutrients[112].

Biotechnological applications of SRB

Sulphuric acid is used in many industrial processes, which results in the occurrence of sulphate in waste water. Sulphate reduction will therefore occur, which is highly undesirable. For example, in anaerobic treatment of agro–industrial waste waters, sulphate reduction results in lower methane yields. In addition, sulphide is toxic, odorous and corrosive. Attempts to avoid sulphate reduction by changing the flow regime in methanogenic bioreactors were not successful. Also, in the petro–chemical industry, sulphate reduction causes problems: hydrogen sulphide formation causes corrosion (BOX 2) and safety problems for personnel who are involved in offshore activities[113]. To avoid hydrogen sulphide formation in oil fields, it has been proposed that nitrate should be injected to stimulate nitrate-reducing activity to oxidize hydrogen sulphide and suppress sulphate reduction by nitrite or by the higher redox potential that is created[114,115].

Sulphate reduction can be applied beneficially to biotechnology, such as the removal of heavy metals from groundwater and waste water. This application takes advantage of differences in the chemical properties of metal sulphates and sulphides[116,117]. Metal sulphates (cadmium, cobalt, copper, iron, nickel and zinc) are highly soluble, but the corresponding metal sulphides have low solubility. Thus, by sulphate reduction, metals can be precipitated, recovered and reused. This concept has been applied to immobilize metals from surface water and process water from mining industries. Organic waste materials (for example, straw) are often used to immobilize heavy metals in lake sediments[118,119]. Defined substrates, such as lactate, ethanol, methanol and hydrogen-rich gas, are often preferred as electron donors for sulphate reduction. Based on the THIOPAQ system (Paques; see Further information), a process for sulphate reduction and oxidation of the excess sulphide was developed to remove heavy metals (FIG. 5). The sequence of conversions is provided in Equations 1–3.

$$SO_4^{2-} + 8\,[H] + H^+ \rightarrow HS^- + 4\,H_2O \qquad (1)$$

$$HS^- + Me^{2+} \rightarrow MeS\downarrow + OH^- \qquad (2)$$

$$HS^- + 0.5\,O_2 \rightarrow S^\circ\downarrow + OH^- \qquad (3)$$

This process is in operation at a zinc smelter (Nyrstar, The Netherlands) to treat the zinc sulphate-containing process water. Sulphate reduction takes place in a full-scale (500 m^3) sulphate-reducing gas-lift reactor. Synthesis gas, which is formed by steam-reforming natural gas, is the electron donor for sulphate reduction. The gas that enters the reactor is composed of 76%

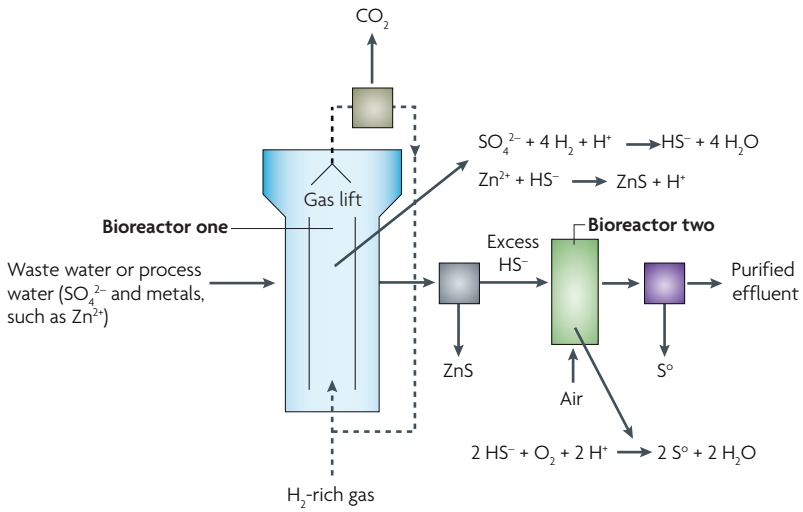


Figure 5 | **Schematic overview of the THIOPAQ process to remove sulphate and heavy metals from waste water.** Sulphate is reduced to sulphide by sulphate-reducing bacteria in bioreactor one using hydrogen as an electron donor. Subsequently, the sulphide is used to precipitate the heavy metals. An excess of sulphide is converted to elemental sulphur by sulphide-oxidizing bacteria in bioreactor two. The precipitated metal sulphides and elemental sulphur can be reused.

hydrogen, 20% carbon dioxide, 3% nitrogen and 1% carbon monoxide. The zinc sulphide that precipitates with the sulphate-reducing biomass is collected in a settler and reused in the roasting process. More than 95% of the dry weight of the sludge in the bioreactor is zinc sulphide.

The microbial community that is present mainly consists of heterotrophic SRB that belong to the genera *Desulfovibrio* and *Desulfomicrobium*, but low numbers of methanogens and homoacetogens are also present[120]. The dominant SRB require acetate as a carbon source. When acetate (or another organic carbon source) is not supplied, as in the full-scale reactor, these sulphate reducers will depend on acetate formation by homoacetogens. One of the dominant sulphate reducers, *Desulfovibrio paquesii*, which is also abundantly present in other sulphate-reducing bioreactors[121], was isolated and classified as a novel species[122].

Another biotechnological application for SRB is the removal and reuse of sulphur compounds from waste water and off gases. Under oxygen-limitation, sulphide-oxidizing bacteria mainly produce elemental sulphur instead of sulphate (Equation 3). This feature is applied to remove hydrogen sulphide from natural gas or biogas[123,124]. When combined with an anaerobic step, it is possible to treat water and gas that contain oxidized sulphur compounds. The oxidized sulphur compounds are reduced to sulphide, which is then partially oxidized to elemental sulphur. One example of this application is flue-gas desulphurization (FGD). Lime or limestone wet scrubbing is a commonly applied FGD technology. Biotechnological FGD (Bio-FGD) is an alternative that makes use of the following conversions of the sulphur cycle.

In the first step, sulphur dioxide is scrubbed from the flue gas with an alkaline solution to form sulphite (Equation 4).

$$SO_2 + OH^- \rightarrow HSO_3^- \quad (4)$$

The presence of oxygen in the flue gas results in the unavoidable oxidation of part of the sulphite to sulphate (Equation 5).

$$HSO_3^- + 0.5\,O_2 \rightarrow SO_4^{2-} + H^+ \quad (5)$$

In an anaerobic bioreactor, sulphite and sulphate are reduced by SRB to sulphide (Equations 6,7).

$$HSO_3^- + 6\,[H] \rightarrow HS^- + 3H_2O \quad (6)$$

$$SO_4^{2-} + 8\,[H] \rightarrow HS^- + 3H_2O + OH^- \quad (7)$$

Then, in a micro-aerobic reactor, sulphide is partially oxidized to elemental sulphur by autotrophic sulphide-oxidizing bacteria (Equation 8).

$$HS^- + 0.5\,O_2 \rightarrow S° \downarrow + OH^- \quad (8)$$

During sulphide oxidation, alkalinity is produced, which can be led back to entrap sulphur dioxide. Alkalinity is only lost via the bleed stream. Therefore, Bio-FGD requires a low input of lime or limestone. At the laboratory scale, the feasibility of Bio-FGD has been demonstrated[125,126]. In China, a full-scale Bio-FGD has recently been initiated by Paques (see Further information). This Bio-FGD is operated using a citric acid waste stream as electron donor for sulphate and sulphite reduction. Citrate is not a common electron donor for SRB. Furthermore, initial experiments in our laboratories indicate that citrate is not the direct electron donor for SRB: it is first fermented to acetate and formate by *Trichococcus* and *Veillonella* species. Thus, acetate and formate are the direct electron donors for sulphate reduction. The preferential use of acetate in sulphate reduction requires long-term operation of the bioreactors[70]. Several sludge samples from different sulphate-reducing bioreactors that are in operation contain high numbers of acetate-degrading SRB[81].

Concluding remarks and future perspectives

In this Review, we have discussed how SRB are ubiquitous in anoxic habitats, where they use sulphate as a terminal electron acceptor in the degradation of organic compounds. However, their energy metabolism is not restricted to sulphate reduction and SRB can use a wide range of other electron acceptors. In addition, they also have fermentative metabolism. An important physiological feature of SRB is that they can compete with methanogens or grow syntrophically with them depending on the availability of sulphate. These microorganisms are not only versatile in their metabolism, but also in the environmental conditions in which they thrive. The use of different molecular methods has demonstrated that their diversity is enormous and that there are still many

Table 2 | **General genomic features of different sulphate-reducing microorganisms***

Domain	Phylum	Class	Genome size (bp)	G+C (%)	Number of genes	Predicted CDSs	Genes with function prediction	Genes without function prediction	CDS (%)	Number of 16S rRNAs	Number of tRNAs
***Archaeoglobus fulgidus* DSM 4304**											
Archaea	Euryarchaeota	Archaeoglobi	2,178,400	49	2,519	2,468	1,798	670	98	1	46
***Caldivirga maquilingensis* IC-167**											
Archaea	Crenarchaeota	Thermoprotei	2,077,575	34	1,986	1,943	1,307	63	98	0	42
***Desulfotomaculum reducens* MI-1**											
Bacteria	Firmicutes	Clostridia	3,608,104	42	3,424	3,324	2,334	990	97	8	71
***Desulfovibrio vulgaris* subsp. *vulgaris* strain Hildenborough**											
Bacteria	Proteobacteria	Deltaproteo-bacteria	3,773,159	63	3,640	3,545	2,216	1,329	97.4	5	68
***Desulfovibrio vulgaris* subsp. *vulgaris* DP4**											
Bacteria	Proteobacteria	Deltaproteo-bacteria	3,661,391	63	3,189	3,103	2,297	806	97.3	5	68
***Desulfovibrio desulfuricans* G20**											
Bacteria	Proteobacteria	Deltaproteo-bacteria	3,730,232	58	3,865	3,784	2,302	1,482	97.9	4	66
***Desulfotalea psychrophila* LSv54**											
Bacteria	Proteobacteria	Deltaproteo-bacteria	3,659,634	47	3,331	3,234	1,739	1,495	97	7	64
***Synthrophobacter fumaroxidans* MPOB**											
Bacteria	Proteobacteria	Deltaproteo-bacteria	4,990,251	60	4,162	4,098	2,809	1,289	98.5	2	51

*Data from the Integrated Microbial Genomes (IMG) database (see Further information). CDS, coding sequences; rRNA, ribosomal RNA.

uncultured representatives. Apart from their importance in nature, SRB, together with sulphur-oxidizing micro-organisms, can be successfully exploited in the sustainable clean-up of industrial waste streams.

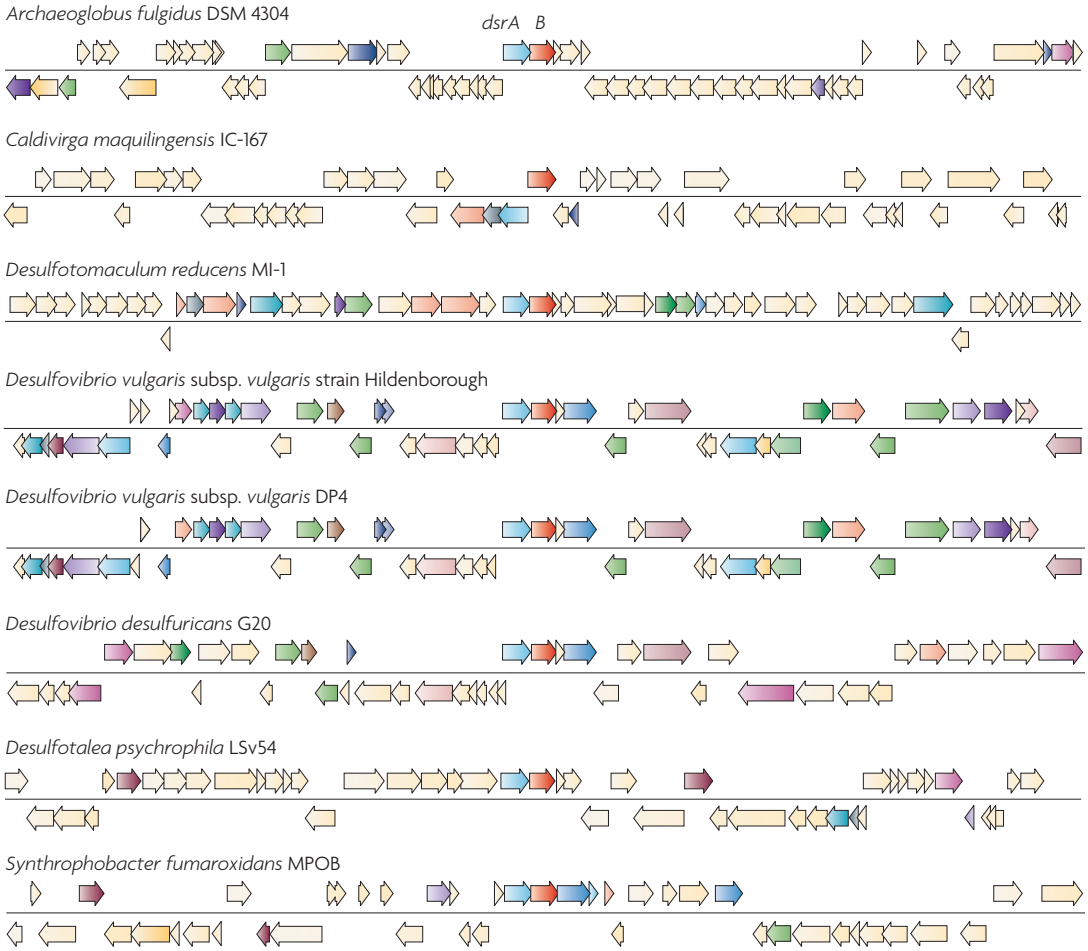
Although we have generated a huge amount of information on the diversity, physiology and biochemistry of SRB, we think that we have only scratched the surface. So far, diversity studies have been mainly descriptive, and the physiology and biochemistry of SRB have been studied primarily with just a few model organisms, such as *Desulfovibrio desulfuricans*, *Desulfovibrio vulgaris* and *Desulfovibrio gigas*. Therefore, future research should move away from descriptive studies and focus on explanations and predictions, using ecological concepts and innovative technologies, such as meta-transcriptomics and meta-proteomics.

Although difficult, isolation of microorganisms is still necessary to obtain detailed insights into their physiology, behaviour and interactions with other organisms, as well as for biotechnological purposes. Novel high-throughput technologies might increase the success of isolating ecologically important community members. Recently, Ingham and co-workers[127] described the development of the micro-Petri disk, a million-well disposable chip for culturing and high-throughput screening microorganisms. The use of this revolutionary tool might result in the isolation of different novel SRB.

One of the greatest challenges in microbial ecology is to identify the function of microorganisms in their natural habitats. MAR–FISH[128] and SIP[129] have been used successfully for this purpose. However, Li *et al.*[130] recently described a new approach, SIMSISH (secondary ion mass spectrometry *in situ* hybridization), that combines probe-based hybridization with isotope measurements at the single-cell level using a NanoSIMS instrument. If this approach can also be combined with the *in situ* detection of mRNA[131], it will soon be possible to study the ecophysiology of SRB, particularly those that have not yet been isolated, in greater detail.

The complete genomes of different SRB — *Archaeoglobus fulgidus* VC-16[132], *Caldivirga maquilingensis* IC-167, *Desulfovibrio vulgaris* subsp. vulgaris strain Hildenborough[133], *Desulfotalea psychrophila*[134], *Desulfovibrio desulfuricans* G20, *Desulfotomaculum reducens* MI-1 and *Syntrophobacter fumaroxidans* MPOB — have been sequenced (TABLE 2), and the genomes of other SRB, for example, *Desulfobacterium autotrophicum*, *Desulfovibrio magneticus*, *Thermodesulfovibrio yellowstonii* and *Thermodesulfobacterium commune*, are currently being sequenced. Comparative analysis of these genomes will provide detailed information on the energy and carbon metabolism of these organisms, and on the diversity and evolution of the enzymes that are involved in these processes (BOX 3). Moreover, these sequences

Box 3 | **Genomics of sulphate reducers**



The complete genome sequences of eight sulphate reducers have been deposited in public databases to date — *Archaeoglobus fulgidus* DSM 4304 (Euryarchaeota), *Caldivirga maquilingensis* IC-167 (Crenarchaeota), the Gram-positive bacterium *Desulfotomaculum reducens* MI-1 (Firmicutes) and five Gram-negative Deltaproteobacteria, *Desulfovibrio vulgaris* subsp. *vulgaris* strain Hildenborough, *Desulfovibrio vulgaris* subsp. *vulgaris* DP4, *Desulfovibrio desulfuricans* G20, *Desulfotalea psychrophila* LSv54 and *Syntrophobacter fumaroxidans* MPOB. The genomes of these sulphate-reducers have different features (TABLE 2). The genomes of the two archaea, *A. fulgidus* (~2.2 Mb) and *C. maquilingensis* (~2.1 Mb), are much smaller than those of the sulphate-reducing bacteria (~3.6–4.9 Mb) and have a lower number of transfer RNAs. Comparative analysis of the clusters of orthologous group (COG) profiles (see the figure) shows a low correlation value of 0.30 or less between the sulphate-reducing archaea and the sulphate-reducing bacteria. Intermediate values (0.54–0.74) were found among the six bacteria, whereas high correlation values (0.91–0.99) were found among the three *Desulfovibrio* strains. The low similarity between the genomes of *A. fulgidus* and *D. psychrophila* was also observed by Rabus *et al.*[134], who found that only genes that encode proteins which are involved in sulphate reduction and some common hypothetical proteins were shared, which indicated that only a small fraction of genes are necessary for sulphate reduction. Comparative analysis of the genomes of other sulphate reducers and closely related microorganisms is needed to confirm this assumption.

The figure shows the gene neighbourhood of *dsrAB* genes in different sulphate-reducing bacteria. Genes in the same colour (except for pale yellow) are from the same COG group.

open up the possibility for functional genomics. DNA microarrays have been used to study the expression of genes under different environmental conditions, such as temperature, salinity[135] and pH, and proteomics has been used to study the oxygen stress response[136]. With these tools in hand, we can not only obtain important information on the niche differentiation of SRB, but also predict their behaviour in engineered ecosystems, thereby allowing their performance in the removal of sulphur compounds from waste streams to be improved. SRB have been studied successfully for more than a century, but the novel opportunities that have been created by the genomics revolution will generate enormous opportunities for microbiologists to obtain detailed insights into the ecology and biotechnology of these important microorganisms.

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FURTHER INFORMATION
Gerard Muyzer's homepage: http://www.muyzer.eu
Genomes OnLine Database: http://www.genomesonline.org/
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The Integrated Microbial Genomes (IMG) Database: http://imgweb.jgi-psf.org/cgi-bin/w/main.cgi
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