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The genome of the Gram-positive metal- and sulfate-reducing bacterium *Desulfotomaculum reducens* strain MI-1

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Summary

Spore-forming, Gram-positive sulfate-reducing bacteria (SRB) represent a group of SRB that dominates the deep subsurface as well as niches in which resistance to oxygen and dessication is an advantage. *Desulfotomaculum reducens* strain MI-1 is one of the few cultured representatives of that group with a complete genome sequence available. The metabolic versatility of this organism is reflected in the presence of genes encoding for the oxidation of various electron donors, including three- and four-carbon fatty acids and alcohols. Synteny in genes involved in sulfate reduction across all four sequenced Gram-positive SRB suggests a distinct sulfate-reduction mechanism for this group of bacteria. Based on the genomic information obtained for sulfate reduction in *D. reducens*, the transfer of electrons to the sulfite and APS reductases is proposed to take place via the quinone pool and heterodisulfide reductases respectively. In addition, both H_2-evolving and H_2-consuming cytoplasmic hydrogenases were identified in the genome, pointing to potential cytoplasmic H_2 cycling in the bacterium. The mechanism of metal reduction remains unknown.

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Introduction

The genus *Desulfotomaculum* contains sulfate-reducing bacteria (SRB) that form heat-, oxygen- and desiccation-resistant endospores (Widdel and Bak, 1992). Because of their ability to form spores, members of this genus thrive in habitats where anoxic conditions are not maintained permanently and where desiccation occasionally occurs. As a result, *Desulfotomaculum* and the closely related genus *Desulfosporosinus* tend to be the dominant SRB in subsurface environments disturbed by human activities, such as mines, as well as sites where the groundwater level is subject to significant seasonal fluctuations. Phylogenetic studies of the bacterial communities in deep mines in Japan (Ishii *et al*., 2000) and South Africa (Moser *et al*., 2003) illustrate the importance of *Desulfotomaculum* spp. in those environments. A separate investigation 4 km deep in a South African mine revealed that species of *Desulfotomaculum* and *Methanobacterium* dominate the microbial communities in those environments (Moser *et al*., 2005). Recently, the relevance of *Desulfotomaculum* and other Gram-positive spore-forming bacteria, particularly *Desulfosporosinus* and *Clostridium* has come to the fore for the bioremediation of U(VI)-contaminated sites and it appears as though these organisms could play an important role at these sites following electron donor amendments (Chang *et al*., 2001; Madden *et al*., 2007; N'Guessan *et al*., 2008).

Desulfotomaculum reducens MI-1 was isolated from marine sediments heavily contaminated with chromium from Mare Island Naval Shipyard (Tebo and Obraztsova, 1998). It can use a wide range of organic compounds as electron donors, including short-chain fatty acids such as propionate, butyrate and valerate, alcohols such as methanol, ethanol, n-propanol and n-butanol as well as lactate, pyruvate and glucose. More importantly, it can use a large number of electron acceptors for growth: sulfate,

thiosulfate, dithionite, as well as elemental sulfur. It is one of the few SRB able to grow by coupling the oxidation of organic compounds to the reduction of Fe(III) to Fe(II) (Tebo and Obraztsova, 1998).

As is typical of many SRB, *D. reducens* ferments pyruvate. However, a recent characterization of *D. reducens* has unveiled unusual metabolic properties. The most striking result is that spores of *D. reducens* are able to catalyse the reduction of U(VI) when H_2, a product of pyruvate fermentation, is provided as an electron donor and when cell-free medium collected after cell growth (spent medium) is added (Junier *et al*., 2009). Under these conditions, the presence of competing electron acceptors such as nitrate or sulfate does not affect U(VI) reduction (Junier *et al*., 2010). This is significant due to the extensive nitrate contamination found concomitantly with U(VI) in various US Department of Energy (DOE) sites and the prevalence of nitrate outcompeting U(VI) as an electron acceptor for many microorganisms (Elias *et al*., 2003; Luo *et al*., 2005).

Desulfotomaculum reducens MI-1 is an excellent example of a microorganism to be investigated in more detail because its unusual metabolic characteristics make it effectively both a sulfate- and a metal-reducing bacterium. In addition, it is one of the few Gram-positive SRB for which a complete genome sequence is available. The other two are *Desulfotomaculum acetoxidans* (a close relative to *D. reducens*) and Candidatus *Desulforudis audaxviator* that was sequenced from DNA obtained from South African mine (Chivian *et al*., 2008). It is one of only three SRB with a genome sequence available and able to couple growth to Fe(III) reduction. The other two are *Desulfotalea psychrophila* (Knoblauch *et al*., 1999) and *Desulfobulbus propionicus* (Holmes *et al*., 2004).

Eighteen draft and finished SRB genome sequences are available to date and were used for comparison: seven *Desulfovibrio* spp. (*Dv. desulfuricans* G20, *Dv. desulfuricans* ATCC 27774, *Dv. piger*, *Dv. salexigens*, *Dv. vulgaris* Miyazaki, *Dv. vulgaris* DP4, *Dv. vulgaris* Hildenborough), *Desulfonatronospira thiodismutans*, *Desulfohalobium retbaense*, *Desulfococcus oleovorans*, *Desulfobacterium autotrophicum*, *Desulfobulbus propionicus*, *Desulfomicrobium baculatum*, *Desulfotalea psychrophila*, Candidatus *Desulforudis audaxviator*, *Desulfonispora thiosulfatigenes* and two *Desulfotomaculum* spp. (*D. reducens* and *D. acetoxidans*). The latter four are Gram-positive.

In this article, we present the complete genome sequence of *D. reducens* strain MI-1. Using manual annotation, metabolic information as well as the comparison to genomes of the eighteen above-mentioned SRB, we describe general features of the *D. reducens* genome including genetic components involved in central metabolic pathways, sulfate reduction, hydrogen metabolism and metal reduction.

Results and discussion

General genome features

The finished genome of *D. reducens* consists of a single circular chromosome, 3 608 104 base pairs (bp) long, with a G+C content of 43% (Table 1 and Fig. 1). 71 tRNAs were identified. In addition to the 69 standard tRNAs, two are for nonstandard aminoacids: pseudouridine (dred_R0046) and selenocysteine (dred_R0069). The genome contains eight almost identical copies of the three rRNAs (5S, 16S and 23S) but in addition, we found 16S and 5S rRNA genes that were slightly longer than the ones pertaining to operons (1629 bp instead of 1612 bp for 16S rRNA and 205 bp instead of 117 bp for 5S rRNA). A combination of automated annotation and extensive manual curation predicted 3324 protein-coding sequences (CDS), of which, 2334 (70%) were assigned functions.

Central and electron donor metabolism

Central metabolic pathways such as the Embden-Meyerhoff-Parnas (glycolysis) pathway and the reductive pentose phosphate pathway are present. The glycolysis pathway (Fig. S1) and the reductive pentose phosphate pathway (Fig. S2) are complete. A partial reductive citric acid cycle is also present that allows the transformation of oxaloacetate to succinate via malate and fumarate and succinyl-coA to isocitrate via α-ketoglutarate. However, it is unclear whether the succinate to succinyl-coA step is possible because the succinyl-coA synthetase normally

Table 1. *Desulfotomaculum reducens* strain MI-1 genome statistics.

Characteristic	Value
Chromosome size (bp)	3 608 104
G+C ratio (%)	43.28
Coding density (%)	85.5
No. of predicted protein coding gene	3 324
No. of predicted proteins unique to *D. reducens (%)*	515 (15.5)
Number of rRNA operons	8
Number of tRNA genes	71
No. of predicted proteins with putative function (%)	2 334 (70.2)
No. of predicted proteins with unknown function (%)	990 (29.8)
Genes coding transmembrane proteins	510
BLASTP comparison against completed microbial genome database (No. of top hits)	
Firmicutes	2 553
Clostridia	2 375
Bacilli	178
Deltaproteobacteria	50
Syntrophomonadaceae	32

% GC statistics are for protein coding sequences only. Criteria for BLASTP match inclusion: (i) BLASTP *e*-value 1e – 5 or lower; (ii) alignment covering > 70% of both query and subject.

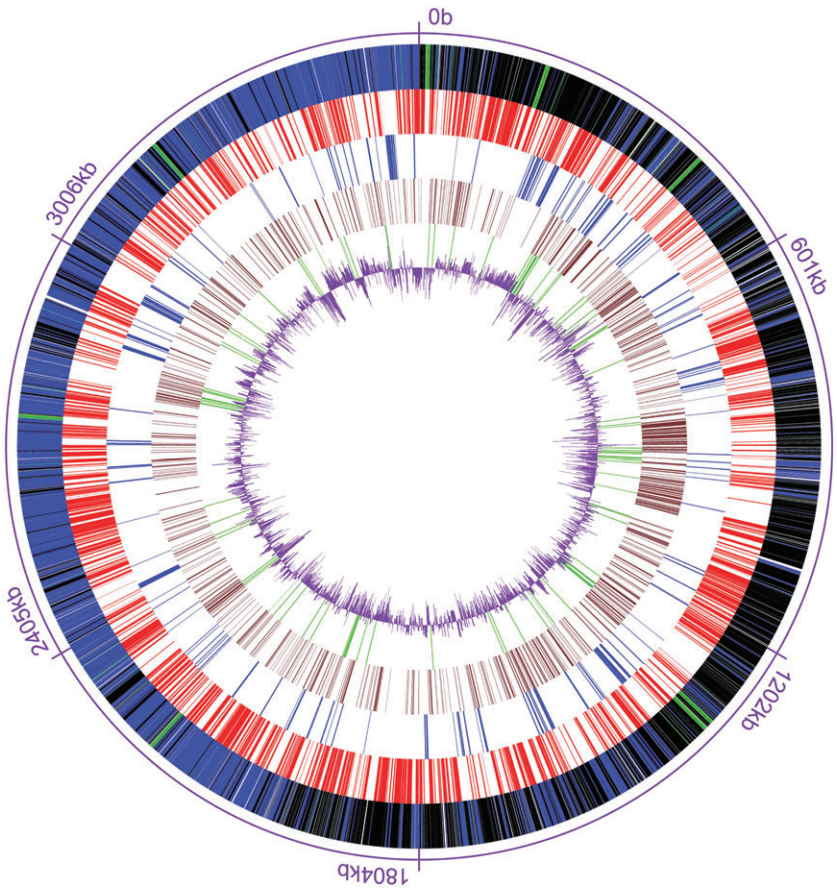

Fig. 1. Circular representation of the genome of *Desulfotomaculum reducens* strain MI-1. From the outside inwards: Genes on forward strand (blue) and genes on reverse strand (black) and tRNA genes (green), genes with best blast hit from *D. acetoxidans* (red), genes with best blast hit from *Desulfitobacterium hafniense* Y51 (blue), hypothetical proteins (brown), transposases and integrases (green), and G+C content (purple).

active in this pathway (E.C. 6.2.1.5) is absent from the *D. reducens* genome (Fig. S3). Without this enzyme, the partial citric acid pathway does not allow the formation of α-ketoglutarate from oxaloacetate, suggesting that glutamate metabolism is lacking in *D. reducens*. In fact, the organism does not grow in the absence of yeast extract, suggesting that it is a fastidious bacterium (Fig. S4).

A characteristic of *D. reducens* is its ability to utilize a large number of electron donors: lactate, pyruvate, glucose, short-chain fatty acids such as propionate, butyrate and valerate, and alcohols such as methanol, ethanol, n-propanol or n-butanol (Tebo and Obraztsova, 1998). Fig. 2 shows the pathways involved in the oxidation of butyrate (green) and ethanol (purple).

Pyruvate is an intermediate in the oxidation of several electron donors (propionate, lactate, glucose) in addition to being itself an electron donor and fermentative substrate. The pathways for the metabolism of propionate, lactate and glucose via pyruvate are shown in yellow in Fig. 2. An interesting feature of the *D. reducens* genome is that it encodes genes for all three potential pyruvate transformation mechanisms (Fig. S5; Fig. 2) (White, 2000). Decarboxylation by the pyruvate dehydrogenase (PDH) (dred_1893) yields acetate, CO_2 and NADH; decarboxylation by the pyruvate-ferredoxin oxidoreductase (PFOR) (dred_0047-50) yields acetate, CO_2 and H_2 and decarboxylation by the pyruvate-formate lyase (PFL) (dred_2750-53) yields acetate and formate. Experimental evidence for activity of the former two is available (Junier *et al*., 2009; Junier *et al*., 2010). In order to produce H_2 from the fermentation of pyruvate, an H_2-evolving hydrogenase is involved (see hydrogen metabolism section).

Acetate is the product of the oxidation of all electron donors because *D. reducens* is an incomplete oxidizer. Thus, acetyl-CoA is also an intermediate in many pathways. Surprisingly, we were unable to identify a phosphotransacetylase to catalyse the formation of acetyl-phosphate from acetyl-CoA in the genome of *D. reducens* (Fig. S5) despite comparing the phosphotransacetylase from *D. acetoxidans* to the entire *D. reducens* genome using BLASTP. Nonetheless, an acetate kinase (*E.C.* 2.7.2.1; dred_2094) was identified and is predicted to catalyse the transformation of acetyl-phosphate to acetate with the production of ATP. We also identified an acetyl-CoA ligase (E.C. 6.2.1.13; dred_2081) that produces acetate and ATP from acetyl-CoA directly and that is likely to be involved in energy production from the acetyl-CoA intermediate (Fig. S5).

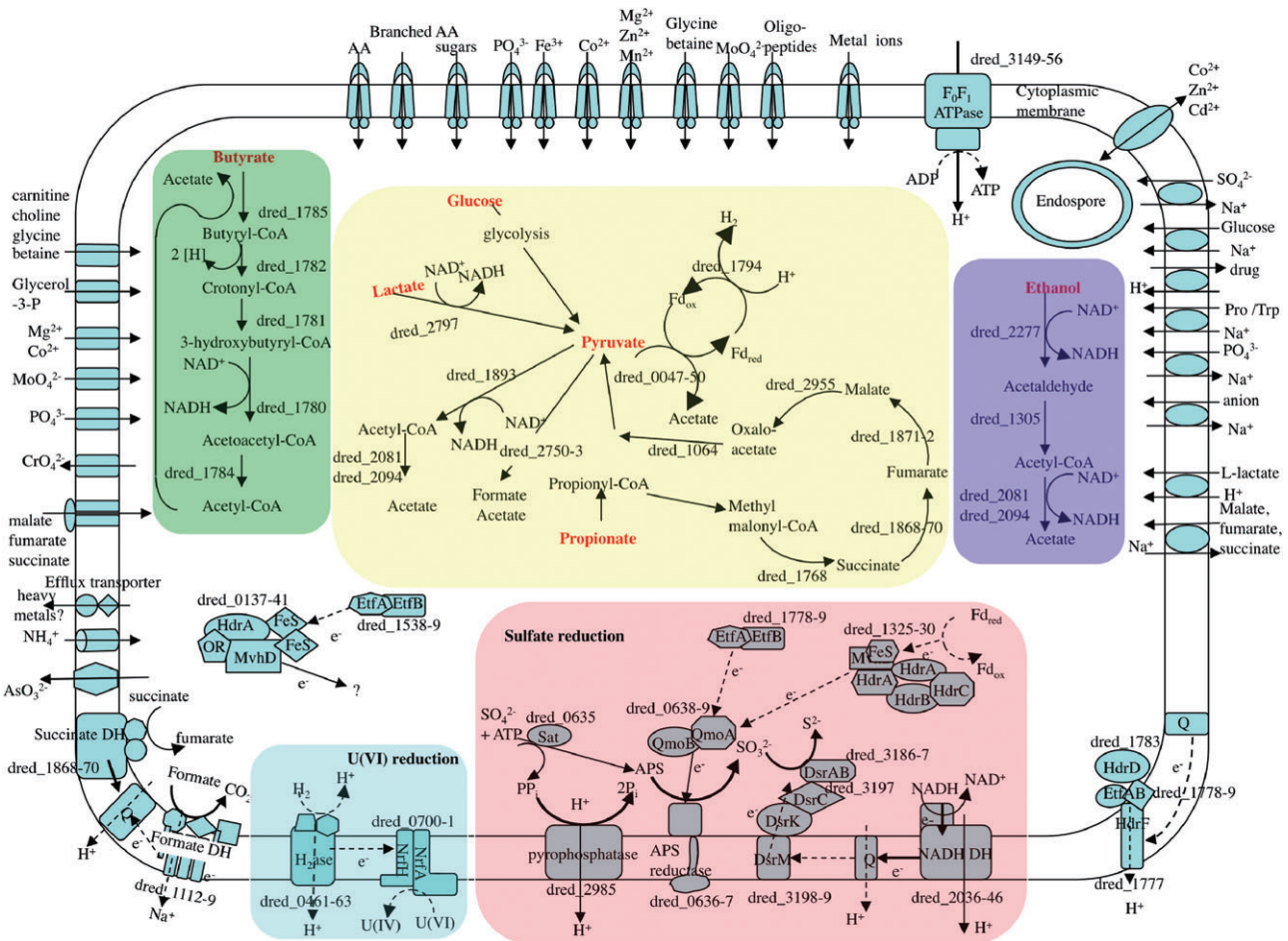

Fig. 2. Metabolic reconstruction of *D. reducens* based on known growth substrates and metabolic capacities. Arrows indicate metabolic flows: dashed lines represent putative electron flow. The sole protein shown in dark blue contains selenocysteine. Abbreviations are: DH, dehydrogenase; H_2ase, hydrogenase; DsrMK, menaquinol oxidizing complex; DsrABC, sulfite reductase; NrfHA, nitrite reductase; Hdr, heterodisulfide reductase; MvhD, methyl viologen reducing hydrogenase, delta subunit; Sat, sulfate adenyl transferase; QmoAB, Hdr- and Mvh-containing protein complex; FeS, iron sulfur protein; Fd, ferredoxin, EtfAB, electron-transfer flavoprotein; OR, oxidoreductase; PPi, pyrophosphate.

Two complete non-identical copies of the aerobic-type carbon monoxide dehydrogenase (CoxMSL) are present (dred_2774-6 and dred_1502-4). This protein is a dimer of heterotrimers: it contains a molybdoprotein, a flavoprotein and an Fe-S protein and catalyses the oxidation of CO coupled to the reduction of an electron acceptor (Schubel *et al.*, 1995). This aerobic-type carbon monoxide dehydrogenase (CODH) was recently identified in the complete genomes of two other anaerobes: *Carboxydothermus hydrogenoformans* (Luo *et al.*, 2005) and *Moorella thermoacetica* (moth_1958-60) (Pierce *et al.*, 2008). This suggests that the aerobic-type CODH may function with other electron acceptors than O_2 (e.g., sulfate). We tested *D. reducens* for its ability to utilize CO as an electron donor in the presence of sulfate as an electron acceptor with or without an additional electron donor (lactate). We found that CO was inhibitory to sulfate reduction in the presence of lactate and did not support sulfate reduction in the presence of CO alone (Fig. S6) suggesting no direct involvement of CODH in bulk CO oxidation. Two studies (Voordouw, 2002; Pereira *et al.*, 2008) suggest that CO may be produced during sulfate reduction in *Desulfovibrio vulgaris*. The transcriptomic study (Pereira *et al.*, 2008) suggests that CO may be produced during pyruvate decarboxylation and is consistent with CO serving as a physiological electron donor for energy-producing processes at low concentrations. Thus, even if the aerobic type CODH does not support growth with CO as an electron donor and CO is toxic to cells at the concentrations considered, CODH may be involved in the cycling of low levels of CO within the cell during sulfate reduction (see sulfate section).

Sulfate catabolism

Unlike other types of respiration where the terminal reductases are located on the cytoplasmic (or the outer)

membrane, sulfate reduction is biochemically challenging because of the cytoplasmic localization of the terminal reductases [adenosine phosphosulfate (APS) reductase and sulfite reductase]. This means that the direct translocation of protons across the membrane coupled to electron transport cannot take place. A model for the pathway of dissimilatory sulfate reduction involving 'hydrogen cycling' was developed for *Desulfovibrio vulgaris* (Odom and Peck, 1981). The general scheme is predicated on the production of H_2 that diffuses freely into the periplasm and carries with it both electrons and protons. In the H_2 cycling model, hydrogen gas in the periplasm donates electrons to periplasmic hydrogenases that transfer them to periplasmic c_3 cytochromes, releasing protons into the periplasm and generating a proton motive force. The hydrogen cycling model has been expanded to include formate and CO as potential intermediates shuttling electrons from the cytoplasm to the periplasm and is termed 'redox cycling' (Pereira *et al*., 2008). Similarly to hydrogen cycling, periplasmic formate dehydrogenases oxidize formate and release protons, electrons and CO_2 into the periplasm (Pereira *et al*., 2008). Cytoplasmic CO is converted to periplasmic CO_2 and H_2 via a membrane-spanning CODH-hydrogenase complex (Voordouw, 2002). Subsequently, the electrons that have been accumulated in the periplasmic c_3 cytochrome pool are transferred back into the cytoplasm to the APS and sulfite reductases through transmembrane complexes (e.g., Qmo, Rnf, Dsr, Hmc) (Heidelberg *et al*., 2004).

Desulfotomaculum reducens is a Gram-positive SRB and, thus, lacks a periplasmic space as well as periplasmic proteins. As a result, the electron flow during sulfate reduction must differ from that in *Dv. vulgaris*. In this section, we propose a mechanism by which *D. reducens* obtains energy from sulfate reduction in the absence of a periplasm (shown in red in Fig. 2).

Activation of sulfate to APS. The sulfate adenyltransferase (dred_0635) releases pyrophosphate during the conversion of sulfate to APS. A fourteen transmembrane helix (TMH) proton-translocating pyrophosphatase (Fig. 2) is encoded in the genome of *D. reducens* (dred_2985) as well as in the genome of all three other Gram-positive SRB. In contrast, only a soluble pyrophosphatase is found in Gram-negative SRB. The transmembrane pyrophosphatase may allow the translocation of protons during the cleavage of the pyrophosphate diphosphate bond, suggesting that the activation of sulfate may contribute to the establishment of a proton motive force. Its presence in all four Gram-positive SRB sequenced to date is consistent with a strategy to obtain energy from sulfate reduction in the absence of periplasmic proteins.

Reduction of APS to sulfite. Adjacent to the sulfate adenyltransferase is the APS reductase (dred_0636-7) that is homologous to APS reductases from other SRB. However, the alpha subunit (dred_0637) of the APS reductase in *D. reducens* was predicted to be membrane-anchored and outside-facing using the transmembrane helix (TMH) prediction algorithm TMHMM (http://www.cbs.dtu.dk/services/TMHMM). Out of the eighteen SRB genomes, *D. reducens* is the only one for which the APS reductase alpha subunit is predicted to be membrane-anchored (with a probability > 85%). Note that the *Desulfonispora* sp. genome sequence does not cover this region and thus, the APS reductase from that organism was not included in this analysis. Bioinformatic evidence of the membrane-anchored localization for the *D. reducens* APS reductase will need to be confirmed by biochemical analyses as single transmembrane helix predictions are not as reliable as multi-helix predictions. However, there is a precedent for this observation. Immunocytochemical evidence shows the association of the APS reductase with the cytoplasmic membrane in the thermophilic SRB *Desulfovibrio thermophilus* whereas it confirms the cytoplasmic localization of the protein in *Dv. vulgaris* and *Dv. gigas* (Kremer *et al*., 1988).

Associated with the adenyltransferase and the APS reductase are a heterodisulfide reductase (Hdr) (dred_0638) and the fusion protein of a heterodisulfide reductase and the delta subunit of the methyl-viologen-reducing [Ni-Fe] hydrogenase (Mvh) (dred_0639). The latter two ORFs (dred_0638 and dred_0639) are conserved in all SRB sequenced to date (Fig. 3) and represent two of the three genes of the QmoABC complex in *Dv. vulgaris*. QmoABC is a transmembrane complex that, in *Dv. vulgaris*, allows the transfer of electron from the periplasmic c_3 cytochrome pool to the terminal reductases in the cytoplasm (Haveman *et al*., 2004). While the gamma subunit (QmoC) of QmoABC includes TMHs in all Gram-negative SRB, the three examples of Gram-positive SRB (*D. reducens*, *D. acetoxidans* and Candidatus *Desulforudis* – the *Desulfonispora* sp. draft sequence does not include the appropriate region) lack TMHs. In *D. reducens*, QmoC is completely absent. Because the Qmo operon is adjacent to the APS reductase, it has been suggested that it may be involved in transferring electrons from periplasmic *c*-type cytochromes to this cytoplasmic reductase in *Dv. desulfuricans* (Pires *et al*., 2003; Haveman *et al*., 2004). The fact that there is no transmembrane subunit in the Qmo complex in the three Gram-positive SRB for which sequence is available suggests that the Qmo complex is unlikely to catalyse transmembrane electron transfer in those bacteria. Nonetheless, it is possible that QmoAB in *D. reducens* serves as an electron donor to APS reductase if electrons are donated to the complex from a cytoplasmic source such

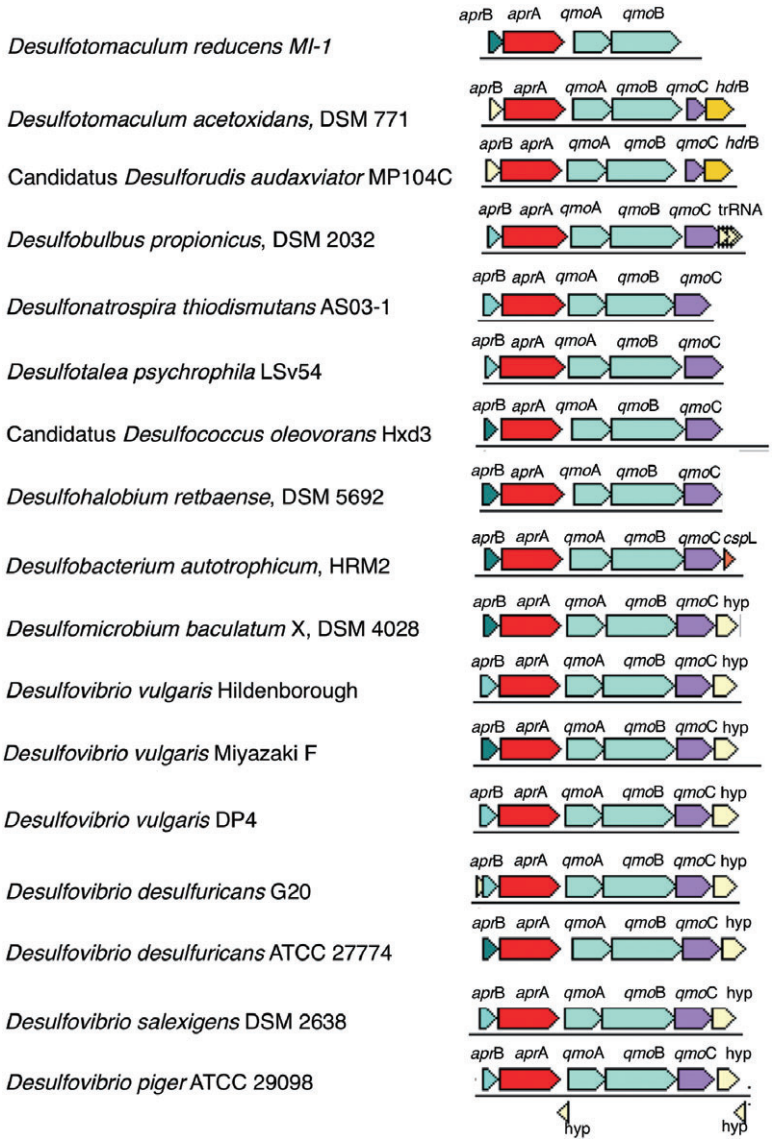

Fig. 3. *apr*BA and *qmo*ABC organization for all available SRB genome sequences. *apr*BA, beta and alpha subunit of the APS reductase; *qmo*ABC, alpha, beta and gamma subunits of the quinone-interacting membrane-bound oxidoreductase. QmoC is the transmembrane subunit but is absent in *D. reducens* and soluble in *D. acetoxidans* and *D. audaxviator*. *csp*L, cold shock protein; hyp, hypothetical protein; *hdr*B, heterodisulfide reductase; *qmo*AB, genes encoding a heterodisulfide reductase and a methyl-viologen reducing hydrogenase protein complex.

as electron transfer flavoproteins (EtfAB) or heterodisulfide reductase (Hdr) (Fig. 2).

Reduction of sulfite to sulfide. The sulfite reductase (dred_3186-7) in *D. reducens* is homologous to sulfite reductases from all other SRB. In *Desulfovibrio* spp. and presumably other Gram-negative SRB, a transmembrane complex, the Dsr complex, is postulated to transfer periplasmic electrons to the sulfite reductase (Heidelberg *et al*., 2004). The equivalent complex is dubbed Hme complex in *Desulfobacterium autotrophicum* (Strittmatter *et al*., 2009). The Dsr complex is a five-protein complex (DsrMKJOP) (Pires *et al*., 2006) whose encoding genes show almost perfect synteny across all 14 sequenced Gram-negative SRB (Fig. 4). In *Dv. desulfuricans*, DsrM is an integral membrane cytochrome *b* and DsrK is a soluble protein homologous to the HdrD subunit of the heterodisulfide reductase (Pires *et al*., 2006). Using the Ortholog Neighborhood Viewer tool in JGI-IMG (http://img.jgi.doe.gov) for *Dv. vulgaris* Hildenborough's *dsr*MKJOP against all SRB genomes, we were able to identify orthologous genes for *dsr*MK but not for *dsr*JOP in *D. reducens* (Fig. 4). These genes were annotated as the gamma subunit of a nitrate reductase (dred_3199) and a putative reductase (dred_3198). The gamma subunit of the nitrate reductase (dred_3199, *dsr*M) is a *b*-type cytochrome and is predicted to have five transmembrane helices and to pertain to a protein family (PF02665) that receives electrons from the quinone pool. While there is some similarity between dred_3198-9 and *dsr*MK from *Dv. vulgaris* Hildenborough, it is clear that there is a dichotomy between these genes and associated proteins in Gram-positive bacteria and their counterparts in Gram-negative bacteria. In fact, a BLAST analysis of the

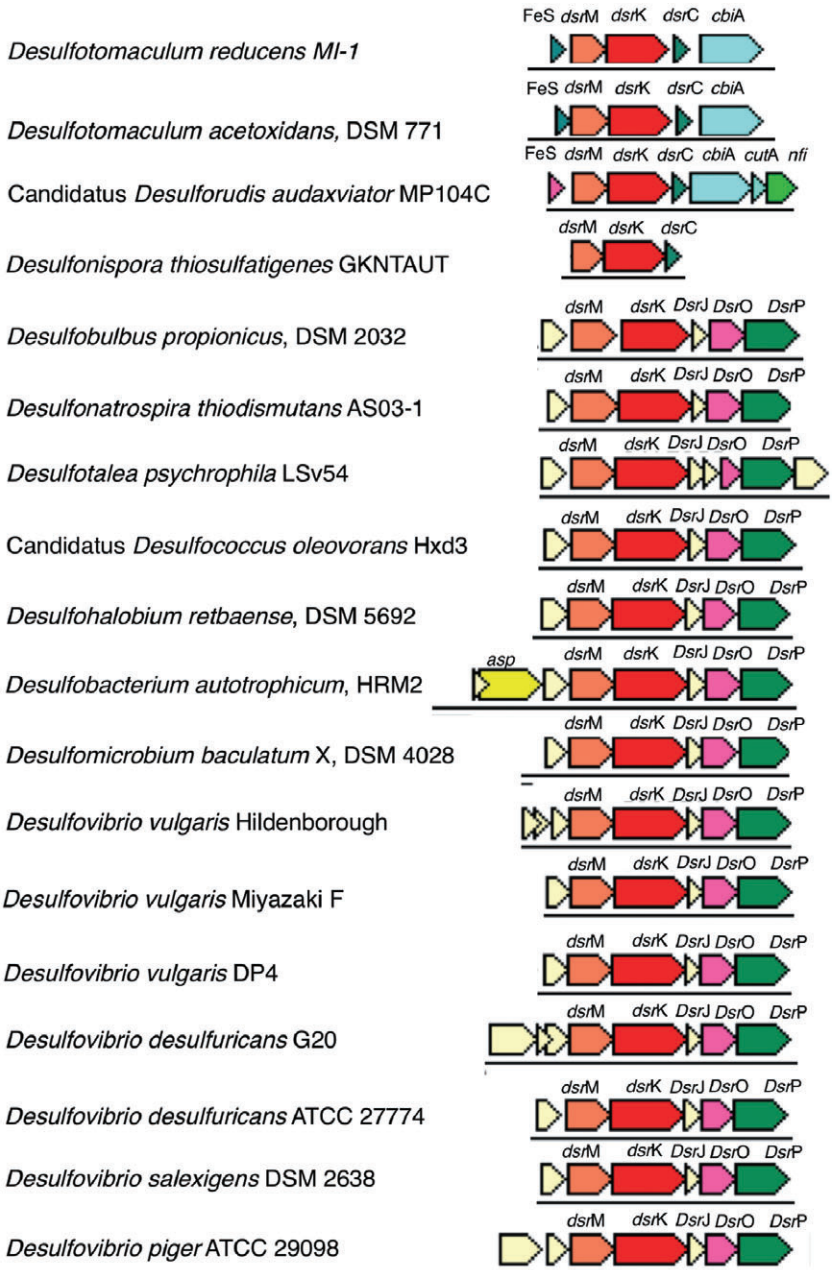

Fig. 4. Gene organization for the *dsr* operon for all available genome sequences. *cbi*A, cobyrinic acid synthase; *cut*A, copper toxicity protein; FeS, iron sulfur protein; *nfi*, endonuclease V; *asp*, aspartyl aminopeptidase. Unlabelled genes are hypothetical proteins.

membrane-spanning protein encoded by dred_3199 yields no hits for Gram-negative SRB. It has been suggested that the Dsr complex is made up of two modules DsrMK and DsrJOP and that the DsrMK module might be involved in menaquinol oxidation as well as the reduction of a cytoplasmic substrate (Pires *et al.*, 2006). That description of the activity of DsrMK, in the absence of DsrJOP, is consistent with its potential electron transport role (from the quinone pool to a cytoplasmic protein) in *D. reducens* and other Gram-positive SRB (Fig. 2).

Adjacent to *dsr*MK, arranged as part of what appears to be the same operon, the *D. reducens* genome (and that of the other three sequenced Gram-positive SRB) encodes a soluble protein (dred_3197) that corresponds to the gamma subunit of the dissimilatory sulfite reductase (DsrC) (Fig. 4). In *D. vulgaris*, DsrC has recently been implicated in serving as an electron shuttle between the membrane-bound DsrMKJOP complex and the cytoplasmic DsrAB sulfite reductase (Oliveira *et al.*, 2008). In that study, DsrC was found to interact with DsrK (the cytoplasmic catalytic subunit) and to form a persulfide-containing intermediate that once dissociated from DsrAB was released as sulfide. We hypothesize that, in *D. reducens* and other Gram-positive SRB, DsrC may similarly transfer

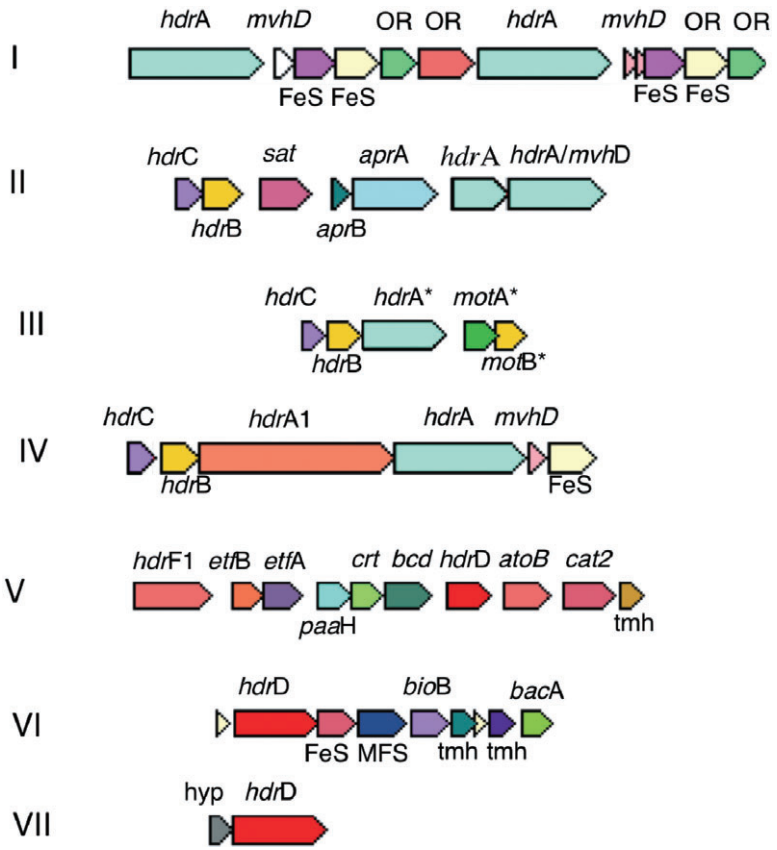

Fig. 5. Seven *hdr* loci in *D. reducens*. *etf*AB, electron transfer flavoprotein; *paa*H, 3-hydroxybutyryl-CoA dehydrogenase; *crt*, 3-hydroxybutyryl-CoA dehydratase; *bcd*, butyryl-CoA dehydrogenase; *ato*B, acetyl-CoA C-acetyltransferase; *cat*2, 4-hydroxybutyrate coenzyme A transferase; *bio*B, biotin synthetase; *bac*A, undecaprenyl-diphosphatase; FeS, iron-sulfur protein; *mvh*D, methyl viologen hydrogenase, delta subunit; MFS, major facilitator superfamily protein; OR, oxidoreductase; tmh, transmembrane helices-containing protein; *hdr*A, *hdr*B, *hdr*C, *hdr*D, *hdr*F, different heterodisulfide reductase subunits; *hdr*F1, transmembrane heterodisulfide reductase; *hdr*A1, NADH-dependent heterodisulfide reductase. Locus I is dred_0137 to dred_0148; Locus II is dred_0633 to dred_0639 (includes *qmo*AB which are dred_0638-9); locus III is dred_0358 to dred_0354; locus IV is dred_1325 to dred_1330; locus V Is dred_1777 to dred_1786; locus VI is dred_0427 to dred_0433 and locus VII is dred_0689 to dred_0690. The asterisk '*' indicates a selenocysteine-containing protein.

electrons from DsrK to DsrAB, which in turn, is involved in the reduction of sulfite to sulfide. The overall electron transfer would then take place from the quinone pool to DsrM, DrsK, DsrC and finally DsrAB (Fig. 2).

Electron flow to APS and sulfite reductases. Other transmembrane electron transfer complexes present in *Dv. vulgaris* (e.g., HmcABCDEF or RnfABCDGE) are not found in *D. reducens* or other Gram-positive SRB, which is consistent with the presence of a distinct sulfate reduction mechanism in Gram-positive SRB. In *D. reducens*, the electrons are generated via the oxidation of organic substrates and transit via NADH, H_2, formate, CO as well as various reduced soluble electron transport proteins such as ferredoxin (dred_2206) and electron transfer flavoproteins (EtfAB) (dred_1778-9, dred_1538-9, dred_0572-3 and dred_0367-8) (Fig. 2).

Formate is oxidized via formate dehydrogenase (dred_1112-19) that includes a ten-TMH transmembrane subunit (dred_1116) annotated as a polysulfide reductase and that is part of a protein family (PF03916) that is known to participate in electron exchange with the quinone pool. The oxidation of formate to CO_2 could be coupled to sodium translocation (Fig. 2) as the last ORF in the operon (dred_1119) encodes a sodium-dependent transporter. This ORF is slightly upregulated during lactate-dependent sulfate reduction (data not shown), suggesting the formation of formate as an intermediate of lactate oxidation. The electrons are presumably passed on to the quinone pool. Similarly, NADH is oxidized by a quinone-interacting, proton-translocating NADH dehydrogenase (DH) (dred_2036-46) that also passes electron to the quinone pool (Fig. 2). From the quinone pool, the electrons are transferred to DsrM and on to sulfite reductase, as suggested above.

Heterodisulfide reductase. The genome of *D. reducens* encodes numerous heterodisulfide reductases (Hdr) found in seven loci (Fig. 5). A recent analysis of the genome of *Desulfobacterium autotrophicum* HRM2 showed nine *hdr* loci in that organism. Many of the cytoplasmic Hdr in *Db. autotrophicum* were presumed to be involved in (i) the transfer of electron from transmembrane complexes to the sulfite reductase, (ii) the reduction of the disulfide bond of the DsrABC complex, and (iii) the reduction of ferredoxin (Strittmatter *et al*., 2009). In *D. reducens*, the *hdr* loci include several gene configurations: *hdr*A associated with *mvh*D (loci I, II and IV), *hdr*A, *hdr*B and *hdr*C in close proximity (loci II, III, IV), a membrane-spanning *hdr*F associated with *etf*AB and with *hdr*D (locus V) and *hdr*D alone (loci VI and VII) (Fig. 5). Locus I consists of two identical repeats of the same

sequence (dred_0137 to dred_0141 and dred_0143 to dred_0148) separated by a gene encoding a protein of unknown function (dred_0142).

In two loci (II and IV), the presence of all three subunits of Hdr (HdrABC) along with the delta subunit of an Mvh (a [NiFe] H_2ase) is reminiscent of the Mvh : Hdr complex in the methanogen *Methanothermobacter thermoautotrophicus* (Setzke *et al*., 1994). This complex is thought to transfer electrons from H_2 to an electron acceptor interacting with the Hdr (Mander *et al*., 2004). The main difference between the methanogen and *D. reducens* is the absence of the other subunits of Mvh : *Methanothermobacter marburgensis* has three (Stojanowic *et al*., 2003), including two (MvhA and MvhG) that correspond to hydrogenase modules conserved in all [NiFe] H_2ases, but *D. reducens* only has the delta subunit (MvhD) that is unique to Mvh (Table S1). The delta subunit has been implicated in the transfer of electrons from Mvh to Hdr (Stojanowic *et al*., 2003). By analogy, electrons derived from a soluble cytoplasmic protein could be transferred to MvhD and on to HdrA (locus I) or HdrABC (locus IV) and to the APS reductase (Fig. 2).

Locus V combines the 4-TMH membrane-spanning *hdr*F (dred_1777) with electron-transfer flavoproteins (dred_1778-9) and an *hdr*D subunit (dred_1783) (Fig. 5). It is reminiscent of HdrDE in *Methanosarcina barkeri* (Heiden *et al*., 1994) but lacks HdrE, the membrane-anchoring *b*-type cytochrome. In *Methanosarcina mazei*, this complex is a proton pump that is part of the electron transport chain and receives electrons from hydroxyphenazine and uses them to reduce the CoenzymeM–CoenzymeB complex (Ide *et al*., 1999; Baumer *et al*., 2000). In *D. reducens*, the membrane-spanning domain pertains to HdrF and it is likely that this subunit receives electrons from the quinone pool and passes them on to the electron-transfer flavoproteins (Fig. 2). It is also possible that proton–translocation occurs during the electron transfer (Fig. 2).

If the current proposed scheme for sulfate reduction in *D. reducens* (Fig. 2) is confirmed, it would represent a significant departure from the accepted model of 'redox cycling' for sulfate reduction by Gram-negative SRB. In the proposed scheme (shown in red in Fig. 2), electrons from electron donors (such as lactate or butyrate) would be transferred to the Qmo complex from intermediate electron-carrying proteins such as heterodisulfide reductase and/or electron transfer flavoproteins, and Qmo would pass the electrons on to APS reductase, which in turn, would reduce APS to sulfite. Electrons could also be transferred from electron donors to electron-carrying compounds such as H_2, formate and NADH. Those electrons would be transferred to the quinone pool by proteins such as the membrane-bound hydrogenase, formate DH and NADH DH. From the quinone pool, electrons could be transferred to the DsrC subunit of the sulfite reductase via the DsrMK complex and sulfite reduced to sulfide. The generation of a proton motive force, which relies on the presence of periplasmic H_2 in Gram-negative bacteria, would occur via the translocation of protons through pyropohosphatase, formate DH, membrane-bound H_2ase, the quinone pool as well as NADH DH. Given that many of the proteins proposed to be involved in sulfate reduction in *D. reducens* have homologues in the other sequenced Gram-positive SRB (i.e., pyrophosphatase, DsrMK, QmoAB, HdrABC, MvhD), *D. reducens* may serve as a model for sulfate reduction in Gram-positive SRB.

Hydrogen metabolism

In general, the consumption and the evolution of H_2 are mediated by hydrogenases (H_2ases), redox metalloenzymes that catalyse the reversible reaction $H_2 \leftrightarrow 2H^+ + 2e^-$ (Vignais *et al*., 2001; Vignais and Colbeau, 2004; Meyer, 2007). These enzymes have been classified as either [FeFe], [NiFe] or [NiFeSe] H_2ases according to the composition of their metal sites.

The *D. reducens* genome encodes six hydrogenases (Table 2). This number of hydrogenases is comparable with that in the genomes of organisms that, in contrast to *D. reducens*, are able to conserve energy by using H_2 as a source of electrons. For instance, the genome of *Dv. vulgaris* in which sulfate reduction depends on H_2 oxidation encodes six hydrogenases (Heidelberg *et al*., 2004), and that of *Geobacter sulfurreducens,* which is able to couple H_2 oxidation to growth with Fe(III) as an electron acceptor, has four (Methe *et al*., 2003). All six H_2ases in *D. reducens* are Fe-containing hydrogenases and four of them are encoded by multi-locus operons and represent trimeric H_2ases (Table 2). The other two (dred_1440 and dred_1794) are single-gene hydrogenases.

Trimeric Fe-containing H_2ases. Three (dred_3290-92, dred_1651-53 and dred_1654-56) of the four multimeric [FeFe] H_2ases are cytoplasmic, trimeric hydrogenases composed of a catalytic subunit and two additional subunits and are encoded by non-identical copies of the operon. The genes encoding the catalytic subunit (dred_3290, dred_1651 and dred_1654) contain several FeS binding sites in addition to the active site. The additional subunits are homologous to two peripheral subunits of NADH-ubiquinone oxidoreductase, indicating that these are NAD(P)(H) dependent H_2ases. These trimeric hydrogenases resemble the H_2-evolving [FeFe] H_2ases of the hyperthermophilic bacterium *Thermotoga maritima* that is able to grow by fermenting carbohydrates (Verhagen *et al*., 1999). The three H_2ases were found to be upregulated during sulfate reduction with lactate as an

Table 2. List of hydrogenases identified in the *D. reducens* genome, their characteristics and their expression level during pyruvate fermentation or lactate-dependent sulfate reduction.

						Expression level	
Locus dred_	Product name	Type	Organization	Localization	Size (aa)	Fermentation	Respiration
1440	Hydrogenase	FeFe	Monomeric	Unknown	429	0.63 ± 0.19	1.47 ± 0.61
1794	Hydrogenase	FeFe	Monomeric	Unknown	462	3.37 ± 0.24	0.35 ± 0.03
0461	Hydrogenase	FeFe	Trimeric	Membrane-bound	381	0.94 ± 0.20	1.02 ± 0.04
0462					261	1.14 ± 0.17	0.95 ± 0.06
0463[a]					520	1.39 ± 0.12	0.97 ± 0.03
1651[a]	Hydrogenase	FeFe	Trimeric	Cytoplasmic	593	0.16 ± 0.09	1.88 ± 0.10
1652					569	0.12 ± 0.08	2.06 ± 0.09
1653					158	0.12 ± 0.06	2.34 ± 0.07
1654[a]	Hydrogenase	FeFe	Trimeric	Cytoplasmic	659	0.48 ± 0.06	19.75 ± 0.79
1655					627	0.47 ± 0.16	12.76 ± 0.74
1656					163	0.42 ± 0.10	11.09 ± 0.47
3290[a]	Hydrogenase	FeFe	Trimeric	Cytoplasmic	594	0.39 ± 0.02	2.16 ± 0.54
3291					575	0.09 ± 0.02	3.06 ± 1.05
3292					177	0.11 ± 0.02	2.97 ± 1.08

a. Indicates the catalytic domain-containing subunit in trimeric hydrogenases.

electron donor but not during pyruvate fermentation (Table 2), suggesting that H_2 production may be mediated by these trimeric [FeFe] H_2ases and coupled to H_2 consumption (no H_2 is detected in the medium) during lactate oxidation.

The fourth trimeric [FeFe] hydrogenase (dred_0461-3) is membrane-bound and is encoded by an operon that includes a FeS-cluster-containing component (dred_0462) and a putative hydrogenase cytochrome *b* subunit (dred_0461) that is predicted to contain ten transmembrane helices. This H_2ase is homologous to a potentially H_2-consuming hydrogenase in *Desulfitobacterium hafniense* Y51 (Nonaka *et al*., 2006). Thus, this H_2ase could be involved in the transfer of electrons from H_2 to the quinone pool and – by virtue of its membrane localization – transfer protons to the outside (Fig. 2).

Monomeric Fe-containing H_2ases. These two H_2ases (dred_1440 and dred_1794) are upregulated (Table 2) either during pyruvate fermentation (dred_1794), suggesting the evolution of H_2, or during sulfate reduction (dred_1440) suggesting the consumption of H_2.

Phylogenetic study of the catalytic subunit. A phylogenetic study of the catalytic subunit of the six [FeFe] hydrogenases (Fig. 6) reveals that there are two major clusters. One corresponds to fast-evolving hydrogenases (the branches are long and there is more evolutionary distance between adjacent species) and the other to slow-evolving hydrogenases (shorter branches with less evolutionary distance between adjacent species). It is readily apparent that the trimeric H_2ases are in the slow-evolving branch whereas their monomeric counterparts are in the fast-evolving branch. This is consistent with the need for evolution in concert of the three subunits for the trimeric case whereas the monomeric H_2ases can evolve independently.

The overall picture that emerges from the above sections is that the trimeric cytoplasmic [FeFe] H_2ases are responsible for the production of H_2 during sulfate reduction, the trimeric [FeFe] membrane-spanning H_2ase consumes H_2 and translocates H^+ and the monomeric [FeFe] H_2ases either produce (dred_1794) or consume (dred_1440) H_2.

Metal reduction

Vegetative cells of *D. reducens* are able to reduce soluble Fe(III) with pyruvate, lactate or butyrate but not H_2 as an electron donor, in a process that supports growth (Fig. S7). This is an unusual ability in SRB as there are only four other SRB known to couple Fe(III) reduction to growth: *D. psychrophila* (Knoblauch *et al*., 1999), *Desulfosporosinus lacus* (Ramamoorthy *et al*., 2006), *Desulfosporomusa polytropa* (Sass *et al*., 2004) and *Desulfobulbus propionicus* (Holmes *et al*., 2004). Furthermore, vegetative cell suspensions (Tebo and Obraztsova, 1998) as well as spores (Junier *et al*., 2009) of *D. reducens* were shown to reduce U(VI) with butyrate and H_2 as an electron donor, respectively.

The biochemical mechanism of microbially mediated iron reduction has been studied in some detail in two bacterial genera: *Geobacter* and *Shewanella*. In *Geobacter* spp., direct contact between Fe(III) oxides and bacterial cells is required and the production of conductive 'nanowires' facilitates the transfer of electrons to the solid phase (Reguera *et al*., 2005). The transfer of electrons from the NADH dehydrogenase to the terminal electron acceptor during iron catabolism requires five *c*-type cytochromes and a type IV pilus (Weber *et al*., 2006).

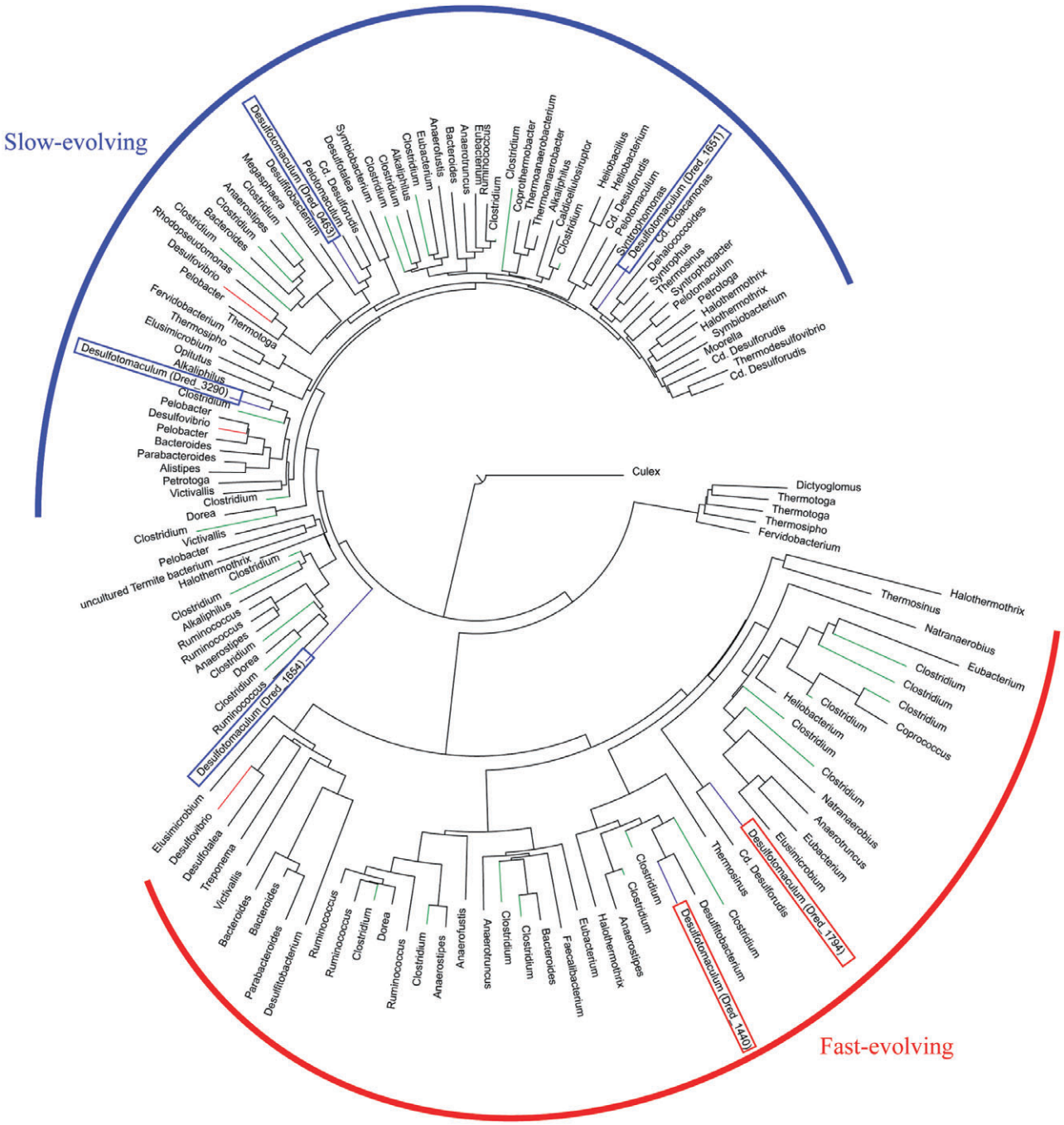

Fig. 6. Neighbour-joining phylogenetic tree of Fe-only hydrogenases based on aligments of the H cluster domain. Clades consisting entirely of the same genus were condensed to a single leaf. The branches corresponding to the different Fe-only hydrogenase genes in *Desulfotomaculum reducens* are boxed in blue for trimeric hydrogenases and in red for monomeric hydrogenases.

Shewanella oneidensis MR-1 also produces conductive appendages (Gorby *et al*., 2006) and also requires *c*-type cytochromes for soluble and insoluble Fe(III) reduction (Beliaev *et al*., 2001). In addition, the use of endogenous and exogenous soluble extracellular electron shuttles has been well documented to mediate the reduction of Fe(III)-oxides in both genera (Newman and Kolter, 2000; Nevin and Lovley, 2002).

As for microbial U(VI) reduction, in *S. oneidensis*, the pathways for iron and U(VI) reduction are similar and involve several *c*-type cytochromes (Beliaev *et al*., 2001; Bencheikh-Latmani *et al*., 2005). U(VI) reduction in *G.*

sulfurreducens involves other *c*-type cytochromes than those involved in Fe(III) reduction (Shelobolina *et al*., 2007).

In contrast, the genome of *D. reducens* is strikingly cytochrome-poor. The only *c*-type cytochrome present is a triheme cytochrome c_{552} that is encoded by two genes (dred_0700 and dred_0701). This is an integral membrane *c*-type cytochrome annotated as the two-subunit nitrite reductase (NrfHA). This protein is likely to transfer electrons from the quinone pool to an unidentified cytoplasmic or extracellular electron acceptor. Evidence for such a process rests in the small subunit (NrfH) that is predicted (via BLAST analysis) to interact with menaquinol. Even though NrfHA is annotated as a nitrite reductase, it is unlikely to be involved in nitrite reduction. Analysis of mRNA from a *D. reducens* culture amended with nitrite during sulfate reduction shows that this gene has a higher qualitative level of expression in the absence than in the presence of nitrite (Fig. 7C). This is in sharp contrast to the effect of nitrite on the expression of the physiological nitrite reductase in *Dv. vulgaris* for which genes encoding nitrite reductase were highly upregulated (Haveman *et al*., 2004). Thus, the low nitrite reduction activity observed could be due to the sulfite reductase that is known to have some nitrite reductase activity in *Dv. vulgaris* (Haveman *et al*., 2004). We hypothesize that, in *D. reducens*, NrfHA may play a role in electron transfer directly, or indirectly via the quinone pool, to electron acceptors other than nitrite, for example U(VI) (shown in blue, Fig. 2) or soluble Fe(III).

Inspection of electron micrographs of *D. reducens* after U(VI) reduction suggests that the process is associated with the cell wall because the accumulation of U(IV) occurs both inside and outside the cell membrane (Fig. 8). NrfHA is an outside-facing, transmembrane *c*-type cytochrome. Due to its localization, we hypothesize that this protein may be involved in U(VI) reduction.

Several studies have considered U(VI) and Fe(III) reduction by the two SRB *Dv. vulgaris* and *Dv. desulfuricans*. The mechanism of reduction was found to involve both periplasmic (Payne *et al*., 2002) and cytoplasmic (Li and Krumholz, 2009) proteins. Uranium reduction in *Dv. desulfuricans* with H_2 as an electron donor was found to involve the transfer of electrons from a periplasmic hydrogenase via the periplasmic c_3 cytochromes to U(VI) (Payne *et al*., 2002). In *Dv. vulgaris* Hildenborough, Fe(III) and U(VI) reduction was found to also involve periplasmic H_2ases and c_3-type cytochromes (Elias *et al*., 2004). A more recent study has shown that it is a periplasmic [FeFe] H_2ase, not a [NiFe] H_2ase, that is involved in the reduction of soluble Fe(III) (Park *et al*., 2008). By analogy, the H_2-consuming, membrane-bound [FeFe] hydrogenase in *D. reducens* (Table 2) could be involved in U(VI) reduction. Paralleling the mechanism described in *Desulfovibrio* spp., the hydrogenase could be transferring electrons from H_2 to the *c*-type cytochrome NrfHA, which would serve as the terminal reductase (shown in blue, Fig. 2).

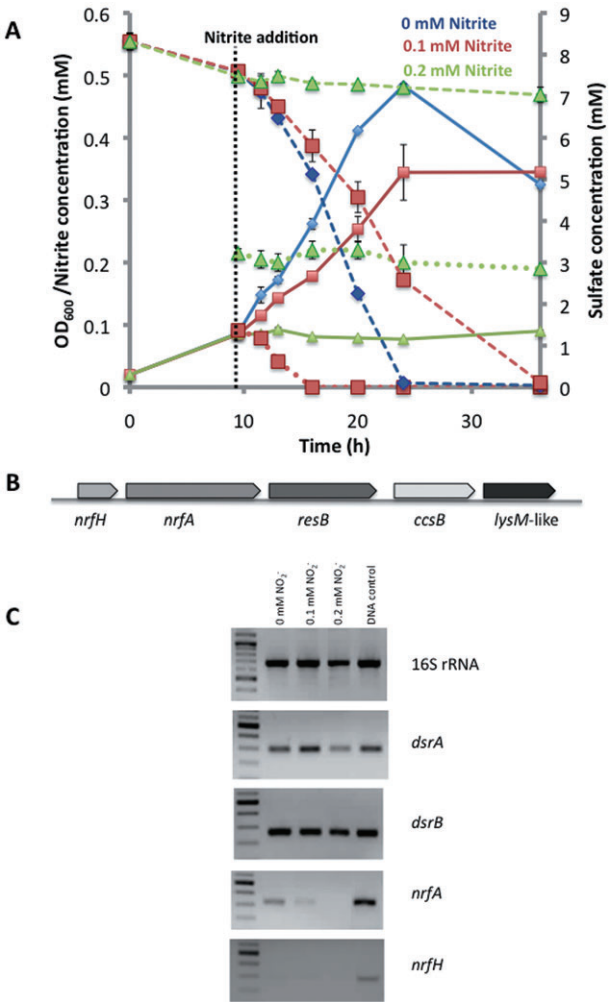

Fig. 7. Effect of nitrite on growth of and sulfate reduction by *D. reducens*.
A. Cell growth as OD_{600} (closed symbols and solid lines) sulfate (closed symbols and dashed lines) and nitrite (open symbols and dashed lines) reduction. The addition of 0.1 mM nitrite inhibits growth and sulfate reduction, but the cells recover after nitrite is removed from solution. In contrast, 0.2 mM nitrite shuts down growth and sulfate reduction.
B. Nitrite reductase operon.
C. Reverse transcription PCR results for *dsr*AB and *nrf*HA after addition of nitrite. NrfA is expressed in the absence of nitrite but to a lesser extent in its presence. NrfH is not detectable in any of the cases considered.

In addition to the H_2ases, a cytoplasmic thioredoxin, a thioredoxin reductase and associated oxidoreductase were found to participate in U(VI) reduction in the cytoplasm of *Dv. desulfuricans* G20 (Li and Krumholz, 2009). In an effort to determine whether a similar mechanism could be involved in *D. reducens*, we evaluated the pres-

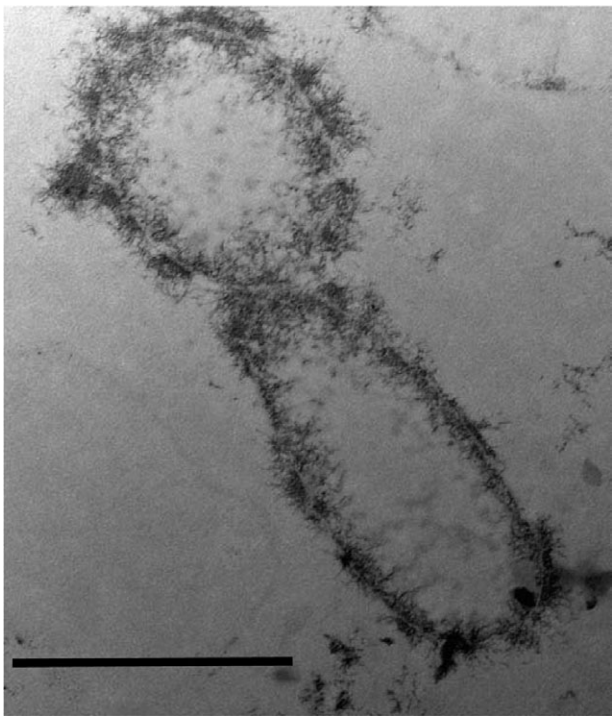

Fig. 8. Electron micrograph of thin sections of a *D. reducens* culture grown fermentatively with pyruvate in the presence of 100 μM U(VI) and stained with Sato lead. Scale bar is 1 μm.

ence of thioredoxins in its genome. There are three ORFs annotated as thioredoxins in the *D. reducens* genome (dred_0762, dred_0904 and dred_2669), but only one (dred_2669) is associated with a thioredoxin reductase (dred_2670). A third gene (dred_2668) that includes nine TMH and has no known function is present as part of this apparent operon. This thioredoxin could possibly be involved in U(VI) reduction, but no direct evidence for this is available at the present time.

Conclusions

Desulfotomaculum reducens is one of two cultivated Gram-positive SRB for which a complete genome is available. Due to the absence of a periplasm, the mechanism of sulfate-reduction in this SRB does not correspond to that identified in *Desulfovibrio vulgaris* and likely involves the transfer of electrons directly from the quinone pool to the DsrMK complex and to the sulfite reductase (DsrABC). In addition, it is proposed that the cytoplasmic QmoAB complex transfers electrons from cytoplasmic proteins to the APS reductase. Other proteins such as heterodisulfide reductases and electron-transfer flavoproteins are likely involved in electron transfer and we propose that they may provide electrons to the Qmo complex. However, a detailed mechanism cannot be surmised by genome analysis. Finally, a number of proton-translocating transmembrane proteins – notably a pyrophosphatase conserved in all four Gram-positive SRB sequenced to date – suggest the production of a proton motive force without periplasmic oxidation of H_2. Because many of the proteins involved in the *D. reducens*-specific sulfate-reduction pathway have homologues in the genomes of other Gram-positive SRB, *D. reducens* may represent a good model for sulfate-reduction in Gram-positive SRB.

Experimental procedures

DNA extraction and purification

Genomic DNA was extracted from an overnight culture of *D. reducens* that was grown in Widdel medium (Widdel and Bak, 1992) with sulfate (10 mM) as an electron acceptor and lactate (20 mM) as an electron donor. DNA extraction was carried out using CTAB (hexadecyltrimethyl ammonium bromide). The cells were treated with 100 mg ml^{-1} lysozyme, followed by proteinase K (10 mg ml^{-1}) and the product amended with a solution of 0.3 M CTAB and 0.7 M NaCl and a chloroform:isoamyl alcohol (24:1) solution. After centrifugation of the mixture, the supernatant was transferred to a new tube and amended with a phenol:chloroform:isoamyl alcohol (25:24:1) solution. After a second centrifugation, the supernatant was transferred and the DNA precipitated with isopropanol. A final treatment with RNAse A (Qiagen, Valencia, CA) was used to remove residual RNA.

Sequencing

The genome of *D. reducens* MI-1 was sequenced at the Joint Genome Institute (JGI) using a combination of 3 kb, 8 kb and 40 kb (fosmid) DNA libraries. All general aspects of library construction and sequencing performed at the JGI can be found at http://www.jgi.doe.gov/. Draft assemblies were based on 40 920 total reads. All three libraries provided 11× coverage of the genome. The Phred/Phrap/Consed software package (http://www.phrap.com) was used for sequence assembly and quality assessment (Ewing and Green, 1998; Ewing *et al.*, 1998; Gordon *et al.*, 1998). After the shotgun stage, reads were assembled with parallel phrap (High Performance Software, LLC). Possible mis-assemblies were corrected with Dupfinisher (Han and Chain, 2006) or transposon bombing of bridging clones (Epicentre Biotechnologies, Madison, WI, USA). Gaps between contigs were closed by editing in Consed, custom primer walk or PCR amplification (Roche Applied Science, Indianapolis, IN, USA). A total of 2 634 additional reactions were necessary to close gaps and to raise the quality of the finished sequence. The completed genome sequences of *D. reducens* contains 43 940 reads, achieving an average of 12-fold sequence coverage per base with an error rate less than 1 in 100 000.

Annotation

Genes were identified using a combination of Critica (Badger and Olsen, 1999) and Glimmer (Delcher *et al.*, 1999), fol-

lowed by a round of manual curation, which resulted in adjustments of the start codons and insertion of missed genes and pseudogenes. tRNAs were predicted using the tRNAScan-SE tool (Lowe and Eddy, 1997). Automatic product name assignment was made based on the results obtained from searches against curated databases. Signal peptides were identified using the SignalP 3.0 (Bendtsen *et al.*, 2004) and TMHMM (Krogh *et al.*, 2001) at default values. Manual curation of the automatic annotation occurred within the IMG-ER (http://merced.jgi-psf.org/cgi-bin/er/main.cgi) system. The sequence data described here have been deposited in GenBank (CP000612).

Phylogenetic analysis of Fe-Fe hydrogenases

The hydrogenase phylogeny was based on the H cluster, which is shared by all members of the hydrogenase family (Vignais *et al.*, 2001). The position of the H cluster in one sequence was determined by scanning for Pfam PF02906 (H cluster) with Prosite. The sequences were then aligned with Muscle (Edgar, 2004), and the alignment was trimmed so as to keep only the H cluster. A phylogenetic tree was then constructed using BioNJ (Gascuel, 1997), using a eukaryote (*Culex quinquefasciatus*) as an outgroup. Clades consisting entirely of the same genus were condensed to a single leaf.

Nitrite reduction as a detoxification strategy

To test nitrite reduction, a culture was grown in WLP medium supplemented with yeast extract, 0.05%; $NaHCO_3$, 30 mM; 1,4-piperazinediethane sulfonic acid disodium salt monohydrate (PIPES), 20 mM; sodium sulfate, 10 mM, lactic acid 20 mM. The culture was incubated for 9.5 h until the onset of growth and sulfate reduction. Aliquots of the culture (20 ml) were transferred to 50 ml serum bottles previously flushed with N_2. The cultures were then supplemented with 0, 0.1 or 0.2 mM of sodium nitrite. Growth was quantified by measuring optical density at 600 nm. Samples for SO_4^{2-} and NO_2^- were collected, filtered (PVDF 0.2 μm filters) and analysed by ion chromatography (DX-500, Dionex, Sunnyvale, CA, USA) using an IonPac AS12A column and a bicarbonate (30 mM) eluent. A sample for RNA extraction was collected at 3.5 h of incubation after the addition of nitrite. RNA was extracted using the RNAeasy Qiagen Kit with on-column DNAse treatment. The quantity and quality of the RNA were evaluated with a Nanodrop spectrophotometer (Thermo Scientific). Reverse transcription PCR (RT-PCR) was carried out for the following genes: 16S rRNA, *nrf*H and *nrf*A. For RT-PCR primers specific for *D. reducens* were designed using Primer-BLAST: 16S rRNA, DR140 (5′-TAG ACC GGG ATA ACA GCT G-3′) and DR842 (5′-ATA CCC GCA ACA CCT AGC AC-3′); *nrf*H, nrfHF (5′- CAT TAT GGA TCC CTG GGT TG -3′) and nrfHR (5′-GTC CTG ACC ACG GTC ATT CT-3′), and *nrf*A, nrfAF (5′-AGC CCC GGA GTC ACT TTT AT-3′) and nrfAR (5′-CAT GAC ACT GGG CAC ATA CC-3′). First-strand synthesis was carried out using SuperScript III from Invitrogen. PCR was carried out using NEB *Taq* DNA polymerase.

Hydrogenase expression data

For expression analysis Widdel medium was used. The medium was dispensed (100 ml) into 200 ml glass serum bottles and autoclaved. The following solutions were added from sterile anaerobic stocks (final concentration): yeast extract, 0.05%; $NaHCO_3$, 30 mM; 1,4-piperazinediethane sulfonic acid disodium salt monohydrate (PIPES), 20 mM. For fermentative growth only 20 mM of pyruvic acid was added to the medium. For sulfate respiration 20 mM sulfate (as sodium sulfate) and 20 mM lactic acid were included in the medium. The final pH of the medium was 7.2 ± 0.2. Growth in the cultures was monitored by OD_{600}. The experiments were run in four biological replicates. The cultures were sampled (16 ml) for RNA extraction at mid-exponential phase. Samples were collected in an RNAse-free 50 ml Falcon tube by centrifugation at 7000 *g* for 7 min and resuspended in 400 μl of 3 mg ml^{-1} lysozyme in TE buffer (pH 8.0) and mixed by vortexing. After digestion for 10 min at room temperature, 1.4 ml of Buffer RLT (Qiagen) containing freshly added 0.01% v/v β-mercaptoethanol was added to the sample and mixed vigorously by vortexing. The homogenized cell lysates were stored at –80°C. After all the samples were collected, the cell lysates were thawed for 15 min at 37°C in a water bath to dissolve salts. The samples were separated into four equal aliquots of 450 μl. RNA extraction was carried out as described above. RNA from the four aliquots was combined and precipitated using 0.1 volume of 1 M sodium acetate and 2.5 volume cold 95% ethanol and incubated over-night at –20°C. RNA was collected by centrifugation for 15 min at 12 000 *g* at 4°C and washed with 75% ethanol. RNA was dried at 37°C for 20 min and resuspended in 30 μl RNAse-free water. RNA concentration and quality were re-measured using the Nanodrop and the Bioanalyzer (Agilent). A total of 20 μg of RNA per sample was sent to Nimblegen Roche for cDNA synthesis and hybridization onto custom-designed 4x77K microarrays. Normalized RMA signals were used for the analysis using the software GeneSpring GX v7.3. Per gene normalization was applied when the data were loaded into a customized one-colour experiment in the analysis software. Expression data were extracted using the option 'advanced find gene'. Average values were calculated from the four biological replicates.

Electron microscopy

Samples for electron microscopy were prepared by fixing the cells in gluteraldehyde and dehydrating them sequentially in pure grade Ethanol (Fluka). The fixed dehydrated cells were then pelleted and immobilized in LR-white resin (EMS, Hatfield, PA, USA) that was polymerized at 60°C. This procedure was carried out inside an anaerobic chamber to prevent sample oxidation. Thin sections of the resin were cut using a microtome, placed on copper grids (Quantifoil Micro Tools GmbH, Jena) and stained with Sato lead. The samples were observed in a FEI CM20 microscope (Eindhoven, Netherlands). Images were recorded on a Gatan 797 slow scan CCD camera (1024 pixels × 1024 pixels × 14 bits) and processed with the Gatan Digital Micrograph 3.11.0 software (Gatan, Pleasanton, CA, USA).

Access to the genome is provided by the DOE Joint Genome Institute (JGI) at the Integrated Microbial Genome (IMG) site: http://img.jgi.doe.gov/cgi-bin/pub/main.cgi

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Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1. Genes involved in glycolysis in *D. reducens*.
Fig. S2 Genes involved in the reductive pentose phosphate pathway in *D. reducens*.
Fig. S3 Genes involved in the partial reductive TCA cycle in *D. reducens*.
Fig. S4 Growth of *D. reducens* in the presence and absence of yeast extract.
Fig. S5 Genes involved in pyruvate metabolism in *D. reducens*.
Fig. S6 Inhibition of sulfate reduction in *D. reducens* by CO.
Fig. S7 Fe(III) reduction by *D. reducens*.
Table S1. Genes with homology to the delta subunit of methyl-viologen hydrogenase.

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