Biochimica et Biophysica Acta 1837 (2014) 1148–1164

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Review

The "bacterial heterodisulfide" DsrC is a key protein in dissimilatory sulfur metabolism☆

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ARTICLE INFO

Article history:
Received 1 December 2013
Received in revised form 7 March 2014
Accepted 13 March 2014
Available online 22 March 2014

Keywords:
Dissimilatory sulfur metabolism
Sulfate reducing bacteria
Sulfur oxidizing bacteria
DsrC
CCG domain
Heterodisulfide

ABSTRACT

DsrC is a small protein present in organisms that dissimilate sulfur compounds, working as a physiological partner of the DsrAB sulfite reductase. DsrC contains two redox active cysteines in a flexible carboxy-terminal arm that are involved in the process of sulfite reduction or sulfur[1] compound oxidation in sulfur-reducing[2] or sulfur-oxidizing[3] organisms, respectively. In both processes, a disulfide formed between the two cysteines is believed to serve as the substrate of several proteins present in these organisms that are related to heterodisulfide reductases of methanogens. Here, we review the information on DsrC and its possible physiological partners, and discuss the idea that this protein may serve as a redox hub linking oxidation of several substrates to dissimilative sulfur metabolism. In addition, we analyze the distribution of proteins of the DsrC superfamily, including TusE that only requires the last Cys of the C-terminus for its role in the biosynthesis of 2-thiouridine, and a new protein that we name RspA (for regulatory sulfur-related protein) that is possibly involved in the regulation of gene expression and does not need the conserved Cys for its function. This article is part of a Special Issue entitled: 18th European Bioenergetic Conference.

1. Introduction

Heterodisulfide reductases (Hdr) are enzymes present in methanogenic archaea that catalyze the reduction of the heterodisulfide, CoM-S-S-CoB, formed in the last step of methanogenesis, to the corresponding low molecular weight thiols coenzyme M (CoM-SH) and coenzyme B (CoB-SH) [1,2]. In methanogens with cytochromes, Hdr is a membrane-bound enzyme, HdrED, which uses the quinone-like cofactor methanophenazine as electron donor. In hydrogenotrophic methanogens (without cytochromes), Hdr is a soluble protein, HdrABC, that forms a complex with a hydrogenase (MvhADG) [3,4] or a formate dehydrogenase [5]. Although the CoM-S-S-CoB heterodisulfide is found only in methanogens, proteins related to Hdr show a much more widespread distribution, suggesting that energy conservation coupled to disulfide/thiol conversions may be a more general feature [6]. A great diversity of Hdr-like proteins has been found for example in sulfate reducing organisms (SRO) [7–13], including several subunits of respiratory membrane complexes [14]. A small protein, DsrC (also known as DsvC), is believed to be the "bacterial heterodisulfide" that is the substrate for some of these proteins. The term "bacterial heterodisulfide" is not entirely correct since DsrC is also present in some Archaea, but we use it to stress the analogy to the CoM-S-S-CoB heterodisulfide of methanogens. Here, we review the current knowledge on the function of this protein, including its distribution and genetic arrangement in different organisms, and discuss the suggestion that DsrC may be a redox hub in dissimilatory sulfur metabolism. In addition, we describe a new protein belonging to the DsrC superfamily that is possibly involved in gene regulation.

2. General features of DsrC

DsrC is a protein of 12–14 kDa harboring no cofactors and characterized by the presence of a highly conserved C-terminal region containing two strictly conserved cysteine residues. One of these Cys is the penultimate residue at the C-terminus (which we name here as Cys_A) and the other is found ten residues upstream (which we name here as Cys_B). DsrC was first reported as a previously unidentified subunit of the dissimilatory sulfite reductase (DsrAB), as the three polypeptides were found to co-purify in several *Desulfovibrio* spp. [15], and an

Abbreviations: SRO, Sulfate reducing organisms; SOB, Sulfur oxidizing bacteria; Dsr, Dissimilatory sulfite reductase; Hdr, Heterodisulfide reductase; RspA, Regulatory sulfur-related protein; HTH, Helix-turn-helix

☆ This article is part of a Special Issue entitled: 18th European Bioenergetic Conference.

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[1] Sulfur is used here as a general term for sulfur compounds. We use the term "elemental sulfur" when we explicitly refer to sulfur of zero oxidation state.

[2] "Sulfur-reducing organisms" or simply "reducing organisms" are used as general terms for organisms that reduce sulfur compounds in a dissimilatory metabolism, including sulfate, sulfite, thiosulfate or organosulfonates.

[3] "Sulfur-oxidizing organisms" or simply "oxidizing organisms" are used as general terms for organisms that oxidize reduced sulfur compounds in a dissimilatory metabolism, including sulfide, thiosulfate or elemental sulfur.

http://dx.doi.org/10.1016/j.bbabio.2014.03.007
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$\alpha_2\beta_2\gamma_2$ composition was thus proposed for this reductase. However, the cloning of the *dsrC* gene in *Desulfovibrio vulgaris* Hildenborough revealed that it was found in a different transcriptional unit than the *dsrAB* genes, and that their expression was not co-regulated during growth [16]. Reports of purified dissimilatory sulfite reductases lacking the DsrC protein, from the hyperthermophilic archaea *Archaeoglobus fulgidus* [17] and *Pyrobaculum islandicum* [18], provided further evidence that DsrC was probably not a subunit, but rather an interacting partner of DsrAB, and that the complex between the two proteins was for some reason more stable in *Desulfovibrio* spp. Dissociation of DsrC from this complex in *Desulfovibrio desulfuricans* was also reported [19]. It was later shown that, both in *D. vulgaris* Hildenborough and in *Desulfomicrobium norvegicum*, the dissimilatory sulfite reductase is isolated in several oligomeric forms binding two, one or no DsrC molecules per $DsrA_2B_2$ unit [20]. Recently, we reported that the majority of DsrC in cell extracts of *D. vulgaris* is actually not associated with DsrAB [21]. Nevertheless, reports of co-localization of *dsrC* and *dsrAB* genes in several organisms, such as the sulfur oxidizer *Allochromatium vinosum* (where a large *dsr* gene cluster, *dsrABEFHCMKLJOPNRS*, was reported [22,23] — see Fig. 5 below), the thiosulfate reducer *P. islandicum* [18] and the sulfate reducer *Thermodesulforhabdus norvegica* [24], confirmed the generality of a physiological interaction between DsrAB and DsrC.

An important role of DsrC in cellular metabolism is suggested by the fact that *dsrC* is a highly expressed gene in the model sulfate reducer *D. vulgaris*, at similar or even higher levels than genes for other proteins involved in sulfate reduction [21,25–27]. Curiously, in lactate/sulfate conditions, the *dsrC* expression is higher in the stationary than in the exponential phase [16,26,28], whereas in formate/sulfate the reverse is observed [28,29]. In *A. vinosum*, a *dsrC* deletion mutant was genetically unstable and could not be grown or maintained even in the absence of sulfur compounds [30], in contrast to other *dsr* genes, such as *dsrMKJOP*, which were only essential for oxidation of elemental sulfur globules [31,32]. In this organism, the *dsrAB* and *dsrC* genes were recently shown to be upregulated in the presence of several reduced sulfur compounds, and particularly with sulfide [33]. A high *dsrC* mRNA level is also observed in metatranscriptomic data, including samples from marine oxygen minimum zones, where *dsrC* is one of the most abundantly transcribed sulfur energy metabolism genes [34], and samples from intracellular symbionts of the coastal bivalve *Solemya velum* [35].

Several 3D structures of DsrC have been determined, including two NMR structures from *Pyrobaculum aerophilum* [36] and *A. vinosum* [30], and an X-ray structure from *A. fulgidus* [37]. All structures present a similar globular shape with the exception of the C-terminal arm, which adopts an extended and disordered configuration in the solution structures (Fig. 1), whereas it is in a well-defined retracted position in the crystal structure. The two conserved Cys in this arm are found in close proximity in the crystal structure but not actually forming a disulfide bond [37]. In the presence of oxidizing agents, DsrC forms a dimer, but the oxidized monomer with a disulfide bond between Cys_A and Cys_B could be produced by treatment with arginine [21]. The dynamic nature of the C-terminal arm, together with its high amino acid conservation suggested it as a site of protein–protein interaction [36], which was later confirmed in the crystal structure of the *D. vulgaris* DsrAB–DsrC complex (see below) [38]. The globular part of DsrC presents a helix-turn-helix (HTH) structural motif [30,36] (Fig. 1). This is one of the most common motifs involved in protein binding to DNA, but it is also observed in protein–protein interactions [39]. The DsrC structure is most similar to that of the Tet repressor TetR [36], which belongs to the tetrahelical bundle group of HTH proteins. The suggestion that DsrC could be involved in transcriptional regulation [36] was tested in *A. vinosum* and it was shown that DsrC can bind to a putative *dsr* promoter region located upstream of the *dsrA* gene, supporting a possible regulatory function of DsrC [40].

DsrC belongs to a wider family of bacterial proteins designated TusE/DsrC/DsvC family, which are also present in organisms that do not dissimilate sulfur compounds (see Section 4), and in which the cysteines of the C-terminal arm may not be conserved. This includes *Escherichia coli* TusE (previously known as YccK), which was shown to participate in a sulfur-relay system in the form of protein-bound persulfides for the thiouridylation of glutamate, glutamine and lysine transfer-RNAs, involving the sulfurtransferases TusA, TusBCD and TusE [41]. In *E. coli* TusA accepts activated sulfur from the cysteine desulfurase IscS. Then, TusA transfers this sulfur to the TusD subunit of the TusBCD complex. The persulfurated TusD transfers the sulfur to TusE, which interacts with thiouridylase MnmA for 2-thiouridine formation. This role is dependent on the presence of the conserved TusE Cys_A. The thiolation of the uridine base is crucial for precise codon detection and recognition by the cognate aminocyl-tRNA synthase. However, other proteins than TusE can perform thiouridine biosynthesis, since it is clear that 5-methyl-2-thiouridine tRNA derivatives are universally present [42], but the Tus proteins are not conserved in all domains of life, or even in Bacteria. Very recently, a similar sulfur transfer system was shown to

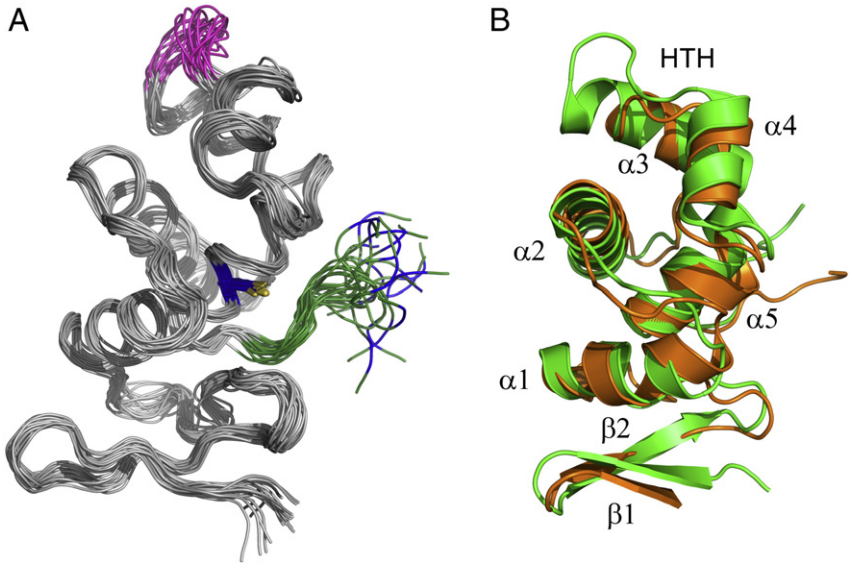


Fig. 1. Three-dimensional structure of DsrC. A) Superimposition of several NMR structures of *A. vinosum* DsrC represented as ribbons. The flexible carboxy-terminus is colored in green, the two conserved Cys are in blue, the sulfur atom of the Cys_B is in yellow, and the HTH-structural motif is in pink. B) Cartoon representation of the superposition of DsrC from *A. vinosum* (green, PDB code: 1YX3) [30] and from *D. vulgaris* (orange, PDB code: 2V4J) [38]. Images were prepared with PyMOL (www.pymol.org).

operate in *A. vinosum* involving the DsrEFH complex (homologous to the TusBCD complex) and DsrC [43]. This process occurs in sulfur-oxidizing bacteria (SOB) (see below), but not in SRO where DsrEFH is not present [32]. In addition, the Tus proteins were shown to be implicated in the maintenance of the intracellular redox state of *E. coli*, due to the reducing capacity of the active cysteines and persulfide moieties of these proteins [44].

3. Role of DsrC in sulfite reduction

The DsrAB sulfite reductase is one of the most important enzymes in dissimilatory sulfur metabolism. The *dsrAB* genes have a widespread distribution, being present not only in SRO and in SOB (where the enzyme presumably works in the reverse direction), but also in several organisms that reduce sulfite, thiosulfate or organosulfonates, in organisms that disproportionate sulfur compounds and also in some syntrophic bacteria [45–47]. Through its operation in SRO, the DsrAB sulfite reductase is believed to be a major player in determining the sulfur isotope fractionation preserved in geological records and used to reconstruct the redox history of Earth's surface [48]. The enzyme, which has an $\alpha_2\beta_2$ ($DsrA_2B_2$) composition, contains a coupled siroheme-[4Fe–4S] as cofactor and belongs to a superfamily that includes also assimilatory sulfite reductases, and nitrite reductases [46,49,50]. Despite years of intensive study, the reaction mechanism of DsrAB has long been a matter of controversy, because, in contrast to the assimilatory sulfite reductase that reduces sulfite directly to sulfide, DsrAB produces a mixture of products in vitro, composed mainly of trithionate and thiosulfate [51,52]. This led to the suggestion that the reduction of sulfite to sulfide proceeds in three steps involving a thiosulfate and a trithionate reductase [53]. However, this proposal was disputed because the DsrAB product composition is strongly dependent on the reaction conditions, which are usually far from those likely to be present in vivo [54,55]. In particular, the production of trithionate and thiosulfate, relative to sulfide, is increased in the presence of high sulfite and low electron donor concentrations [55,56]. The so-called trithionate pathway is also unlikely to be of physiological relevance because there was never consistent evidence for trithionate reductase, and thiosulfate reductases are absent in many SRO [10].

A major step in understanding the DsrAB mechanism came with the determination of two crystal structures of the enzymes from *D. vulgaris* Hildenborough [38] and *A. fulgidus* [57], which resolved years of dispute over the cofactor composition and showed that only one catalytic siroheme-[4Fe–4S] cofactor is present per $\alpha\beta$ unit, bound to DsrB, whereas the equivalent cofactor bound by DsrA seems to have a structural role. The *D. vulgaris* structure was particularly important because the DsrAB protein is present in a $\alpha_2\beta_2\gamma_2$ complex with DsrC, which is absent in the structure from *A. fulgidus*. The *D. vulgaris* structure revealed that the C-terminal arm of DsrC projects inside the DsrAB complex in a way that brings its last conserved cysteine (Cys_A) right next to the catalytic site, where a sulfite molecule is present [38] (Fig. 2A and B). This conspicuous set up led Oliveira et al. [38] to propose a mechanism (Fig. 3) where sulfite is reduced by four electrons to an S^0 valence state that would bind to DsrC Cys_A resulting in a persulfide as a key intermediate. Internal reaction of this persulfide with the other conserved Cys (Cys_B) from the DsrC C-terminal arm would release H_2S, generating a disulfide bond in DsrC ($DsrC_{ox}$), which the authors proposed to be the "heterodisulfide" substrate of the membrane-bound complex DsrMKJOP (described in point 6.2). A similar interaction between DsrAB and DsrC was observed in the structures of the DsrAB–DsrC complexes from *Desulfovibrio gigas* [58] and *Desulfomicrobium norvegicum* [20]. In the *D. gigas* structure the C-terminal arm of DsrC is present in three configurations, including one with the terminal Cys_A positioned right next to a sulfite molecule bound to the siroheme iron (Fig. 2B), and another where the arm is retracted bringing the two Cys in close contact, but not forming a disulfide bond (Fig. 2C). This shows that the transition between the extended and retracted configurations can occur while DsrC is still associated with DsrAB, as proposed by Oliveira et al. [38]. In the *Desulfovibrio* and *Desulfomicrobium* structures [20,38,58], the majority of molecules have the C-terminal Cys_A of DsrC covalently bound to the siroheme (through a covalent bond between the Cys-sulfur and a heme carbon), which obviously inactivates this protein. This Cys-heme cross-link is most likely the result of non-physiological oxidative radical reactions at the siroheme during aerobic purification, but still it indicates that the complex between DsrAB and DsrC is more stable in these organisms than in *A. fulgidus*, where obviously the two proteins dissociate before any such side-reaction can occur.

A detailed mechanism of the reduction of sulfite by DsrAB, not considering DsrC, was also proposed by Parey et al. who suggested that the six-electron reaction proceeds through three two-electron steps and the intermediary formation of [S^{II}] and [S^0] species at the active site [59]. They suggested that these two intermediates could react further with sulfite to generate trithionate and thiosulfate, respectively (Fig. 3). The DsrAB–DsrC complex structures and the mechanism proposed by Oliveira et al. [38] suggest that DsrC is essential for the final conversion of the [S^0] intermediate to sulfide. Taken together, these observations strongly indicate that the in vitro conditions usually used to test the activity of DsrAB – very high sulfite concentrations and absence of DsrC (or inactivity in the case of *Desulfovibrio* spp.) – are responsible for the

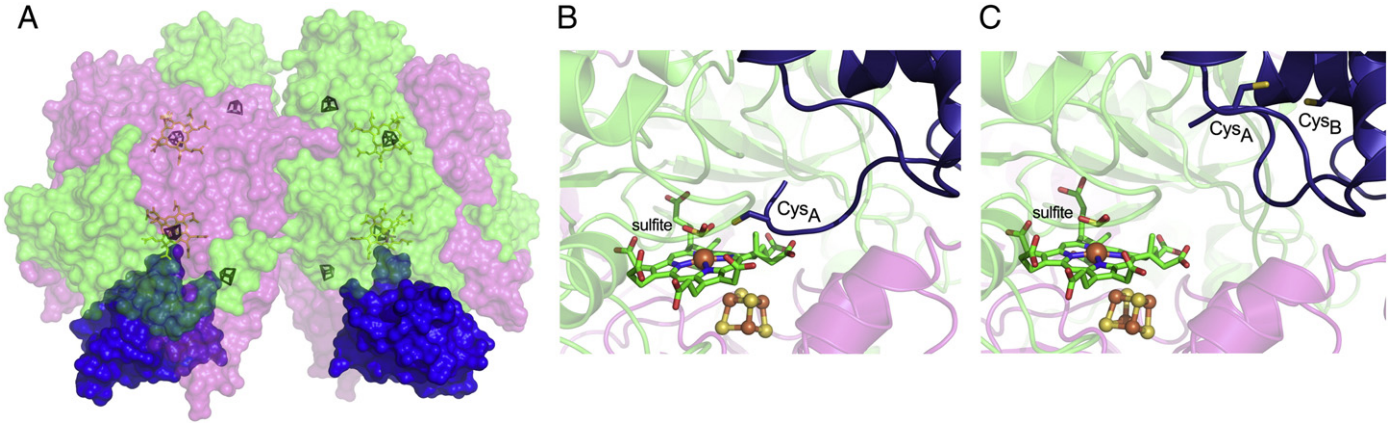


Fig. 2. Structure of the DsrAB sulfite reductase in complex with DsrC. A) Molecular surface representation of the $\alpha_2\beta_2\gamma_2$ DsrAB/C complex from *D. vulgaris* (PDB code: 2V4J) [38]. DsrC (in blue), DsrA (in green) and DsrB (in pink) are semi-transparent for better visualization of the DsrC C-terminal insertion between DsrA and DsrB, reaching the catalytic siroheme. In each DsrAB unit the lower cofactor corresponds to the siroheme-[4Fe–4S] active site and the top one to the non-catalytic sirohydrochlorin-[4Fe–4S]. B and C) Close-up view of the *D. gigas* DsrAB/C catalytic site (PDB code: 3OR2) in cartoon representation [58]. A sulfite molecule is present at the siroheme. The images show two different positions observed for the C-terminal arm of DsrC, which can be found next to the siroheme (B) or in a retracted position where Cys_A and Cys_B come close together (C). This last structure is not deposited in the PDB and was kindly provided by the authors [58]. For clarification the Cys are in stick representation; in (C) the distance between the two Cys sulfurs is 4.2 Å.

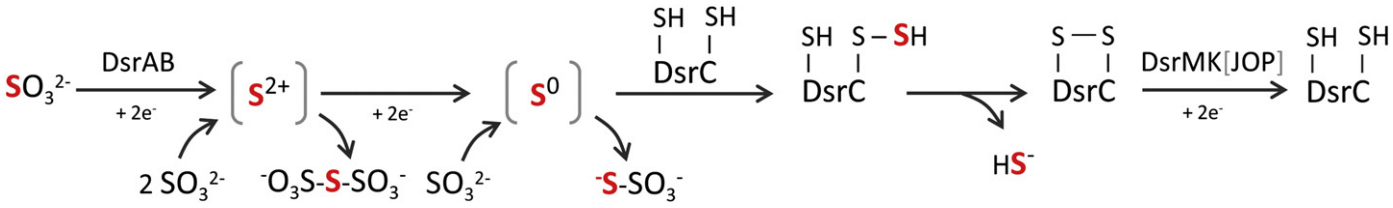


Fig. 3. Schematic representation of the mechanism for sulfite reduction in SRO involving DsrAB, DsrC and DsrMK(JOP), based on the proposals of Oliveira et al. [38] and Parey et al. [59]. The first two intermediates in a S^{II} and S^0 state remain bound at the DsrAB catalytic site, and may react with excess sulfite to produce trithionate and thiosulfate. DsrC is essential for converting the S^0 intermediate to sulfide, and DsrMK[JOP] is involved in restoring the oxidized form of DsrC to the reduced state, a function that is possibly performed also by other HdrB/D-related proteins.

formation of trithionate and thiosulfate as main products, which are formed by further reaction of sulfite with the partially reduced intermediates, under conditions of excess sulfite (Fig. 3). Electron donor limitation, which experimentally results in higher production of the thionates, probably results in an increased life time of the [S^{II}] and [S^0] intermediates, facilitating their reaction with sulfite. These side reactions are possible because the active site of DsrAB can accommodate more than one sulfite molecule, in contrast to the assimilatory enzyme. Parey et al. also reported that the *A. fulgidus* DsrAB enzyme can also reduce trithionate and thiosulfate [59], and we confirmed that the *D. vulgaris* enzyme does the same. In *A. fulgidus*, the absence of DsrC makes the catalytic siroheme extremely solvent-accessible due to the presence of the large channel where the DsrC C-terminal binds, facilitating access of substrate molecules to the active site. In contrast, in the DsrAB structures where DsrC is present this channel is occupied and the substrate reaches the siroheme through another narrower substrate channel [20,38], which, nevertheless, does not prevent production of the thionate products.

The involvement of DsrC in sulfite reduction has very important bioenergetic implications in SRO, because it links this process with chemiosmotic coupling through the DsrMKJOP complex. The mechanism proposed suggests that two of the electrons required to reduce sulfite come from the menaquinone pool via DsrC. If the reduction of the oxidized form of DsrC is electrogenic, this provides a coupling site for the reduction of sulfite. Other possible physiological partners for reduction of $DsrC_{ox}$ are discussed below. In addition, a very important question that remains to be answered is the identification of the physiological electron donor to DsrAB, providing the four electrons required to reduce sulfite to the zero valence intermediate. Further studies are obviously required to fully elucidate the operation of DsrAB, but we have nowadays a much better picture of how it may work.

4. Role and occurrence of DsrC in sulfur oxidation

The DsrC protein is of eminent importance not only for dissimilatory sulfate/sulfite/thiosulfate reduction, but it also plays a prominent role in the composite Dsr pathway of reduced sulfur compound oxidation [31,60]. This pathway involves the accumulation of sulfur globules as a transient product during the oxidation of sulfide, thiosulfate, polysulfides or elemental sulfur. It occurs in many environmentally important photo- and chemolithotrophic bacteria. Low-molecular-weight organic persulfides have been proposed as carrier molecules transferring sulfur from the periplasmic or extracellular sulfur globules into the cytoplasm where it is further oxidized by the Dsr proteins. Recently, it was shown that the DsrEFH protein is an acceptor for persulfidic sulfur imported into the cytoplasm and that the sulfur is then transferred to DsrC [43], just as it has been shown for the related TusBCD and TusE proteins from *E. coli* [41]. DsrE binds sulfur specifically to the conserved Cys78 residue. Persulfurated DsrEFH then serves as an effective sulfur donor for DsrC, which is exclusively persulfurated at the C-terminal Cys_A and not at Cys_B [43]. DsrC finally transfers the sulfur to the dissimilatory sulfite reductase (DsrAB) acting in the oxidative direction, i.e. in reverse of the reaction catalyzed in sulfate reducers (see last figure). The oxidation of sulfite, the product of the DsrAB catalyzed reaction in sulfur oxidizers, to the final product sulfate is performed either indirectly by APS reductase and ATP sulfurylase via adenosine-5′-phosphosulfate (APS), or directly via the cytoplasmically oriented membrane-bound iron–sulfur molybdoenzyme SoeABC [61].

Structural data on DsrAB from a sulfur oxidizer is currently not available, however, given the high sequence similarity of the proteins from sulfur oxidizers and sulfate reducers, the general arrangement of DsrC and DsrAB in the complex should be similar. In its persulfidic state, DsrC could bring sulfane sulfur in contact with the catalytic siroheme of DsrAB where the sulfur is then oxidized. According to the current model DsrC might dissociate from DsrAB in a persulfonated form. DsrC from *A. vinosum* can bind sulfite [43] and this reaction is reversible. A sulfonate group bound to Cys_A could therefore be reductively released as sulfite by the formation of a disulfide bridge between Cys_A and Cys_B. Such a scenario is reasonable as the long known reaction of disulfide bonds with sulfite ($RS-SR + SO_3^{2-} \leftrightarrow RS^- + RS-SO_3^-$) is fully reversible at pH values above 7.0 [62].

Just as proposed for the situation in sulfate reducers, the intramolecular disulfide in DsrC could serve as a substrate of the membrane-bound DsrMKJOP complex (described below). Experimental support for this suggestion was obtained through the proof of direct interaction of *A. vinosum* DsrC with DsrK [63].

Neither DsrEFH nor DsrC have rhodanese (thiosulfate:cyanide sulfurtransferase) or glutathione persulfide:cyanide sulfurtransferase activity, i.e. these two proteins are incapable of mobilizing sulfur from persulfidic carrier molecules and therefore need donor proteins [43]. In *A. vinosum*, the TusA homologue Alvin_2600 is an obvious candidate for direct interaction with, and sulfur transfer to, DsrEFH. In this organism the *tusA* gene is flanked in the same direction of transcription by two genes encoding a rhodanese-like protein (*rhd*, Alvin_2599) and a transmembrane protein of the DsrE superfamily (Alvin_2601). Relative mRNA levels for both the *tusA* and the *dsrE2* gene increased during growth on sulfide and thiosulfate compared to malate [33]. The observed changes match the increases in relative mRNA levels found for genes encoding the sulfur-binding proteins of the Dsr pathway, DsrEFH and DsrC, as well as DsrAB itself, other components of the Dsr system, sulfur globule proteins and also the enzymes of the two different cytoplasmic sulfite oxidation pathways. Notably, the *rhd-tusA-dsrE2* arrangement occurs not only in all currently genome sequenced phototrophic sulfur oxidizers harboring the Dsr system, but also in a wide array of chemo- and further phototrophic sulfur oxidizers that do not contain DsrC or the Dsr pathway. In these organisms, the gene cluster *hdrC1B1A1hyphdrC2B2* is present that encodes a possible heterodisulfide-reductase like protein complex (Table 1). This complex was predicted to be responsible for the oxidation of organic persulfides that are formed as intermediates during the oxidation of externally available elemental sulfur to sulfite in the acidophilic chemolithotroph *Acidithiobacillus ferrooxidans* [64,65]. In fact, the mutually exclusive occurrence of the Dsr and the Hdr-like complex proteins (Table 1) strongly supports the notion that the putative Hdr-like complex is involved in a process functionally replacing the Dsr system. In *A. ferrooxidans* the *rhd-tusA-dsrE2* genes are situated immediately upstream of the genes

Table 1
Occurrence of genes encoding Dsr and Hdr-like complexes, *dsrC*-related genes and the *rhd-tusA-dsrE2* gene cluster in genome-sequenced sulfur oxidizing prokaryotes.

Organism/group	*rhd*	*tusA*	*dsrE2*	*dsrC/tusE/rpsA* copies	*dsr* complex*	*hdr* complex†
Bacteria						
Alphaproteobacteria						
Hyphomicrobiaceae						
Hyphomicrobium denitrificans ATCC 51888	No	YP_003754839	YP_003754829	0	No	YP_003754830-835
Rhodomicrobium vannielii ATCC 17100	No	YP_004010984	YP_004010983	1	YP_004010978-966	No
Rhodospirillaceae						
Magnetospirillum gryphiswaldense MSR-1	No	CAM75689	CAM75687	3	CAM75808-797	No
Magnetospirillum magneticum AMB-1	No	YP_421179	YP_421178	1	YP_422730-41****	No
Magnetococcaceae						
Magnetococcus marinus MC-1	No	YP_864721	YP_864720	1	YP_866063-5/ 5618-24/3983-5	No
Betaproteobacteria						
Burkholderiaceae						
Burkholderiales bacterium JOSHI_001	No	WP_009551576	WP_009551575	2	WP_009551564-551	No
Gallionellaceae						
Sideroxydans lithotrophicus ES-1	No	YP_003524327	YP_003524326	6	YP_003524306-292	No
Hydrogenophilaceae						
Sulfuricella denitrificans skB26	WP_009206414	WP_009206413	WP_009206412	5	WP_009207522-535**	YP_008546724-27††
Thiobacillus denitrificans ATCC 25259	YP_314331	YP_314332	YP_314333	9	YP_316243-230**	No
Thiobacillus thioparus DSM 505	WP_018508572	WP_018508573	WP_018508574	10	WP_018507220-207**	No
Gammaproteobacteria						
Acidithiobacillaceae						
Acidithiobacillus caldus SM-1	YP_004749705	YP_004749704	YP_004749703	0	No	YP_004749702-697
Acidithiobacillus ferrivorans SS3	YP_004784899	YP_004784898	YP_004784897	0	No	YP_004784896-891
Acidithiobacillus ferrooxidans ATCC 23270	YP_002426938	YP_002426937	YP_002426936	0	No	YP_002426935-930
Acidithiobacillus ferrooxidans ATCC 53993	YP_002220598	YP_002220597	YP_002220596	0	No	YP_002220595-589†††
Acidithiobacillus thiooxidans ATCC 19377	WP_010637272	WP_010637274	WP_010637276	0	No	WP_010637278-286
Chromatiaceae						
Allochromatium vinosum DSM 180 T	YP_003444541	YP_003444542	YP_003444543	5	YP_003443222-236***	No
Lamprocystis purpurea DSM 4197	no	WP_020506580	WP_020506581	6	WP_020504936-923**	No
Marichromatium purpuratum 984	WP_005225002	WP_005225001	WP_005225000	3	WP_005223286-308**	No
Thiocapsa marina 5811	WP_007192879	WP_007192878	WP_007192877	7	WP_007193787-773***	No
Thiocystis violascens DSM 198	YP_006414236	YP_006414235	YP_006414234	6	YP_006412728-740	No
Thioflavicoccus mobilis 8321	YP_007244989	YP_007244988	YP_007244987	8	YP_007242648-660	No
Thiorhodococcus drewsii AZ1	WP_007039535	WP_007039534	WP_007039533	4	WP_007040750-736***	No
Thiorhodovibrio sp. 970	WP_009147444	WP_009147445	WP_009147446	5	WP_009147786-773**	No
Ectothiorhodospiraceae						
Alkalilimnicola ehrlichii MLHE-1	No	YP_742508	No	2	YP_742489-502	No
Ectothiorhodospira sp. PHS-1	No	WP_008932742	WP_008932743	0	No	WP_008932744-749
Halorhodospira halophila DSM 244	No	YP_001003503	No	1	YP_001003517-529	No
Thioalkalivibrio nitratireducens DSM 14787	YP_007218369	YP_007218368	No	8	YP_007216031-018**	YP_007218367-362
Thioalkalivibrio sp. K90mix	YP_003459881	YP_003459882	No	0	No	YP_003459883-888
Thioalkalivibrio sulfidophilus HL-EbGr7	YP_002514285	YP_002514284	No	5	YP_002514252-265**	YP_002514283-278
Thioalkalivibrio thiocyanodenitrificans ARhD 1	WP_018232042	WP_018232041	No	6	WP_018232018-031**	No
Thioalkalivibrio thiocyanoxidans ARh 4	WP_006745883	WP_006745882	No	5	WP_006747914-901**	No
Thiorhodospira sibirica ATCC 700588	No	WP_006787555	WP_006787554	0	No	WP_006787553-548
Thiotrichaceae						
Thiothrix disciformis DSM 14473	No	WP_020393794	WP_020393578	4	WP_020397046-032**	No
Thiothrix nivea DSM 5205	No	WP_002708738	WP_002708739	6	WP_002710145-156****	No
Sulfur-oxidizing symbionts						
Candidatus Ruthia magnifica str. Cm	No	YP_903482	YP_903481	3	YP_904057-045	No
Candidatus Vesicomyosocius okutanii HA	No	YP_001219073	YP_001219072	3	YP_001219625-612**	No
Chlorobi						
Chlorobiaceae						
Chlorobaculum parvum NCIB 8327	YP_001997653	No	No	1	YP_001997654-667	No
Chlorobaculum tepidum TLS	NP_661737	NP_661741	NP_661755	2	NP_661745-751	No
Chlorobium chlorochromatii CaD3	YP_380251	YP_380250	YP_380252	1	YP_380249-233	No
Chlorobium ferrooxidans DSM 13031	No	WP_006367306	No	0	Only a *dsrA-like* gene present: WP_006367305	No
Chlorobium limicola DSM 245	YP_001942753	YP_001942125	YP_001942126	1	YP_001942754-771	No
Chlorobium phaeobacteroides BS1	YP_001960259	YP_001960258	YP_001960238	1	YP_001960257-241	No
Chlorobium phaeobacteroides DSM 266	YP_910618	YP_910619	YP_910617	1	YP_910620-636	No
Chlorobium phaeovibrioides DSM 265	YP_001129565	YP_001129566	YP_001129564	1	YP_001129567-583	No
Chloroherpeton thalassium ATCC 35110	No	No	YP_001995564	2	No	YP_001995056-054††††
Chlorobium luteolum DSM 273	YP_373965	YP_373966	YP_373964	1	YP_373967-983	No
Pelodictyon phaeoclathratiforme BU-1	YP_002019137	YP_002019136	YP_002019138	1	YP_002019135-118	No
Prosthecochloris aestuarii DSM 271	YP_002014738	YP_002014739	YP_002014772	1	YP_002014740-756	No
Aquificae						
Aquificaceae						
Aquifex aeolicus VF5	No	No	NP_213270/271	0	No	NP_213272-278†††††
Hydrogenivirga sp. 128-5-R1-1	No	WP_008286378	WP_008286380/379	0	No	WP_008286381-86

Table 1 (*continued*)

Organism/group	*rhd*	*tusA*	*dsrE2*	*dsrC/tusE/rpsA* copies	*dsr* complex*	*hdr* complex†
Aquificae						
Hydrogenobacter thermophilus TK-6	No	YP_003433531	YP_003433530/529		No	YP_003433528-23
Hydrogenobaculum sp. HO	No	YP_007500438	YP_007500437/436	0	No	YP_007500435-30
Hydrogenobaculum sp. Y04AAS1	No	YP_002121749	YP_002121748/747	0	No	YP_002121746-41
Thermocrinis albus DSM 14484	No	YP_003474109	YP_003474108/107	0	No	YP_003474106-101
Archaea						
Crenarchaeota						
Sulfolobaceae						
Acidianus hospitalis W1	No	YP_004458872	YP_004458873/874	0	No	YP_004458871-866
Metallosphaera cuprina Ar-4	No	YP_004409272	YP_004409271/270	0	No	YP_004409273-278
Metallosphaera sedula DSM 5348	No	YP_001191627	YP_001191628/629	0	No	YP_001191626-621
Sulfolobus acidocaldarius DSM 639	No	YP_255044	YP_255045/046	0	No	YP_255043/29-34
Sulfolobus islandicus M.14.25	No	YP_002829162	YP_002829164/163	0	No	YP_002829161-156
Sulfolobus solfataricus P2	No	NP_342591	NP_342590/589	0	No	NP_342592-597
Sulfolobus tokodaii str. 7	No	NP_377858	NP_377860/859	0	No	NP_377857-852

*The *dsr* gene cluster comprises *dsrABCEFHMKLJOPN* if not stated otherwise, in case of the *Chlorobi* the cluster consists of *dsrNCABLUEFHTMKJOPVW*.
**_dsrA-dsrR_. ***_dsrA-dsrS_. ****_dsrA-dsrP_. *****_dsrEFH_ is missing. ******only *dsrB*.
†The cluster consists of *hdrC1B1A1-hyp-hdrC2B2*.
††_hdrBCAA_ are present. †††Instead of *hdrC2* this cluster contains a pseudogene. ††††Only *hdrC1B1A1* are present. †††††_hdrB1_ is present with two copies.

for the putative Hdr-like complex. All of these genes were found to exhibit elevated relative mRNA levels under sulfur-oxidizing versus iron-oxidizing conditions [65,66].

In Table 1 only sulfur-oxidizing prokaryotes are compiled. As evident from Table 1, genes encoding a *hdr*-like complex occur in sulfur-oxidizing crenarchaota of the order Sulfolobales, while *dsr* genes are absent from this organism group. Other archaeal sulfur oxidizers outside the Sulfolobales are *Natronorubrum thiooxidans*, a member of the Euryarcheota, for which a genome sequence is not available, and possibly *Aeropyrum pernix* and some species of the genus *Pyrobaculum*. While lithoautotrophic growth on thiosulfate with oxygen or nitrate as electron acceptors has been reported for *Pyrobaculum aerophilum* [67], the addition of thiosulfate to growth media has a mere stimulatory effect in the other organisms. In fact, the potential pathways of sulfur compound oxidation in *Pyrobaculum* and *Aeropyrum* species have never been elucidated. In contrast, it is well established that *P. islandicum* is capable of using sulfite as the terminal electron acceptor and that it contains a dissimilatory sulfite reductase operating in the reductive direction [18]. According to our analyses, the gene content of all genome-sequenced *Pyrobaculum* species is more indicative of sulfite/thiosulfate reduction and the genus is therefore not listed in Table 1. While the genes *dsrAB*, *dsrC*, and *dsrMKO* are present, *dsrEFH* and *dsrL* are absent. The latter genes occur in all sulfur oxidizers using the Dsr pathway. *A. pernix* does not contain *dsr* genes at all and *hdr*-like genes are neither present in *Pyrobaculum* nor in *Aeropyrum* species. The mechanism of thiosulfate oxidation in *Pyrobaculum* species and *A. pernix* remains enigmatic because none of the genome sequenced species encode thiosulfate:quinone oxidoreductase, any of the proteins of the Sox multienzyme complex [60] or thiosulfate dehydrogenase [68], Furthermore, neither genes for tetrathionate hydrolase nor genes for sulfur oxygenase reductase are present.

Our extended analyses of arrangements of genes involved in sulfur oxidation confirm not only a tight linkage of *rhd-tusA-dsrE2* with genes encoding major components of the sulfur oxidation machinery in many cases, but also indicate a near ubiquitous occurrence of these genes in sulfur oxidizers. A rhodanese-like protein, TusA and DsrE2 thus appear to be common elements in sulfur oxidizers. We propose the rhodanese-like protein as the enzyme mobilizing sulfur from low molecular weight organic perthiols and transferring it via TusA (and possibly also via DsrE2), either via DsrEFH and DsrC to DsrAB (as depicted in last figure) or directly to the Hdr-like complex. DsrAB then functions as the sulfite producing entity and the Hdr-like complex could also be involved in sulfite formation, possibly with participation of further, so far unidentified, proteins. As also evident from Table 1, *dsrC* or *dsrC*-homologous genes appear to be almost completely absent in sulfur oxidizers containing the Hdr-like complex. The green sulfur bacterium *Chloroherpeton thalasssium* is currently the only exception to this rule. In this organism two *tusE*-like genes (see below) are present.

Overall, the present evidence supports the mechanisms of DsrAB action discussed above. Nevertheless, it is also possible to envisage alternative mechanisms for the DsrAB oxidation or reduction pathways, involving DsrC-bound sulfur intermediates throughout the entire reaction sequence. These intermediates may include not only cysteine persulfide and persulfonate states, but also persulfenic and persulfinic states. However, DsrC from SRO does not bind sulfite (our unpublished results), and such binding is also not observed in the DsrAB/DsrC 3D structures, which disfavours the possibility of DsrC-bound intermediates right from the start of the reaction. Further studies are required to fully elucidate these mechanisms.

5. The DsrC/TusE/RspA superfamily

To get further insights into the DsrC/TusE protein family we studied their distribution in different organisms. We confirmed that all organisms that have the *dsrAB* genes also have *dsrC*, supporting the idea that DsrC is essential for the function of DsrAB. The reverse, however, is not true, i.e. some organisms have *dsrC*-homologous genes but not *dsrAB*, which prompted us to analyze the sequences and gene organization of this family of proteins in more detail.

Proteins related to DsrC/TusE belong to the so-called "TusE/DsrC/DsvC family" (PFAM accession number PF04358, with 1238 entries), and the respective genes are usually annotated as "DsrC-like protein", "DsrC family protein", or often "sulfur relay protein". We separated the proteins from this family into three groups on the basis of the functional Cys residues located at the C-terminus: 1) the proteins that have the two conserved Cys at the C-terminus spaced by 10 amino acids ($C_BX_{10}C_A$ motif), which we consider to be DsrC proteins; 2) Proteins having only Cys_A, which we consider to be TusE proteins, since only this Cys is required for their function; 3) Proteins lacking Cys_A or lacking both Cys_A and Cys_B. The proteins in group 3 cannot be true DsrCs, which we assume require the two Cys for activity, or TusEs, which require Cys_A [41]. We named these proteins as RspA for **r**egulatory **s**ulfur-related **p**roteins. These proteins are found mainly in SOB, with a few exceptions in SRO. Given the HTH motif present in this family, we propose that the RspA proteins may be involved in gene regulation, a function that may not require the conserved Cys. Moreover, the loop of the HTH structural motif located between helices 3 and 4 is longer by about six amino acids

in RspA proteins, which could be related to a DNA-binding function (interestingly the same is observed in a few DsrC and TusE proteins).

Initially, we performed an alignment of DsrC sequences to generate an unrooted neighbor-joining (NJ) dendrogram, in order to analyze the proteins from those organisms lacking DsrAB (Fig. 4). To be able to judge for the presence or absence of *dsrAB* genes, only DsrC sequences from completely sequenced genomes were used. We considered only sequences containing both Cys_A and Cys_B in the $C_BX_{10}C_A$ motif. With this methodology a total of 106 DsrC sequences were retrieved from 7 phyla: *Proteobacteria*, *Firmicutes*, *Chlorobi*, *Nitrospira*, *Acidobacteria*, *Crenarchaeota* and *Euryarchaeota*. The topology of the NJ dendrogram clearly shows the presence of two well separated branches for the *Deltaproteobacteria* SRB and SOB, as reported before for DsrAB [45]. The DsrC proteins from the SOB group are all placed together, including organisms from distinct phyla, namely *Proteobacteria* (alpha, beta and gamma classes) and *Chlorobi*. In the case of SRO the situation is clearly different, as described before for DsrAB [45,46], as the DsrC proteins are grouped according to their taxonomic position, independently of the microorganisms being sulfate, thiosulfate or/and sulfite reducers. Therefore, we obtained several groups comprising the *Deltaproteobacteria* SRB, the *Firmicutes*, *Nitrospira*, *Euryarchaeota* (*Archaeoglobus* spp.) and *Crenarchaeota* phyla. While in sulfur oxidizers all the DsrC sequences are very similar, within the group of reducing organisms there is noticeable variation in three regions of the sequences, namely at the N-terminus, in the loop between helices 1 and 2, and in the loop between helices 3 and 4, where the HTH structural motif is located (Fig. S1). For example, the archaeal DsrC proteins (both *Archaeoglobus* and *Crenarchaeota*) have extra amino acids in the first two locations, and the *Archaeoglobus* spp. have a larger loop at the HTH motif.

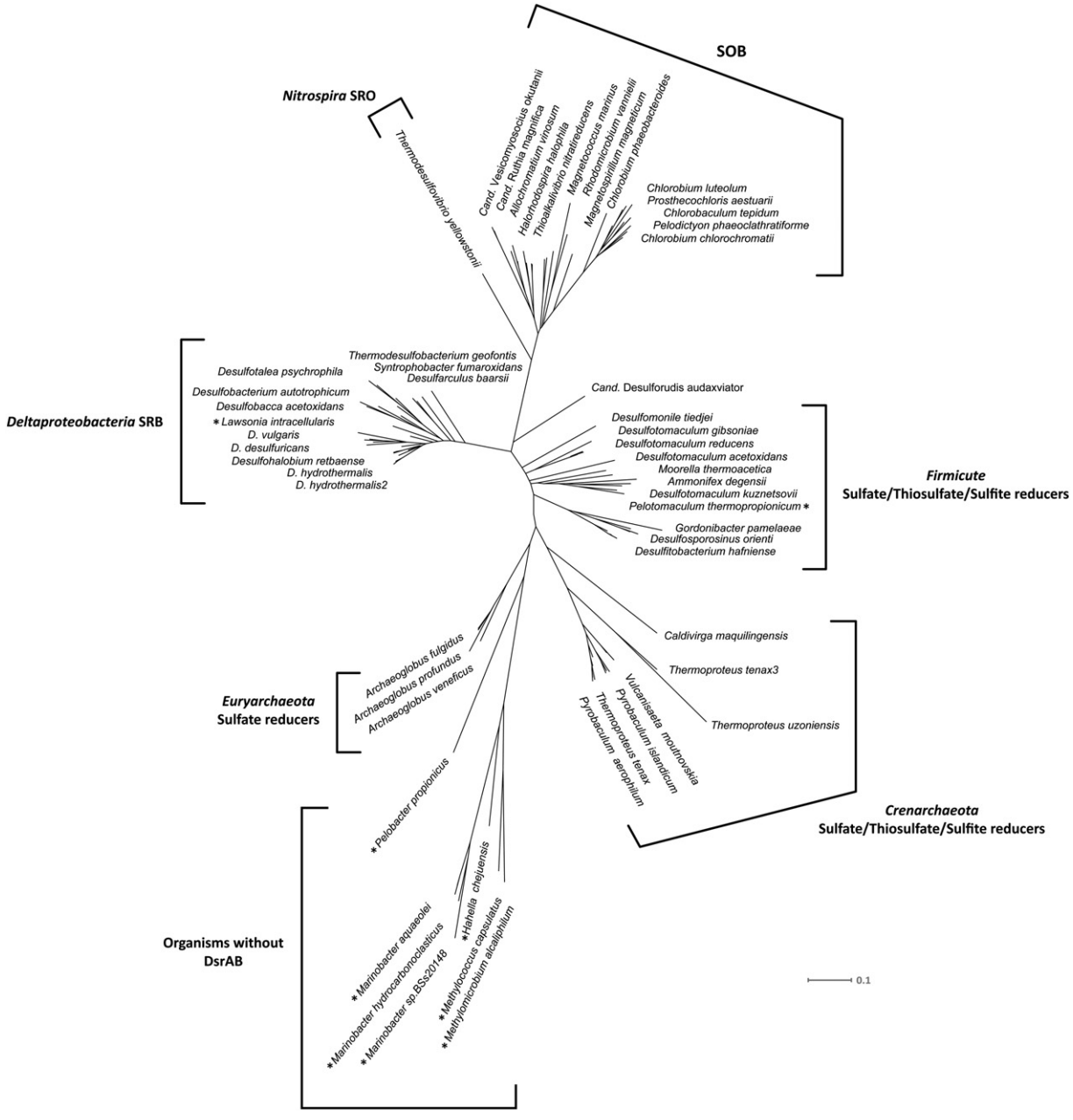


Fig. 4. Unrooted neighbor-joining dendrogram of DsrC. Only sequences containing the $CX_{10}C$ motif at the C-terminus, from fully sequenced genomes, were used. The dendrogram was derived from the alignment of 106 DsrC sequences and was constructed using ClustalX v2.1 with 10,000 bootstraps, 1000 seds and correction for multiple substitutions. The dendrogram was composed in Dendroscope v3.2.9 [113]. The scale bar represents the expected number of changes per sequence position. As an example one organism with a second copy of DsrC (*D. hydrothermalis*) is represented with number 2. Organisms that have the *dsrC* gene but lack the *dsrAB* genes are indicated with (*).

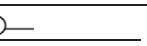
From the 106 DsrC sequences, eleven belong to organisms that do not have the *dsrAB* genes, and the majority of these cluster in a separate group (with the exception of the reducing organisms *Lawsonia intracellularis*, a *Deltaproteobacterium*, and *Pelotomaculum thermopropionicum*, from *Clostridiales*, which fit in the respective phylogenetic groups in the dendrogram). The sequences in this separate group most likely correspond to TusE proteins, since these organisms lack the *dsrAB* genes, although they have the conserved $C_BX_{10}C_A$ motif. In fact, they cluster with *E. coli* TusE (Fig. S2), and both have a larger loop at the HTH motif that is probably engaged in gene regulation. These sequences include a group from the *Gammaproteobacteria* class, like *Marinobacter* spp., *Hahella chejuensis*, *Methylococcus capsulatus* and *Methylomicrobium alcaliphilum*, which curiously have the *dsrEFH* genes located next to *dsrC* despite lacking *dsrAB*. So, we can conclude that the $C_BX_{10}C_A$ motif is a minimum requirement to distinguish a DsrC protein within this family, but a few proteins may have this motif, but in fact functionally operate as TusEs.

TusE is found in organisms that do not have a dissimilatory sulfur metabolism, and also in some SOB. It is present in almost all organisms of the *Gammaproteobacteria* class and it is also present in some organisms of the *Actinobacteria* phylum. In SOB, it is present in the *Betaproteobacteria* and *Gammaproteobacteria* (Table 2). TusE is not found in SRO, but is present in some organisms that are thiosulfate and/or sulfite reducers, such as *Moorella thermoacetica*, *Thermanaeromonas toyohensis* (Fig. 5) and in several *Desulfitobacterium* spp.

The scenario for the presence of the DsrC/TusE/RspA is very distinct in SRO and in SOB. In the genomes of SRO only genes for DsrC proteins are present, with the exception of three SRO that have an RspA protein with none of the conserved Cys (*Desulfurivibrio alkaliphilus*, *Desulfotalea psychrophila* and *Thermodesulfovibrio yellowstonii*). TusE is not present in SRO. Some SRO have a second copy of the *dsrC* gene (*dsrC2*), with a $C_BX_{10}C_A$ motif. The *dsrC2* gene is present in five *Desulfovibrio* spp. (namely in *Desulfovibrio hydrothermalis*, *Desulfovibrio longus*, *Desulfovibrio oxyclinae*, *Desulfovibrio salexigens* and *Desulfovibrio bastinii*), and in two clostridial sulfate reducers (*Desulfotomaculum gibsoniae* and *Desulfotomaculum alcoholivorans*). In these, the *dsrC1* gene is found in the *fd-dsrMK-dsrC1* gene cluster (Fig. 5, with *fd* corresponding to a ferredoxin-coding gene), while the *dsrC2* gene is found in the vicinity of the *sat-aprBA-qmoABC* gene cluster. Although these are essential proteins for the reduction of adenosine 5′-phosphosulfate to sulfite, none contain a HdrB- or HdrD-like subunit, so it is unlikely that DsrC2 works directly with any of these proteins. The DsrC2 sequences are almost identical to the DsrC sequences in each organism, indicating they are paralogues. Thus, they all cluster together within the respective phylogenetic group of SRO (Fig. 4).

In SOB the situation is quite more complex, and two groups of organisms are found: those that have only one copy of the *dsrC* gene, as in

Table 2
Distribution of *dsrC*/*tusE*/*rspA* genes in genome-sequenced SOB containing multiple copies of this gene family. A pictorial representation of this protein family according to the presence of Cys_A and Cys_B, is shown. Dots represent a single gene copy and numbers give the number of paralogs. *rspA* genes in group I are found next to the *dsr* operon; RspA proteins encoded in group II do not have Cys_A, but always have Cys_B; RspA proteins encoded in group III do not have Cys_A, and may have or not Cys_B. Groups II and III form two separate clusters in the dendrogram for this family (Fig. 7).

							
					RspA		
	N_T	DsrC $C_BX_{10}C_A$	"DsrC3" $C_BX_{10}C_A$	TusE	Group I (next to *dsr* operon)	Group II	Group III
Alphaproteobacteria							
Rhodospirillaceae							
Magnetospirillum gryphiswaldense MSR-1	3	●	–	●	–	●	–
Betaproteobacteria							
Burkholderiaceae							
Burkholderiales bacterium JOSHI_001	2	●	–	–	●	–	–
Gallionellaceae							
Sideroxydans lithotrophicus ES-1	6	●	–	●	●	2	●
Hydrogenophilaceae							
Sulfuricella denitrificans skB26	5	●	–	●	●	●	●
Thiobacillus denitrificans ATCC 25259	9	●	–	●	●	4	2
Thiobacillus thioparus DSM 505	10	●	–	●	●	3	4
Gammaproteobacteria							
Chromatiaceae							
Allochromatium vinosum DSM 180 T	5	●	●	–	●	–	2
Lamprocystis purpurea DSM 4197	7	●	●	–	●	●	3
Marichromatium purpuratum 9S4	3	●	–	–	●	●	–
Thiocapsa marina 5811	7	●	●	–	●	●	3
Thiocystis violascens DSM 198	7	●	●	–	●	●	3
Thioflavicoccus mobilis 8321	8	●	●	–	●	●	4
Thiorhodococcus drewsii AZ1	4	●	●	–	●	–	●
Thiorhodovibrio sp. 970	5	●	●	–	●	●	●
Ectothiorhodospiraceae							
Alkalilimnicola ehrlichei MLHE-1	2	●	–	–	–	–	●
Thioalkalivibrio nitratireducens DSM 14787	8	●	–	●	●	2	3
Thioalkalivibrio sulfidophilus HL-EbGr7	5	●	–	●	●	●	●
Thioalkalivibrio thiocyanodenitrificans ARhDl	6	●	–	●	●	●	2
Thioalkalivibrio thiocyanoxidans ARh4	7	●	–	●	●	2	2
Thiotrichaceae							
Thiothrix disciformis DSM 14473	4	●	–	●	●	–	●
Thiothrix nivea DSM 5205	6	●	–	●	●	●	2
Sulfur-oxidizing symbionts							
Candidatus Ruthia magnifica str. Cm	3	●	–	●	●	–	–
Candidatus Vesicomyosocius okutanii HA	3	●	–	●	●	–	–

N_T–total number of genes belonging to the *dsrC*/*tusE*/*rspA* family.

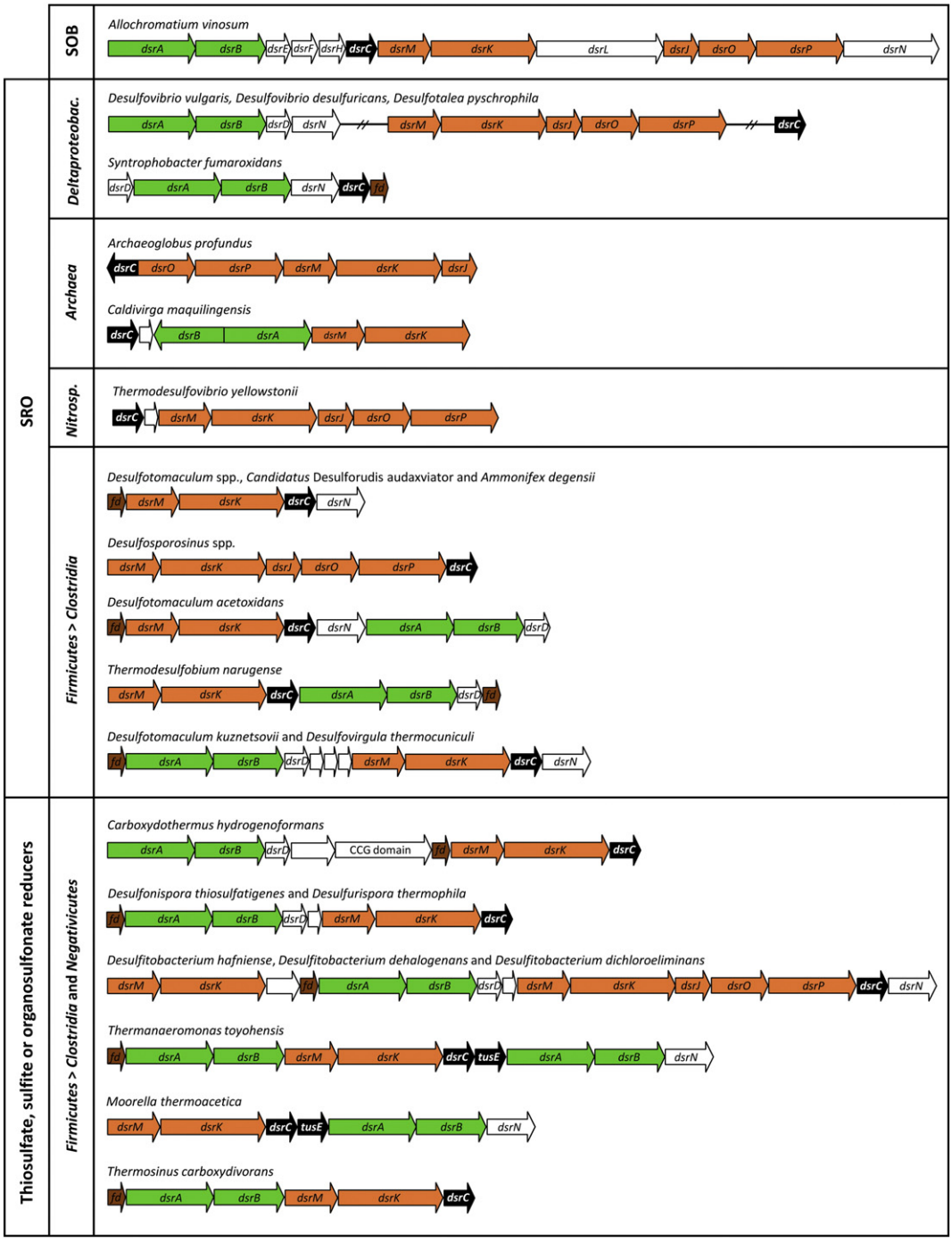


Fig. 5. Examples of *dsrC* gene neighborhoods found close to *dsrAB* or/and *dsrMKJOP* genes in SOB or in sulfate/thiosulfate/sulfite/organosulfonate reducers. *fd*, ferredoxin; *dsrN*, cobyrinic acid a,c-diamide synthase. Adapted from [10].

SRO, and the ones that have multiple copies of the *dsrC*/*tusE*/*rspA* genes. The first group, having a single copy of the *dsrC* gene, include members of *Alphaproteobacteria* and *Chlorobiaceae* (with exception of *Chlorobaculum tepidum* that has a second copy of the *dsrC* gene). SOB belonging to *Betaproteobacteria* and *Gammaproteobacteria* classes, including endosymbionts, have multiple copies of the *dsrC*/*tusE*/*rspA* genes, ranging from two to ten. This variety of *dsrC*-like genes was already noticed in *Thiobacillus denitrificans* with 9 homologues [69] and in *A. vinosum* with 5 homologues [40]. Besides the true *dsrC* gene always located within the *dsr* operon, this group of SOB has in common the existence of *dsrC* homologues not coding for Cys_A, which we name as *rspA* (Table 2). Among the 23 SOB analyzed, only *Alkalilimnicola ehrlichei* and *Magnetospirillum gryphiswaldense* do not have an *rspA* gene. In addition, in 16 out of the 21 SOB genomes, this gene is located downstream of the *dsr* operon (Fig. 6A). This suggests that RspA may participate in the regulation of the *dsr* genes. A sequence alignment of the RspA proteins found downstream of *dsr* operons shows a very high sequence conservation, with C-terminal residues that are different from the ones in DsrC (Fig. 6B and Fig. S3). Noteworthy, the conserved proline (in the $PTGC_AV$ motif), working as a hinge in DsrC and allowing the bending of the C-terminal arm [20], is not present in the RspA sequences.

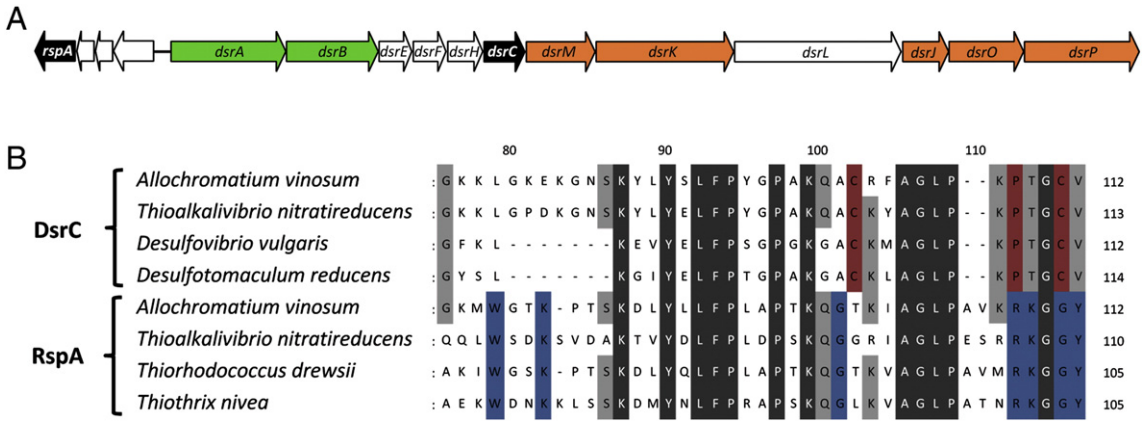


Fig. 6. A *rspA* gene is present next to the *dsr* operon in the majority of SOB analyzed. A) Representation of a typical SOB gene organization including the *rspA* gene close to the *dsr* operon (here from *Thioalkalivibrio sulfidophilus* HL-EbGR7). B) Sequence alignment of the C-terminal residues of DsrC and RspA. Important residues for DsrC function (Cys_A, Cys_B and conserved proline) are in red. Conserved residues in RspA are in blue.

In SOB having more than two *dsrC*/*tusE*/*rspA*-related gene copies (coding for the true DsrC and RspA), a very interesting situation is observed, as they have either a *tusE* gene (not coding for Cys_B) or a *dsrC3* gene (coding for both Cys). This suggests, that in this group of SOB "DsrC3" is not a second copy of a functional DsrC protein, but instead it may replace TusE and be involved in tRNA modifications as well, even though it has Cys_A and Cys_B. TusE-like "DsrC3" is present in all SOB belonging to the *Chromatiaceae* family (with exception of *Marichromatium purpuratum*). In SOB that have additional copies besides a functional *dsrC*, a *dsr*-related *rspA*, and a TusE-like "*dsrC3*" gene, the majority also have additional *rspA* genes (Table 2). Genes for RspAs without either Cys were also found in a few organisms that reduce sulfur compounds, namely in *Desulfobulbus propionicus*, *Desulfonatronum lacustre*, *Desulfotomaculum gibsoniae* and *Thermodesulfobium narugense*.

Overall, from this analysis we conclude that there are probably three physiological functions associated with proteins of the DsrC/TusE/RspA family: sulfite reduction (performed by DsrC), 2-thiouridine biosynthesis (performed by TusE) and possibly gene regulation (performed by RspA). To validate this division, a dendrogram was assembled (Fig. 7) with sequences of the DsrC/TusE/RspA proteins from SOB used to build Table 2. The dendrogram shows a clear separation between the three protein families, and also that RspA proteins can be further divided in three subgroups: i) group I — proteins encoded by genes found close to the *dsr* operon; ii) group II — proteins without Cys_A, but with Cys_B; and iii) group III — proteins without Cys_A that may have or not Cys_B. Therefore, this dendrogram corroborates the existence of the DsrC, TusE and RspA families, and also the divisions proposed in Table 2, including the classification of "DrsC3" as a TusE-like protein.

6. Physiological partners of DsrC

From the overview in Sections 2 and 3 it is evident that the physiological interaction partners of DsrC, apart from DsrAB, fall into two separate categories: The first contains those proteins that act on the disulfide formed between Cys_A and Cys_B, a process important during dissimilatory sulfate reduction as well as during sulfur oxidation. The second category

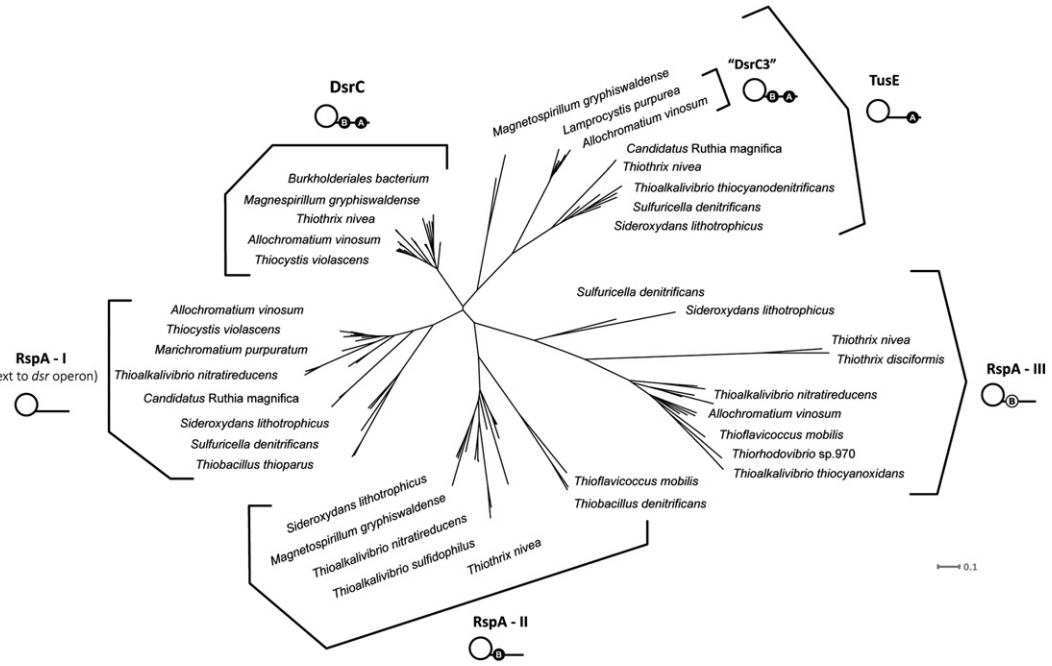


Fig. 7. Dendrogram of DsrC/TusE/RspA proteins from SOB. The dendrogram was derived from an alignment of around 20 sequences of each protein group (97 in total) presented in Table 2. The same pictorial representation according to the presence of Cys_A and Cys_B was used. The dendrogram was built as described in Fig. 4. The scale bar represents the expected number of changes per sequence position.

involves protein-mediated sulfur transfer onto DsrC and is of pivotal importance for dissimilatory sulfur oxidation. Here, we concentrate first on sulfur transfer from DsrEFH to DsrC during sulfur oxidation, and then elaborate on several proteins/protein complexes that are (probably) important for the conversion of the disulfide to the thiol states of DsrC.

6.1. DsrEFH in SOB

As outlined above, in sulfur oxidizers DsrC is loaded with sulfur by its direct interaction partner DsrEFH. DsrEFH from *A. vinosum* is a heterohexameric protein arranged in a $\alpha_2\beta_2\gamma_2$ structure and harbors two conserved cysteine residues in the putative active site cysteines DsrE-Cys_{78} and DsrH-Cys_{20} [70]. While both of these cysteine residues are essential for sulfur oxidation in vivo, sulfur is only bound to DsrE-Cys_{78} in vitro. DsrH-Cys_{20} is not persulfurated upon incubation with sulfide [43]. Persulfurated DsrEFH transfers sulfur atoms specifically to Cys_A of *A. vinosum* DsrC but not to Cys_B, which was shown by the use of DsrC variants lacking either one or both cysteine residues [43]. DsrH-Cys_{20} is not required for sulfur transfer from DsrEFH to DsrC. The reverse reaction, i.e. sulfur transfer from DsrC to DsrEFH was not detected. Persulfurated DsrC very probably serves as a direct substrate for DsrAB in vivo and it is therefore feasible that thiolic DsrC acts as a sulfur trap. This would ensure a constant flow of sulfur atoms necessary for a high turnover rate for DsrAB.

Although structural data is not yet available that would allow detailed insight into the DsrEFH–DsrC interaction, it is well documented that these proteins tightly interact. When combined in solution in their native, non-persulfurated form, DsrEFH and DsrC run as a complex in native polyacrylamide gel electrophoresis and dissociate from each other only very slowly, as shown by Surface Plasmon Resonance [43]. Additional bands that arise upon interaction of the proteins were analyzed in the second dimension, which indicated that $DsrE_2F_2H_2$ associates with either one or two DsrC molecules per heterohexamer. This was confirmed by two independent experimental approaches, Blue-native gel analysis and dynamic light scattering. Interaction of DsrEFH with DsrC is strictly dependent on the presence of DsrE-Cys_{78} and DsrC-Cys_A [30,43]. It is assumed that the interaction of DsrEFH and DsrC is the basis for sulfur transfer between the proteins, and that once loaded with sulfur DsrC dissociates more easily from DsrEFH than observed in the experiments described above, where interaction of the non-persulfurated proteins was studied.

6.2. The DsrMKJOP complex and DsrMK proteins

The *dsrMKJOP* genes were first described in *A. vinosum* as part of the large gene cluster that includes also *dsrAB* and *DsrC* [22,23], and all the genes of the complex were shown to be essential for sulfur globule oxidation [32]. DsrMKJOP is a transmembrane complex with subunits in the periplasm (the triheme cytochrome DsrJ [71] and the polyferredoxin DsrO), in the membrane (the diheme cytochrome *b* DsrM, and DsrP) and in the cytoplasm (the Fe–S protein DsrK). By analogy to other bacterial respiratory complexes, DsrMKJOP resembles an association of two modules, DsrJOP and DsrMK, facing the periplasm and cytoplasm, respectively [10], but the modules form a complex that was isolated from *A. fulgidus* [72] and *D. desulfuricans* [73]. In *A. vinosum*, a complex containing DsrKJO, DsrAB, DsrEFH and DsrC was also isolated [22]. The idea that the disulfide between the two conserved Cys of DsrC could constitute the heterodisulfide-analogous substrate for the DsrK protein was first proposed by Pires et al., [73], based on the similarity of the DsrMK subunits to the membrane-associated HdrED. DsrK and HdrD (the catalytic subunit of Hdr) belong to the CCG protein family (PFAM accession number PF02754) including over 5000 archaeal and bacterial proteins, which contain a conserved cysteine-rich sequence (CXnCCGXmCXXC) [6,7,74]. This Cys motif binds a special $[4Fe–4S]^{3+}$ cluster, which in Hdr is the catalytic cofactor responsible for heterodisulfide reduction [1], and which is also present in DsrK [73]. In the soluble HdrABC protein of hydrogenotrophic methanogens, a similar cofactor is present in the catalytic HdrB subunit [1]. In methanogens with cytochromes (*Methanosarcinales*), HdrED is involved in a redox loop mechanism with the membrane-bound VhoACG hydrogenase, connected by methanophenazine, coupling the heterodisulfide reduction to energy conservation [75]. In hydrogenotrophic methanogens (without cytochromes) the energy coupling mechanism is different and involves the HdrABC complex with a hydrogenase [3] or a formate dehydrogenase [5]. This complex plays a key role in the bioenergetics of these methanogens because it couples the thermodynamically favorable reduction of the heterodisulfide to the thermodynamically unfavorable reduction of ferredoxin (Fd) by either H_2 or formate, through the mechanism of flavin-based electron bifurcation (FBEB) [3,76]. Reduced Fd is required for the reduction of CO_2 by formylmethanofuran dehydrogenase in the first step of methanogenesis [2,77].

The *dsrMKJOP* genes are present in the majority of SRO, but some organisms miss the *dsrJOP* genes of the periplasmic module (e.g. some *Clostridiales*) [10]. We addressed again the distribution of the *dsr* genes previously described [32], and confirmed that all organisms (sulfate/sulfite/thiosulfate reducers and SOB) that have the *dsrAB* genes also have *dsrC* and the *dsrMK* genes. In several organisms, these genes are clustered together (Fig. 5) providing evidence for their physiological interaction. These findings suggest that only the DsrMK module of the complex is essential for sulfite reduction, and that it can work as the minimal functional unit, probably mediating electron transfer between menaquinol and DsrC. A DsrMK protein was already isolated from *Archaeoglobus profundus* [78]. The *dsrEFH* and *dsrL* genes are specific of oxidative sulfur metabolism, and are not found in SRO. In contrast, the *dsrD* gene that appears to be involved in regulation [79], is specific of SRO and is not present in SOB, although it is also not found in some thiosulfate reducers (e.g. *Thermosinus carboxydivorans* and *Thermanaeromonas toyohensis*).

The involvement of the Dsr complex in the sulfite reduction pathway is consensual, but the molecular and mechanistic details of how it operates are not yet known. Understanding the function of the Dsr complex is, in fact, one of the most challenging points both in sulfate/sulfite reducers and in sulfur oxidizers. The DsrJOP module is likely engaged in electron flow between the periplasm and the quinone pool, but how exactly is not clear. The DsrJ cytochrome does not function as electron acceptor for the periplasmic hydrogenases or formate dehydrogenases in SRO [73,80]. This cytochrome includes a heme with unusual His/Cys coordination [71,73], as observed in the SoxXA cytochrome involved in thiosulfate oxidation [81]. This DsrJ Cys is essential for sulfur oxidation in *A. vinosum* [71], but the exact function of DsrJ remains a mystery.

The DsrMK module, due to its similarity to HdrDE and the fact that it can operate individually, is most likely involved in electron transfer between menaquinol and a cytoplasmic substrate, proposed to be $DsrC_{ox}$. This is supported by the fact the DsrM hemes *b* are reduced by menaquinol analogs [73]. Furthermore, DsrC was shown to interact directly with DsrK both in *A. vinosum* [63] and in *D. desulfuricans* (S.S. Venceslau and I.A.C. Pereira, unpublished results). An important point would be the determination of the redox potential of the thiol/disulfide pair of the DsrC catalytic cysteines, which has not been determined yet. In the case of methanogens the $E^{o\prime}$ of CoM-S-S-CoB is −143 mV and that of methanophenazine is -165 mV [82]. In SRO, which contain menaquinone ($E^{o\prime} \sim -70$ mV) this may not have a low-enough potential to reduce the DsrC "heterodisulfide" ($DsrC_{ox}$). This suggests that the process may be more complex than the reduction of $DsrC_{ox}$ by menaquinol. The presence of the DsrJOP and DsrMK modules, both including quinone-interacting proteins (DsrM and DsrP), suggests the possibility of a proton-translocating quinone cycle operating between the two modules, although direct transmembrane electron transfer cannot be ruled out. In the presence of the DsrJOP unit, such quinone cycle may allow quinone electron bifurcation, as in the bc_1 complex, to generate a low potential semiquinone intermediate that can reduce $DsrC_{ox}$. In

many organisms where the DsrJOP module is absent (mainly *Firmicutes*) we find that a ferredoxin gene is often located in the same gene locus as *dsrMK*, *dsrC* and *dsrAB* (Fig. 5) [10]. This suggests that the ferredoxin may serve an alternative function to DsrJOP, possibly as a second electron donor in a confurcation process as suggested for APS reductase and the Qmo complex [83]. Some SRO (e.g. *Desulfomicrobium baculatum* or *Desulfohalobium retbaense*) contain the complete *dsrMKJOP* set of genes and also a ferredoxin gene in the vicinity. Further work is required to elucidate this point.

6.3. Hydrogenases coupled with DsrMK-like proteins (Isp1 and Isp2)

A number of sulfur oxidizers contain the so-called Isp-hydrogenases [84–86]. In these enzymes, the two genes encoding the periplasmically located large and small hydrogenase subunits (HynSL in the case of *A. vinosum* and *Thiocapsa roseopersicina*) embrace two additional open reading frames called *isp1* and *isp2*. Isp1 belongs to the same cytochrome *b* superfamily as DsrM. Isp2 has high sequence similarity to DsrK and the catalytic HdrD subunit of heterodisulfide reductase from *Methanosarcina barkeri* [85]. An unambiguous function has not been elucidated for any Isp-hydrogenase so far. Isp1 and Isp2 are both critical for in vivo H_2-cycling in *T. roseopersicina* and *A. vinosum* [85,87], indicating that both proteins are an integral part of the hydrogenase complex [86]. The final electron acceptor for the HynSL–Isp1–Isp2 complex is unknown. However, the presence of the heterodisulfide-related DsrK-like Isp2 protein led to the proposal that there is a link to sulfur-dependent metabolic pathways [85,86]. This suggestion is substantiated by the observation that *T. roseopersicina* HynSL is expressed in the presence of thiosulfate [85] and that the respective genes from *A. vinosum* exhibit increased relative mRNA levels on sulfide, with values of up to 20-fold higher expression than under photoorganoheterotrophic conditions [33]. Sequence analyses indicate that the HynSL–Isp1–Isp2 complex is able to transfer electrons from hydrogen oxidation in the periplasm via the membrane-bound *b*-type cytochrome onto a cytoplasmic (hetero)disulfide [86]. In principle, the intramolecular DsrC disulfide could be the substrate that is reduced, similar to the situation proposed for the DsrMKJOP complex. However, biochemical evidence supporting this suggestion is completely lacking. We surveyed the sulfur oxidizers listed in Table 1 for the occurrence of the *hynL–isp1–isp2–hynS* cluster and thereby tried to uncover a specific connection with the presence of DsrC-encoding genes. However, a general co-occurrence of the respective genes was not observed. To the contrary, a number of sulfur oxidizers not containing *dsrC*, e.g. *Acidithiobacillus ferrooxidans* and all tabulated members of the *Aquificae*, harbor the genes encoding Isp-hydrogenase, while several bacteria employing the Dsr pathway of sulfur oxidation lack Isp-hydrogenase (e.g. all green sulfur bacteria). We would, however, like to emphasize that Isp-hydrogenase is present in all phototrophic members of the *Chromatiaceae* and that it is not a priori excluded that DsrC serves as a substrate for the hydrogenase complex at least in this organism group. Clearly, more research is necessary to clarify the in vivo role and to identify the substrates of Isp-hydrogenase in the future.

6.4. The Hmc and Tmc membrane complexes in Deltaproteobacterial SRO

In SRO of the *Deltaproteobacteria* class, which contain an abundant pool of cytochromes *c*, two other transmembrane redox complexes are often found, Hmc and Tmc, which include a cytoplasmic subunit also belonging to the CCG/HdrD/DsrK protein family (HmcF and TmcB) [10]. The Hmc complex (HmcABCDEF) has a similar architecture to DsrMKJOP, including also two membrane subunits similar to DsrM/HdrE and DsrP, and a periplasmic polyferredoxin similar to DsrO [14,88]. However, it differs from the Dsr complex in terms of the multiheme cytochrome *c* subunit, which is a sixteen-heme cytochrome in the case of Hmc [89]. The Tmc (TmcABCD) complex has a similar but simpler composition including a periplasmic tetraheme cytochrome *c* [90], a single membrane subunit similar to DsrM/HdrE and two cytoplasmic proteins, namely one similar to HdrD/DsrK and a tryptophan-rich protein with no homologue in the database. The *hmc* genes are usually expressed at low level [26,29,91] and the whole Hmc complex has never been isolated. In contrast, the Tmc complex is expressed at high level and was isolated from *D. vulgaris* Hildenborough, where it was shown to include also a catalytic $[4Fe–4S]^{3+}$ center, as observed in DsrK [92]. The cytochrome *c* subunits HmcA and TmcA (also known as TpIIc$_3$) are efficient electron acceptors of Type I cytochrome c_3, which is reduced by periplasmic hydrogenases and formate dehydrogenases [14]. Curiously, both complexes are usually present in the same organism. The *hmc* and *tmc* genes are never found in the vicinity of *dsrC* and *dsrAB* genes, so no physiological inference can be made from their gene location. However, both complexes have a DsrMK-like module (HmcEF and TmcCB) suggesting the possibility that they could be alternative electron donors to reduce $DsrC_{ox}$ [10].

The Hmc complex does not seem to be involved in sulfate respiration as a *D. vulgaris hmc* deletion mutant could still grow, albeit with slower rate, with lactate or hydrogen and sulfate [93], and the *hmc* genes show a low level of expression in these conditions [26,91,94]. A function for the *D. vulgaris* Hmc complex in syntrophic metabolism was recently highlighted by the inability of the *D. vulgaris hmc* deletion mutant to grow syntrophically with *Methanococcus maripaludis* S2 on lactate, and the higher expression of the *hmc* genes in this condition [95]. However, in *Desulfovibrio alaskensis* G20 conflicting results were obtained as strains with deletions in *hmcB*, *hmcE* and *hmcF* were not impaired in syntrophic growth with *M. maripaludis* on lactate or pyruvate (albeit had a longer lag phase) [96], whereas the Δ*hmcF* strain could not grow syntrophically with *Methanospirillum hungatei* on lactate [97]. So the Hmc complex seems to be involved in electron transfer from cytoplasmic lactate oxidation to periplasmic H_2 generation, eventually via DsrC, if this is a possible electron acceptor for the lactate dehydrogenase (see below).

The expression of the Tmc complex genes is increased in *D. vulgaris* during growth on H_2 [91], and all redox centers of Tmc are reduced in the presence of hydrogenase/TpIc$_3$/H_2 [92]. During growth in lactate/sulfate the *tmc* genes are expressed at a similar level as the *dsrMKJOP* genes, suggesting that they also play a functional role during sulfate respiration [26,91]. A clear phenotype was not found for a *D. vulgaris tmc* deletion mutant [26], which is not totally surprising since other membrane-bound complexes can probably substitute for it. The single membrane subunit of Tmc precludes a quinone-cycle mechanism as may be possible with the Dsr and Hmc complexes, and suggests that the Tmc complex may be a direct transmembrane electron conduit from periplasmic H_2 oxidation to the cytoplasm (in alternative to the Qrc/Qmo couple [98,99]), where its electron acceptor is likely also the $DsrC_{ox}$ protein.

6.5. Other soluble Hdr-like proteins

Besides the proteins associated with membrane complexes described above, there is also a striking number of soluble Hdr-related proteins in SRO [7–12]. Some of these proteins, related to HdrA (the flavin bifurcating subunit), to HdrB and HdrD (the catalytic heterodisulfide-reducing subunits) and/or to HdrE (the membrane cytochrome *b* subunit), are constituted by fusions of these proteins or contain additional modules [7], which led to the proposal of new Hdr proteins, such as HdrF and HdrL proposed by Strittmatter et al. [12], and HdrG proposed by Grein et al. [7]. The presence of Hdr proteins has also been noted in acetogenic bacteria, such as *M. thermoacetica* [100]. In SOB, several Hdr-like proteins are also present, but with a lower distribution than in SRO.

A complete set of *hdrABC* genes is found in many SRO and some SOB, even though these organisms do not contain the heterodisulfide, CoM-S-S-CoB. The *hdrABC* genes are found in two different sets of gene clusters: *hdrABC/floxABCD* and *hdrABC/mvhDGA* [10] (Fig. 8). The *floxABCD*

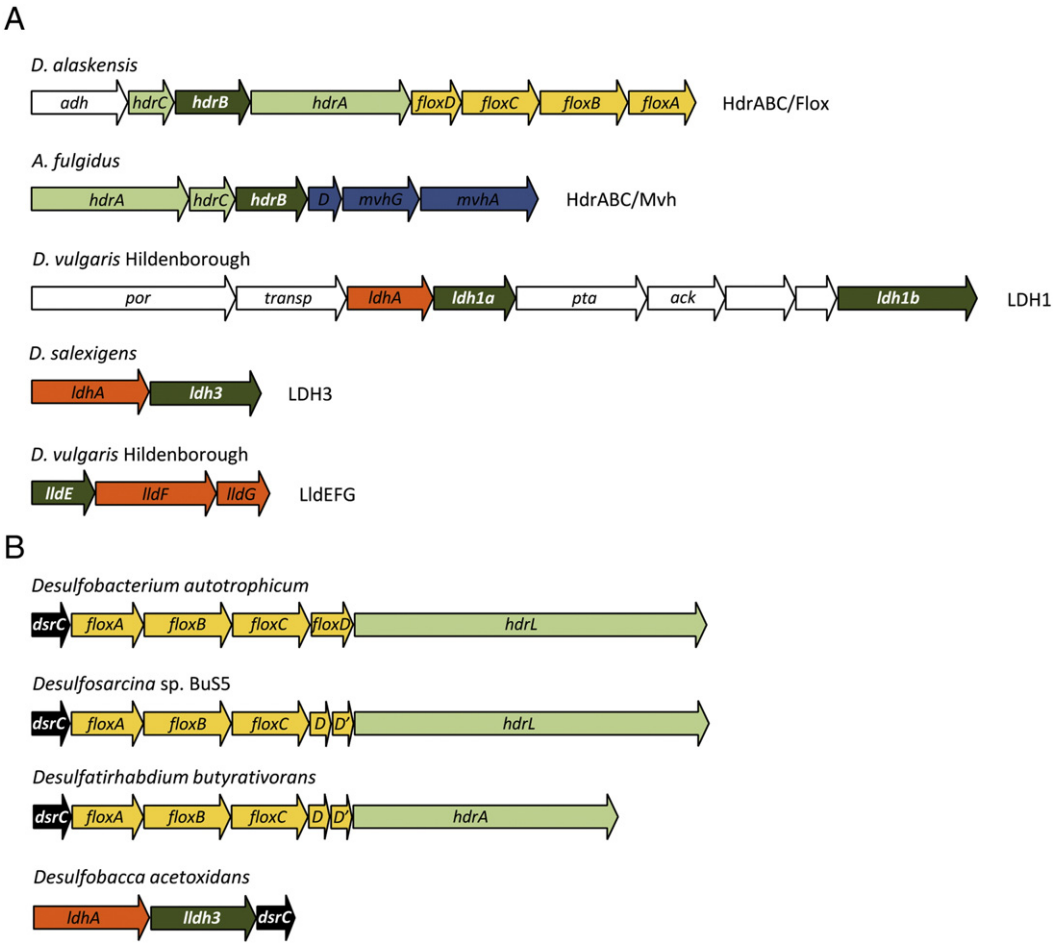


Fig. 8. *hdrB*- or *hdrD*-like genes coding for soluble proteins in SRO. A) Examples of gene loci including *hdrB*- and *hdrD*-related genes (dark green); B) Co-localization of *dsrC* gene with *flox* and *ldh* genes.

genes (for flavin oxidoreductase) code for a NAD(P)H oxidoreductase [10] that seems to be involved in oxidation of NAD(P)H produced by an alcohol dehydrogenase during ethanol oxidation, transferring electrons to HdrABC [25]. A *hdrAD/floxABCD* gene cluster is present in several members of the *Chlorobi* phylum of SOB. The *hdrABC/mvhDGA* gene cluster arrangement, that is characteristic of methanogens, is found in some SRO but not in SOB. As described above, the HdrABC/MvhDGA complex reduces both CoM-S-S-CoB and ferredoxin with H_2, in an electron bifurcating mechanism [3]. The bifurcation of electrons is proposed to take place at the FAD group of HdrA. The presence of the two gene loci *hdrABC/floxABCD* and *hdrABC/mvhDGA* led to the suggestion that also in SRO the mechanism of flavin-based electron bifurcation may be operating, to generate reduced ferredoxin from either NAD(P)H or H_2 [10]. The proposed substrate for HdrB in SRO was again the "heterodisulfide" $DsrC_{ox}$, providing a link to sulfite reduction. A link between FloxABCD and DsrC is supported by the finding of *floxABCD* genes next to a *dsrC* gene in some organisms (Fig. 8). Therefore, we propose that DsrC functions also as an electron carrier linking soluble processes of substrate oxidation (H_2 and ethanol) to sulfite reduction, with energy coupling by electron bifurcation occurring at the HdrABC/MvhDGA and HdrABC/FloxABCD complexes.

Another set of conserved proteins related to HdrB/HdrD is a protein encoded in three putative lactate dehydrogenase (Ldh) gene loci, as described before [10]. In *D. vulgaris* three of the six predicted Ldhs contain such a subunit. The first is part of a large gene cluster involved in oxidation of organic acids [27,101], which includes also genes for pyruvate:Fd oxidoreductase (*por*), a putative lactate permease, the putative Ldh catalytic subunit (*ldhA*), a putative Ldh iron–sulfur subunit that has two CCG domains (named *ldh1a*), phosphate acetyl transferase (*pta*), acetate kinase (ack), and a second larger HdrD-related protein (named *ldh1b*). The second Ldh is a three subunit protein LldEFG (or LutABC) as described in *Bacillus subtilis* [102], *Shewanella oneidensis* [103], and *Campylobacter jejuni* [104], and conserved among a wide range of distantly related bacteria. In fact, the *lldEFG* genes are also present in several members of SOB. The LldE protein is a small HdrB-related protein with two CCG domains [10]. The LldEFG enzyme is membrane-associated although no transmembrane helices are present in any of its subunits. The third Ldh is encoded in a simple two gene locus including the catalytic *ldhA* and the HdrD-like *ldh3* gene.

In *D. vulgaris* growing on lactate/sulfate the *ldh1a* gene is one of the most transcribed *ldh* genes, suggesting a main function in lactate oxidation [26]. In *D. alaskensis* G20 the genes encoding the organic acid oxidation region (including *ldh1a* and *ldh1b*) show increased expression during syntrophic growth on lactate, and deletion mutants of *ldh1a* and *ldh1b* could not grow syntrophically [96,97]. However, these mutants were not impaired in growth with lactate/sulfate, suggesting that other Ldhs can compensate for their absence. Whether or not the Hdr-like subunits of Ldhs can transfer electrons to DsrC is something that should be experimentally tested. The described Ldhs in SRO are membrane-associated proteins [105], but their electron acceptor has not been clearly identified. The LldEFG proteins are believed to transfer electrons directly to the quinone pool. Interestingly, in the SRO *Desulfobacca acetoxidans* [106] the *ldha-ldh3* genes are preceded by a *dsrC* gene (Fig. 8).

Another example of an HdrD-like protein is found next to a gene coding for a molybdopterin aldehyde oxidoreductase (Mop) [107],

and this set of genes is highly conserved among SRO [10]. The Mop protein is a very abundant protein in *D. vulgaris* growing in lactate or formate with sulfate, indicating an important role in energy metabolism [28].

The HdrD-related protein HdrF, first recognized in *Desulfobacterium autotrophicum* HRM2 [12], is a multidomain protein that includes a transmembrane, an iron–sulfur and two CCG domains (see scheme in references [7,12]), resembling a gene fusion of HdrD and HdrE. In SRO and in SOB (3 members of *Chlorobiaceae* family), HdrF was found close to genes coding for an electron-transfer flavoprotein (EtfAB) that can transport electrons to or from HdrF, which in turn can be involved in electron transfer with the quinone pool. Recently, another subset of proteins belonging to the CCG family was identified, and named HdrG, which is most likely an FAD-containing oxidoreductase [7]. HdrG is also a multi-domain protein that includes an FAD-binding domain plus one or two FAD oxidase domains at the N-terminus, an iron–sulfur binding domain and two CCG domains at the C-terminus. HdrG encoding genes were identified in two SOB (*Magnetospirillum magneticum* and *Thioalkalivibrio thiocyanoxidans*). There is also a variant of HdrG, containing two iron–sulfur binding domains instead of one, which is found in several SRO [10] and is widespread in SOB. The HdrF and HdrG proteins were never isolated, so their exact function is unknown. We can speculate that these proteins are involved in electron transfer pathways from NAD(P)H, fatty acid metabolism, or other metabolites, possibly functioning as electron donors to $DsrC_{ox}$ or to the menaquinone pool, since HdrF is membrane-bound.

7. DsrC, a cytoplasmic redox hub in dissimilatory sulfur metabolism?

The multitude of Hdr-related proteins in sulfur-metabolizing organisms speaks strongly for the existence of a "bacterial heterodisulfide" analog in the energy metabolism of these organisms [7–13,22,30,73].

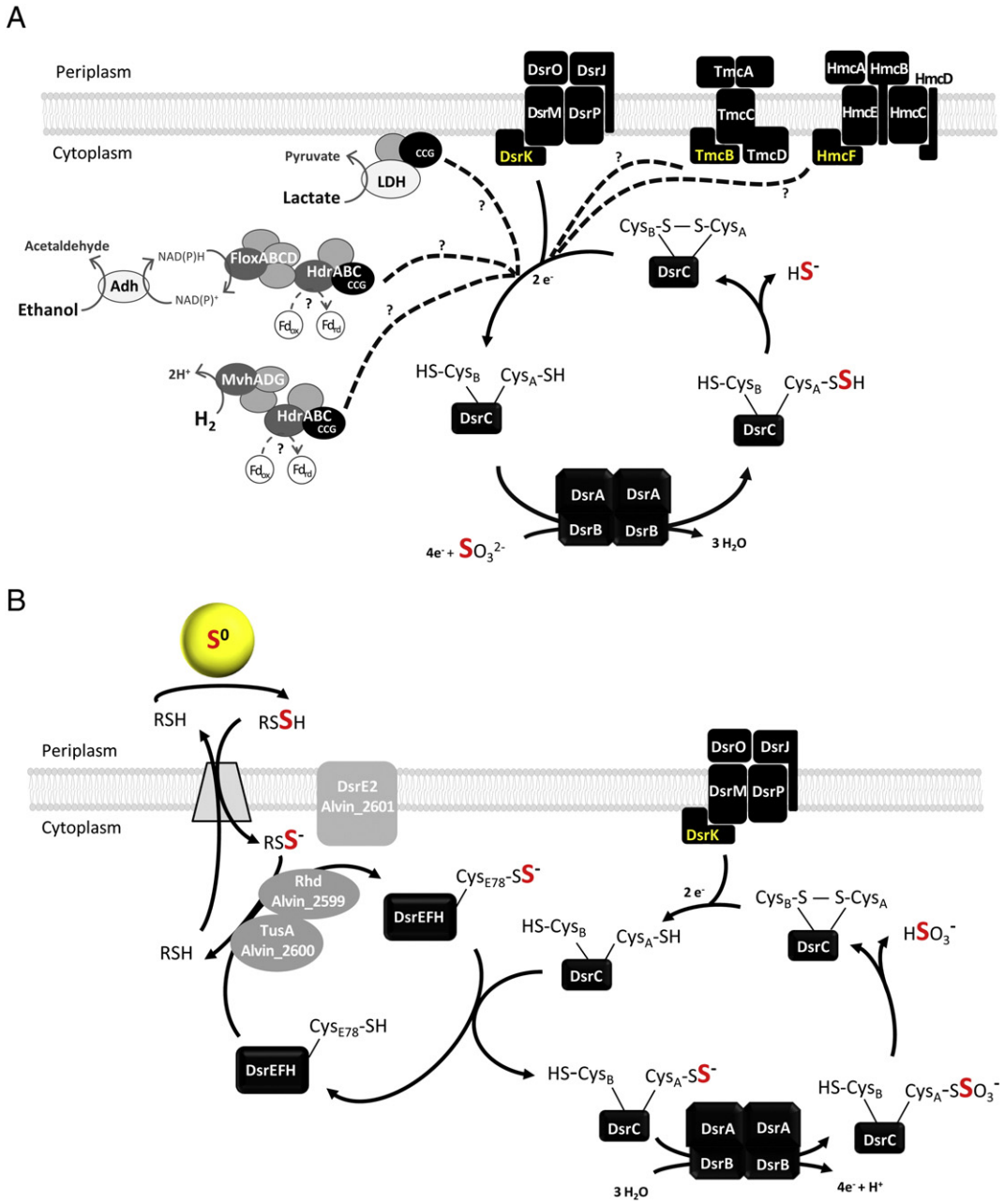


Fig. 9. Integrated models for sulfite reduction in SRO (A), and for sulfur globule oxidation in phototrophic sulfur bacteria (B).

Here, we reviewed evidence to suggest that DsrC, or more specifically the $DsrC_{ox}$ form, is a protein version of the CoM-S-S-CoB heterodisulfide of methanogens. We propose that $DsrC_{ox}$ can potentially be reduced, not only by its most obvious electron donor DsrMK/DsrMKJOP, but possibly also by several HdrB- and HdrD-like proteins present in sulfur-metabolizing organisms (Fig. 9). These putative disulfide reductases include other membrane complexes (HmcABCDEF and TmcABCD) involved in electron transfer with the periplasm and/or menaquinone pool, and also soluble proteins involved in oxidation of several substrates such as ethanol (Adh/FloxABCD/HdrABC), H_2 (MvhDGA/HdrABC), lactate (Ldhs), the β-oxidation pathway and possibly others (Fig. 9). The finding of *dsrC* genes close to genes of some of these proteins adds support to this idea.

However, it should also be kept in mind that the conserved cysteine motif used to identify CCG proteins, is not necessarily an indication that it binds a catalytic $[4Fe–4S]^{3+/2+}$ center, as this motif has also been shown to bind zinc [108]. Furthermore, this domain is also found in subunits of enzymes, such as succinate:quinone oxidoreductase, glycolate oxidase and anaerobic glycerol-3-phosphate dehydrogenase, that are widespread in many organisms not related to sulfur metabolism [6,7]. Many of these proteins are membrane-associated without obvious transmembrane subunits, and it has been proposed that the CCG domain contains amphipathic α-helices that are responsible for the membrane attachment [74,109]. This has been confirmed for *A. vinosum* DsrK, which binds directly to the membrane even in the absence of DsrP or DsrM [63]. Since the lactate dehydrogenase belongs to the same family as these enzymes, it is possible that its Hdr-like subunit is involved in membrane attachment and/or electron transfer to menaquinone, and not really to $DsrC_{ox}$.

So, further experimental evidence has to be obtained before confidently stating that DsrC is the substrate for all these cases. Nevertheless, at least for DsrK and TmcB it has been shown that they contain a $[4Fe–4S]^{3+/2+}$ center [73,92], so the assumption that they reduce a disulfide is more solid. Also, this assumption seems more reasonable in the HdrABC-related proteins. Their occurrence in both methanogens and sulfur metabolizing organisms may reflect a common ancient origin of these organisms [110], and/or their co-localization in the same environments and often syntrophic association [111].

The recent structural data on the DsrAB sulfite reductase provided key insights into how the enzyme operates, which helped to explain not only the in vitro production of thionates [59], but also the key role played by the DsrC protein in the process [38]. Nevertheless, further work is obviously required to fully reveal the mechanism of sulfite reduction and how exactly DsrC works. Elucidating this process has important implications into understanding the sulfur isotope fractionations imparted by biological sulfate reduction, the main environmental process controlling the geological sulfur isotope record [48].

For the membrane-bound complexes the association with DsrC provides a link between chemiosmotic energy conservation and the sulfite reduction step, which has been shown to be associated with proton translocation [112]. For the soluble HdrABC-containing complexes the proposed reduction of $DsrC_{ox}$ may be associated with the new energy coupling mechanism of electron bifurcation performed by HdrABC/MvhDGA or HdrABC/FloxABCD complexes [76]. Overall, the present evidence is quite overwhelming in the argument that DsrC is a central and key protein in the bioenergetics of organisms dissimilating sulfur compounds.

Acknowledgements

This work was supported by PTDC/QUI-BIQ/100591/2008 and PTDC/BBB-BQB/0684/2012 to IACP, and Pest-OE/EQB/LA0004/2011 to ITQB, funded by Fundação para a Ciência e Tecnologia (FCT, Portugal), by the Deutsche Forschungsgemeinschaft (grant Da 351/6-1 to CD), and by the Luso–German Joint Action (A-21/11) funded by DAAD and CRUP. We thank Prof. Chun-Jung Chen of the National Synchrotron Radiation Research Center, Taiwan for providing the coordinates of the *D. gigas* DsrAB/C complex with the retracted DsrC C-terminal arm. We thank Bruno Marreiros for help with sequence processing and Vitor Teixeira for help with PyMOL figures. S.S.V. is recipient of a fellowship (SFRH/BPD/79823/2011) from FCT, Portugal.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2014.03.007.

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