Sulfur globule oxidation in green sulfur bacteria is dependent on the dissimilatory sulfite reductase system

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Green sulfur bacteria (GSB) oxidize sulfide and thiosulfate to sulfate, with extracellular globules of elemental sulfur as an intermediate. Here we investigated which genes are involved in the formation and consumption of these sulfur globules in the green sulfur bacterium *Chlorobaculum tepidum*. We show that sulfur globule oxidation is strictly dependent on the dissimilatory sulfite reductase (DSR) system. Deletion of *dsrM*/CT2244 or *dsrT*/CT2245, or the two *dsrCABL* clusters (CT0851–CT0854, CT2247–2250), abolished sulfur globule oxidation and prevented formation of sulfate from sulfide, whereas deletion of *dsrU*/CT2246 had no effect. The DSR system also seems to be involved in the formation of thiosulfate, because thiosulfate was released from wild-type cells during sulfide oxidation, but not from the *dsr* mutants. The *dsr* mutants incapable of complete substrate oxidation oxidized sulfide and thiosulfate about twice as fast as the wild-type, while having only slightly lower growth rates (70–80 % of wild-type). The increased oxidation rates seem to compensate for the incomplete substrate oxidation to satisfy the requirement for reducing equivalents during growth. A mutant in which two sulfide : quinone oxidoreductases (*sqrD*/CT0117 and *sqrF*/CT1087) were deleted exhibited a decreased sulfide oxidation rate (~50 % of wild-type), yet formation and consumption of sulfur globules were not affected. The observation that mutants lacking the DSR system maintain efficient growth suggests that the DSR system is dispensable in environments with sufficiently high sulfide concentrations. Thus, the DSR system in GSB may have been acquired by horizontal gene transfer as a response to a need for enhanced substrate utilization in sulfide-limiting habitats.

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INTRODUCTION

Phototrophic sulfur bacteria oxidize inorganic sulfur compounds (in particular sulfide, thiosulfate and elemental sulfur) for photosynthetic growth (Brune, 1989; Frigaard & Dahl, 2009; Overmann, 2008). They thrive in anoxic marine and freshwater environments, where sulfide and light occur together. These bacteria are divided into the green sulfur bacteria (GSB; phylum Chlorobi, family *Chlorobiaceae*) and the purple sulfur bacteria (PSB; class Gammaproteobacteria, families *Chromatiaceae* and *Ectothiorhodospiraceae*) (Imhoff, 2008). Although these two major groups share certain ecological functions, their overall physiology and evolutionary origins are very

different. Despite these differences, GSB, PSB and certain chemotrophic prokaryotes (including sulfate reducers, and sulfide- and thiosulfate-oxidizing chemolithotrophs) metabolize inorganic sulfur compounds using homologous enzyme systems that have been exchanged by horizontal gene transfer (e.g. Klein *et al.*, 2001; Meyer *et al.*, 2007). The sulfur metabolism of GSB and PSB has been studied for decades (Brune, 1989; Frigaard & Dahl, 2009; Sakurai *et al.*, 2010). Nevertheless, a solid understanding of the pathways and mechanisms of the oxidation of sulfide, sulfur globules and thiosulfate has still not been achieved.

Genome sequencing has greatly increased our understanding of the sulfur metabolism in GSB (Chan *et al.*, 2008a; Frigaard & Bryant, 2008), and their physiology and evolution in general (Eisen *et al.*, 2002; Frigaard *et al.*, 2003). *Chlorobaculum tepidum* TLS was the first green sulfur bacterium for which genome sequence information was made available (Eisen *et al.*, 2002). This strain grows rapidly on sulfide and thiosulfate, and has been used extensively as a model organism for genetic and physiological studies. A model of the dissimilatory sulfur metabolism in *C. tepidum* based on recent studies is shown in Fig. 1.

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Abbreviations: APS, adenylyl sulfate (adenosine 5'-phosphosulfate); DSR, dissimilatory sulfite reductase; GSB, green sulfur bacteria; PSB, purple sulfur bacteria; Sat, sulfate adenylyltransferase; SOX system, sulfur-oxidizing system; SQR, sulfide : quinone oxidoreductase.

Three supplementary figures and two supplementary tables are available with the online version of this paper.

Fig. 1. Overview of proposed pathways of the thiotrophic sulfur metabolism of *C. tepidum* (Eisen *et al.*, 2002; Frigaard & Bryant, 2008; Sakurai *et al.*, 2010). Abbreviations: APS, adenosine 5'-phosphosulfate; MK, menaquinone; Sat, sulfate adenylyltransferase.

Both GSB and PSB form sulfur globules by incomplete oxidation of sulfide (Frigaard & Dahl, 2009). The exact molecular structure of this sulfur (often referred to as 'elemental sulfur') is still debated, but it is thought to occur as long-chain, zero-valent polysulfanes, probably terminated by organic residues (Prange *et al.*, 2002). In most PSB, such as *Allochromatium vinosum*, the sulfur globules are deposited in the periplasmic space of the cells and are encapsulated by proteins, whereas the sulfur globules of GSB are deposited extracellularly and are not known to be associated with protein. When reducing equivalents are needed (e.g. if sulfide is depleted), the sulfur globules are oxidized completely to sulfate.

The mechanisms involved in the formation and consumption of sulfur globules in PSB and GSB are largely unknown (Frigaard & Dahl, 2009). It has been proposed that a periplasmic pool of oligosulfides or organic thiols serves as an intermediate between sulfur in the globules and the enzymes that oxidize sulfur compounds in the cell (Fig. 1). The only genes known to be essential for oxidation of sulfur globules in PSB are the *dsr* genes, which in *A. vinosum* are located in a single cluster, *dsrABEFHCMKLJOPNRS* (Dahl *et al.*, 2005). These genes encode a dissimilatory sulfite reductase (DSR) system that is homologous to that in sulfate-reducing bacteria but operates in the reverse (oxidative) direction. The Dsr proteins that appear to be common among all DSR-containing bacteria are a sirohaem- or

siroamide-containing sulfite reductase (DsrAB), a transmembrane electron-transporting complex (DsrMKJOP), a putative sirohaem amidase (DsrN), and the DsrC protein. In addition, *A. vinosum* and other sulfide oxidizers (e.g. GSB, *Thiobacillus denitrificans*, *Magnetococcus* sp. MC-1) contain a putative sulfur transferase complex (DsrEFH) and an iron-sulfur flavoprotein with NADH oxidoreductase activity (DsrL). Individual gene inactivation of *dsrA*, *dsrB*, *dsrH*, *dsrM*, *dsrK*, *dsrL*, *dsrJ*, *dsrO* and *dsrP* completely prevents oxidation of sulfur globules in *A. vinosum*, whereas individual gene inactivation of *dsrN* and *dsrR* significantly reduces the oxidation rate of sulfur globules (Dahl *et al.*, 2005; Grimm *et al.*, 2010; Lübke *et al.*, 2006; Pott & Dahl, 1998; Sander *et al.*, 2006).

All GSB capable of growth on sulfur compounds (other than *Chloroherpeton thalassium*) contain *dsr* genes (Frigaard & Bryant, 2008). In *C. tepidum*, the genes are split into two clusters, *dsrNCABL6TMKJOP89* (CT2251–2238) and *dsrCABLEFH* (CT0851–0857), such that *dsrCABL* is duplicated. The published genome sequence of *C. tepidum* contains a frameshift mutation in *dsrB2*/CT2248, providing a possible explanation for the duplication (Eisen *et al.*, 2002). In *Chlorobium* and *Prosthecochloris* strains, all *dsr* genes are organized in a single cluster. Although the DSR system appears to be similar in *A. vinosum* and GSB, there are some notable differences in the components and their evolutionary origin. The *dsr* genes in *C. tepidum* include CT2238,

Table 1. Strains of *C. tepidum* used in this study

Strain	Genotype
CH1	Wild-type (derived from strain TLS)
<i>sqrD</i>	Δ CT0117:: <i>aadA</i> (Sp ^R marker from pSRA81)
<i>sqrF</i>	Δ CT1087:: <i>cat-ermC</i> (Cm ^R -Em ^R marker from pJHJ06)
<i>sqrD sqrF</i>	Δ CT0117:: <i>aadA</i> Δ CT1087:: <i>cat-ermC</i>
<i>dsrU</i>	Δ CT2246:: <i>aadA</i> (Sp ^R marker from pJHJ02)
<i>dsrT</i>	Δ CT2245:: <i>aadA</i> (Sp ^R marker from pJHJ02)
<i>dsrM</i>	Δ CT2244:: <i>aadA</i> (Sp ^R marker from pSRA81)
<i>dsrCABL1</i>	Δ (CT0851–CT0854):: <i>aadA</i> (Sp ^R marker from pSRA81)
<i>dsrCABL2</i>	Δ (CT2247–CT2250):: <i>cat-ermC</i> (Cm ^R -Em ^R marker from pJHJ06)
<i>dsrCABL1/2</i>	Δ (CT0851–CT0854):: <i>aadA</i> Δ (CT2247–CT2250):: <i>cat-ermC</i>
Complemented <i>dsrM</i>	Δ CT2244:: <i>aadA</i> Δ CT0028::(CT2244 <i>ermC</i>)

CT2239, *dsrT*/CT2245 and CT2246, which are not found in *A. vinosum* (Frigaard & Dahl, 2009). The *dsrT* gene is found only in GSB and sulfate reducers (Mussmann *et al.*, 2005; Sander *et al.*, 2006). Phylogenetic studies have shown that the DsrTMKJOP proteins in GSB are closely related to their homologues in sulfate-reducing bacteria, whereas most other Dsr proteins in GSB are closely related to their homologues in PSB (Sander *et al.*, 2006). The *dsrRS* genes found in *A. vinosum* are not found in GSB.

Several enzymes have been proposed to oxidize sulfide in *C. tepidum*, including sulfide:quinone oxidoreductase (SQR) and flavocytochrome *c* (Eisen *et al.*, 2002). In *C. tepidum*, the membrane-bound SQR homologues CT0117 and CT1087 are responsible for some of the sulfide oxidation activity in the cells (Chan *et al.*, 2009). Thiosulfate oxidation in GSB is carried out by the sulfur-oxidizing (SOX) system in a manner similar to that in *A. vinosum* (Azai *et al.*, 2009; Ogawa *et al.*, 2008). In the proposed pathway the sulfone moiety of thiosulfate is oxidized to sulfate and released, whereas the sulfane moiety is released to the putative pool of sulfur globule precursors and subsequently either deposited in sulfur globules or oxidized to sulfite by the *dsr* system (Fig. 1). Oxidation of sulfite to sulfate in *C. tepidum* has been proposed to be carried out by an APS reductase and a Sat (Eisen *et al.*, 2002; Fig. 1).

In the present study we investigate the function and significance of several of the above-mentioned proteins with respect to sulfur metabolism and growth in *C. tepidum*, with a focus on formation and consumption of sulfur globules.

METHODS

Bacterial strains, media and growth conditions. All strains used in this study are listed in Table 1. Our wild-type strain (CH1) was derived from the plating strain of *C. tepidum* TLS (Wahlund *et al.*, 1991), and

was shown to have a restored reading frame of *dsrB2*/CT2248. Unless otherwise stated, *C. tepidum* was grown in liquid CL medium or on solid CP plates (Frigaard *et al.*, 2004) at 42 °C with an incandescent light intensity of 50 μ mol photons m⁻² s⁻¹. Cultivation, manipulation and sampling were performed in an anaerobic chamber with an atmosphere consisting of 5% H₂, 10% CO₂ and 85% N₂ (Coy Laboratory Products). Growth and oxidation rate experiments with various sulfur compounds were performed in sulfur-free CL3 medium supplemented with sterile, anoxic sulfide and thiosulfate stock solutions. One litre of CL3 medium contained 20 ml of salts A3 (per litre: 0.64 g Na₂EDTA·2H₂O, 10 g MgCl₂·6H₂O, 2.5 g CaCl₂·2H₂O, 20 g NaCl), 20 ml of salts B2 (per litre: 25 g NH₄CH₃COO, 20 g NH₄Cl), 20 ml buffer (per litre: 25 g KH₂PO₄, 105 g MOPS), 1 ml trace elements (Wahlund *et al.*, 1991), 50 μ l resazurin (10 mg ml⁻¹) and 20 μ l vitamin B12 (1 mg ml⁻¹). After the solution had been autoclaved at 121 °C for 20 min, the hot medium was cooled for 45 min while bubbling with N₂ under sterile conditions. Inside the anaerobic chamber, 50 ml sterile, anoxic NaHCO₃ solution (2 g) and 1 ml sterile, anoxic sodium ascorbate (1 M) were added. The final pH of the medium was between 6.9 and 7.0. Prior to use, the medium was stored overnight in the anaerobic chamber until it became colourless. Cells of *C. tepidum* used for experiments with sulfur compound transformation were grown overnight (14–18 h) in CL medium and pelleted by centrifugation for 7 min at 720 g (outside the anaerobic chamber). The cell pellet was resuspended in CL3 (inside the anaerobic chamber) before sulfide or thiosulfate was added. Sulfur transformation measurements were performed in cultures (16 ml) in screw-capped tubes placed in a rotating wheel at 42 °C inside the anaerobic chamber.

Quantification of sulfur compounds, protein and BChl *c*. For sulfide analysis, one volume of sample was fixed in 2.5 volumes of 2% (w/v) zinc acetate dihydrate (supplemented with 0.2%, v/v, acetic acid). The sulfide concentrations were determined colorimetrically as described by Cline (1969). Each sulfide concentration in the study is the mean of technical duplicates. Elemental sulfur, BChl *c* and protein were determined on the same sample of pelleted cells. Cell suspension (0.2–1.0 ml) was pelleted by centrifugation (1 min, 5000 g). To remove traces of ascorbate, the pellet was washed with 500 μ l Na₂HPO₄/NaH₂PO₄ (20 mM, pH 7.0) and stored at –80 °C until the measurements were performed. Elemental sulfur, BChl *c* and BChl *d* were extracted by resuspending the cell pellet in methanol, and the concentrations were determined spectrophotometrically in the supernatant at 265, 669 and 659 nm, respectively, using absorption coefficients of 23.9 l g⁻¹ cm⁻¹ (Stal *et al.*, 1984), 86 l g⁻¹ cm⁻¹ and 82.3 l g⁻¹ cm⁻¹ (Stanier & Smith, 1960). The A₂₆₅ was corrected for pigment contribution by subtracting 40% of the A₆₆₉ and A₆₅₉, respectively (this percentage was empirically determined with sulfur globule-free wild-type cells). For protein determination, the methanol-extracted pellet was resuspended in 50 μ l NaOH (0.5 mM), and the protein concentration was determined using a total protein assay kit (TP0300, Sigma) using BSA as standard. High concentrations of sulfur globules did not affect the protein determination. All protein concentrations are means of technical triplicates performed on the same culture. Sulfate (*t*_R=9.2 min) and thiosulfate (*t*_R=19.9 min) were quantified by HPLC using indirect UV detection (detection wavelength 310 nm, reference wavelength 360 nm) and isocratic elution at ambient temperature (~20 °C). The analysis was performed on a model 1100 HPLC system (Agilent) using a PRP-X100 column (dimensions 150 mm × 4.1 mm, particle diameter 5 μ m) (Hamilton) and a mobile phase consisting of *p*-hydroxybenzoic acid (4 mM, pH 8.9) and methanol (2.5%, v/v) in water. To avoid distortion of the sulfate concentration by sulfide oxidation, nine volumes of the sample were mixed with one volume ZnCl₂ (100 mM) inside the anaerobic chamber and centrifuged prior to HPLC analysis.

Amplification and sequencing of the *dsrB* genes. To determine the sequence of *dsrB1* and *dsrB2*, PCR amplicons were prepared using primer pairs 179/180 and 268/271, respectively, since these primers

bind outside the homologous *dsrCABL* regions (Fig. 2a). The specific amplification of each locus was confirmed by sequencing the PCR amplicons using primer 260 (identical to primer CT2245U1; see Supplementary Table S1), since the sequencing reaction extends into the non-homologous region (Fig. 2a). Sequencing of the *dsrB* genes in PCR amplicons obtained in separate experiments with the wild-type ($n=3$) and the *dsrCABL1* mutant ($n=5$) was performed using primer 190 (5'-CATCAACCTACAGGAACGAG-3').

Mutagenesis of *C. tepidum*. Gene inactivation in *C. tepidum* was performed using natural transformation and homologous recombination, as previously described (Frigaard *et al.*, 2004; Frigaard & Bryant, 2001). Gene inactivation constructs were made either by an *in vitro* PCR-based 'ligation' approach or by a cloning-based approach (Frigaard *et al.*, 2004; Jacobsen *et al.*, 2011). Primers used for amplifying the homologous flanking regions of the target gene are listed in Supplementary Table S1, which also describes the mutagenesis strategy used for each mutant.

Plasmids for genetic complementation of the *dsrM* mutant of *C. tepidum* were constructed using the *bchU* gene as expression platform, similar to the manner described by Maresca *et al.* (2008). Two homologous regions (A and B) flanking the start codon of the *C. tepidum bchU* gene were amplified by PCR using primers 486 and 487 for region A (865 bp), and primers 488 and 489 for region B (864 bp) (Supplementary Table S2). The amplified regions were fused by a second round of PCR using primers 486 and 489 and a 1 : 1 mixture of PCR products A and B as template. The 1753 bp PCR product was cloned into pUC19 using *EcoRI* and *HindIII*, resulting in plasmid pCX. Plasmid pCX was engineered such that the start codon region contained an *NcoI* site and a multiple cloning site. The *ermC* cassette encoding erythromycin resistance was amplified with primers 500 and 501 and inserted into pCX using *PstI* and *BamHI*, resulting in plasmid pCX-Em. The *dsrM* gene was amplified from genomic DNA using primers 496 and 510 and inserted into pCX-Em using *NcoI* and *SpeI*, resulting in the plasmid used for complementation of the *dsrM* mutant. A map of the expression construct is shown in Fig. 2(b). Primers are listed in Supplementary Table S2. Correct insertion of the expression construct in the *bchU* locus was verified by PCR (primers 490 and 491) and the production of BChl *d* by the complemented *dsrM* mutant.

Sulfur globule purification. Sulfur globules excreted by the *dsrCABL1/2* mutant of *C. tepidum* were purified and used as growth

substrates for other cultures. Overnight cultures of this mutant grown in CL medium were harvested by centrifugation (10 min, 4200 g). The pellet was resuspended in buffer (20 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.0) containing sucrose (50 %, w/v) and centrifuged (5 min, 3000 g). The pelleted sulfur globules were washed once with buffer (20 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.0) and resuspended in CL3 medium. For experiments with sulfur globule utilization, the stock suspension of sulfur globules was added to CL3 medium, which was supplemented with 0.1 mM sulfide.

RESULTS

Nomenclature of *dsr* and *sqr* genes in GSB

CT2246 in *C. tepidum* encodes a 14 kDa protein with no identified conserved sequence motifs. This gene is conserved in all *dsr* gene clusters in GSB and we suggest naming this gene *dsrU*. Other than GSB, *dsrU* (Mmc1_2160) is found only in the sulfide-oxidizing proteobacterium *Magnetococcus* sp. MC-1 (Schübbe *et al.*, 2009) as part of the cluster *dsrABLU* (Mmc1_2156–2160).

CT2239 and CT2238 are also putative components of the DSR system because they are conserved in all *dsr* clusters in GSB, and because they encode homologues of proteins that are specific to sirohaem biosynthesis (the cofactor of nitrite and sulfite reductases). We suggest naming these genes *dsrV* and *dsrW*, respectively. *Chlorobium ferrooxidans* DSM 13031 contains distant homologues of *dsrV* (ZP_01387072) and *dsrW* (ZP_01387073), but these are part of the assimilatory sulfate reduction *cys* gene cluster (Frigaard & Bryant, 2008).

CT0117 and CT1087 encode different SQR enzymes in *C. tepidum* (Chan *et al.*, 2009). Phylogenetic analyses show that these SQRs and the SQRs from other organisms group in distinct clades (Chan *et al.*, 2009; Frigaard & Bryant, 2008; Theissen *et al.*, 2003). We suggest naming CT0117

Fig. 2. (a) The *dsr* gene clusters in *C. tepidum* (Eisen *et al.*, 2002). Grey indicates regions of high sequence homology. The black triangle indicates the frameshift mutation in the published genome (Eisen *et al.*, 2002). Black arrows indicate binding of primers for PCR and sequencing. Prediction of promoters and terminators was done using FGENESB software (<http://linux1.softberry.com/berry.phtml?topic=fgenesb&group=programs&subgroup=gfindb>). (b) Map of the plasmid pCX-Em used for *dsrM* complementation in *C. tepidum*. Grey indicates regions homologous to the *C. tepidum* genome. The *dsrM* gene was inserted in the *NcoI*/*SpeI* sites and is under the control of the *bchU* promoter (P_{bchU}).

Table 2. Genes related to sulfur metabolism and targeted for inactivation in *C. tepidum*

Gene	Locus tag	Putative function*
<i>sqrD</i>	CT0117	SQR, type SqrD
<i>dsrC1</i>	CT0851	DSR system component, protein DsrC, copy 1
<i>dsrA1</i>	CT0852	DSR, subunit A, copy 1
<i>dsrB1</i>	CT0853	DSR, subunit B, copy 1
<i>dsrL1</i>	CT0854	DSR system component, protein DsrL, copy 1
<i>sqrF</i>	CT1087	SQR, type SqrF
<i>dsrM</i>	CT2244	DSR system component, protein DsrM
<i>dsrT</i>	CT2245	DSR system component, protein DsrT
<i>dsrU</i>	CT2246	Putative DSR system component, protein DsrU
<i>dsrL2</i>	CT2247	DSR system component, protein DsrL, copy 2
<i>dsrB2</i>	CT2248	DSR, subunit B, copy 2
<i>dsrA2</i>	CT2249	DSR, subunit A, copy 2
<i>dsrC2</i>	CT2250	DSR system component, protein DsrC, copy 2

*Chan *et al.* (2009), Eisen *et al.* (2002), Mussmann *et al.* (2005), Sander *et al.* (2006), this work.

and CT1087 as *sqrD* and *sqrF*, respectively, while reserving *sqrA*, *sqrB*, *sqrC* and *sqrE* for other groups of SQRs.

Construction of knockout mutants

Nine knockout strains of *C. tepidum* CH1 were constructed (Tables 1 and 2). The double mutant *sqrD sqrF* was made by transforming the *sqrD* mutant with an *sqrF* knockout construct. The double mutant *dsrCABL1/2* was made by transforming the *dsrCABL1* mutant with a *dsrCABL2* knockout construct. Transformants segregated easily. The genotype of the mutants was confirmed by PCR amplification across the insertion site of the antibiotic-resistance cassette (Frigaard *et al.*, 2004).

Individual inactivation of the *dsrA*, *dsrB*, *dsrC* and *dsrL* copies would require multiple cloning steps due to the duplication of the *dsrCABL* cluster (Fig. 2). Instead, mutants denoted *dsrCABL1*, *dsrCABL2* and *dsrCABL1/2* were constructed that lacked *dsrC1A1B1L1*, *dsrC2A2B2L2* or both clusters, respectively. Mutant *dsrCABL1* was made with a construct containing homologous regions made using primers 180, 181, 178 and 179 (Fig. 2). Mutant *dsrCABL2* was made with a construct containing homologous regions made using primers 268, 269, 270 and 271 (Fig. 2).

dsrB gene sequences of *C. tepidum*

According to the published genome sequence (NC_002932), *C. tepidum* TLS contains two copies of *dsrB* (*dsrB1*/CT0853 and *dsrB2*/CT2248), in which *dsrB2* contains a frameshift mutation leading to a non-functional copy of *dsrB* (Eisen *et al.*, 2002). Sequencing of the *dsrB2*

gene of the wild-type and the *dsrCABL1* mutant revealed that *dsrB2* had reverted to a functional reading frame, such that the sequences of *dsrB2* and *dsrB1* were identical. Due to this genotype, which is different from the published genotype (Eisen *et al.*, 2002), the *C. tepidum* wild-type strain used in this study is denoted CH1.

Sulfide and thiosulfate utilization by wild-type and *dsr* mutants

When sulfide was supplied to *C. tepidum* wild-type it was oxidized to sulfur globules, while a transient increase in thiosulfate could be detected (Fig. 3a, b). After sulfide depletion, the elemental sulfur and the thiosulfate formed were completely oxidized to sulfate. Thiosulfate supplied as sole sulfur source was likewise oxidized completely to sulfate, although no transient increase in elemental sulfur could be detected (Supplementary Fig. S1a). The *dsrM* mutant also oxidized sulfide but permanently accumulated elemental sulfur, and no increase in thiosulfate was observed (Fig. 3c, d). As in the wild-type, thiosulfate was oxidized in the *dsrM* mutant upon depletion of sulfide. However, whereas thiosulfate was completely oxidized to a bimolar amount of sulfate in the wild-type, thiosulfate was oxidized to an equimolar amount of sulfate and elemental sulfur in the *dsrM* mutant (Fig. 3 and Supplementary Fig. S3g; see also below). The *dsrCABL1/2* mutant exhibited the same phenotype as the *dsrM* mutant: sulfide was oxidized to an equimolar amount of elemental sulfur and no formation of sulfate or thiosulfate was observed (Supplementary Fig. S1b, c).

By optical microscopy, the accumulated extracellular sulfur in the *dsrM* and *dsrCABL1/2* mutants appeared as refractive globules similar to the sulfur globules formed by the wild-type, except that the sulfur globules from the *dsr* mutants were larger (diameter ~1–4 µm) than those from the wild-type (diameter ~1 µm) (Supplementary Fig. S2). Sulfur globules produced by cultures of *dsr* mutants also appeared to sediment faster. This behaviour probably contributed to the high SD values obtained in measurements of elemental sulfur concentration in suspensions containing these sulfur globules (e.g. Fig. 4).

Long-term incubations revealed a similar sulfur compound transformation pattern, as expected from the initial time-course experiment. *C. tepidum* wild-type oxidized 2 mM sulfide completely within 24 h to 2 mM sulfate, and 2 mM thiosulfate to 4 mM sulfate (Fig. 4a and Supplementary Fig. S3a). Mutant *dsrM* oxidized sulfide to an approximately equimolar amount of elemental sulfur, while no increase in sulfate could be detected (Fig. 4g). The *dsrM* mutant oxidized thiosulfate to an equimolar amount of sulfate and an approximately equimolar amount of elemental sulfur (Supplementary Fig. S3g). Mutants *dsrCABL1/2* and *dsrT* exhibited the same behaviour with respect to sulfide and thiosulfate oxidation, while mutant *dsrU* behaved in a manner similar to that of the wild-type (Fig. 4 and Supplementary Fig. S3).

Fig. 3. Time-course experiments with the oxidation of sulfide (1.5 mM) in wild-type (a, b) and *dsrM* cultures (c, d) of *C. tepidum*. The initial protein content of the cultures was ~25 µg ml⁻¹. Sulfide (●), sulfur (○), sulfate (△) and thiosulfate concentrations (▲) were measured. The mean and standard deviation of replicate cultures ($n=2$) are shown. The presence of thiosulfate (0.4 mM) at time 0 is due to abiotic formation of thiosulfate during medium preparation. The sulfur recovery (of all sulfur compounds) at the end of the experiment expressed as a percentage of the initial sulfur concentration is shown in the upper-right corner.

These observations support the current model of sulfide and thiosulfate utilization in *C. tepidum* (Fig. 1), and show that sulfur globules are an intermediate component in both sulfide and thiosulfate oxidation. Furthermore, the observations suggest that both the sulfide and the thiosulfate sulfane sulfur enter the same pool of sulfur globule precursors.

Mutants lacking only one *dsrCABL* cluster (*dsrCABL1* and *dsrCABL2*) both exhibited a strongly diminished ability to oxidize sulfur. Both mutants produced a small, yet statistically significant (t test, $P<0.05$, $n=3$) amount of sulfate upon oxidation of sulfide (Fig. 4c, d); however, even after 96 h of incubation sulfur globules were not oxidized (data not shown). These experiments show that oxidation to sulfate is completely abolished only in the double mutant and that both *dsrCABL* clusters are necessary for efficient oxidation to sulfate.

Genetic complementation of the *dsrM* mutant

The *dsrM* mutant was complemented *in trans* with an expression construct of the *dsrM* gene from the wild-type (see Methods). Long-term incubation showed that the complemented mutants regained their ability to produce sulfate from sulfide and thiosulfate, although the consumption rate of the sulfur globules was somewhat slower than in the wild-type and the *bchU* mutant (Fig. 4h and Supplementary Fig. S3h).

Sulfur globule formation and consumption

Sulfide oxidation results in the formation of oligosulfides, which are substrates for sulfur globule formation (Fig. 1). The SQR homologues in *C. tepidum*, SqrD and SqrF, have been shown to catalyse sulfide oxidation and are probably responsible for the formation of oligosulfides (Chan *et al.*, 2009). However, time-course experiments in the present study showed that an *sqrD* *sqrF* double mutant still produced sulfur globules as an intermediate when sulfide was oxidized (Supplementary Fig. S1d, e). This suggests that one or more additional unknown sulfide-oxidizing enzymes produces precursors of sulfur globule formation. Work is in progress to identify this enzyme activity.

Fig. 4. Sulfide utilization (2 mM) by *C. tepidum* strains: (a) wild-type, (b) *dsrCABL1/2*, (c) *dsrCABL1*, (d) *dsrCABL2*, (e) *dsrU*, (f) *dsrT*, (g) *dsrM*, (h) complemented *dsrM* mutant. Incubation times are indicated below the graphs. Growth and metabolic transformation of sulfur compounds had ceased at the end of the experiment. The initial protein content of the cultures was $\sim 100 \mu\text{g ml}^{-1}$. Sulfide (black bars), sulfur (dark-grey bars), thiosulfate (light-grey bars) and sulfate (white bars) concentrations were measured. The sulfur recovery (of all sulfur compounds) at the end of the experiment expressed as a percentage of the initial sulfur concentration is shown in the upper-right corner. The mean and SD of replicate cultures ($n \geq 3$) are shown. The presence of elemental sulfur at time 0 in *dsr* mutants is due to carry-over from the inoculum.

Sulfur globules purified from cultures of *dsr* mutants served as sole sulfur source for other *C. tepidum* cells. When wild-type cells ($30 \mu\text{g protein ml}^{-1}$) were supplemented with sulfur globules ($\sim 3 \text{ mM}$), the sulfur

globules were readily oxidized completely to sulfate within 24 h. This suggests that the formation and structure of the sulfur globules produced by the wild-type and the *dsr* mutants are similar. It also shows that

sulfur globules produced by one cell are freely available to any other cell.

Sulfide and thiosulfate oxidation rates

Oxidation rates were measured on whole cells of the wild-type, three *dsr* mutants (*dsrM*, *dsrT* and *dsrCABL1/2*) and three other mutants lacking genes involved in sulfide oxidation (*sqrD*, *sqrF*, *sqrD sqrF*) (Table 3). Double knockout of the SQR homologues *sqrD* and *sqrF* and single knockout of *sqrD* resulted in a reduced sulfide oxidation rate of about 50 % of that of the wild-type. The single mutant of *sqrF* did not show a statistically significant variation from the wild-type (*t* test, $P < 0.05$). The results show that *sqrD* is responsible for most SQR activity in the cells under the conditions tested, in agreement with results obtained with membrane fractions (Chan *et al.*, 2009). The thiosulfate oxidation rate was unaffected in all *sqr* mutants.

Interestingly, all *dsr* mutants oxidized sulfide and thiosulfate about twice as fast as the wild-type. In these experiments the light intensity was close to saturation (as in all other experiments reported in this work), and the BChl *c* to protein ratio was 0.055 ± 0.010 , w/w, throughout the duration of the experiments and for all strains, which excludes the pigment composition from being responsible for the different oxidation rates. The *dsr* mutants are not able to obtain as many reducing equivalents per unit of consumed substrate (sulfide or thiosulfate) as the wild-type. Thus, the increased substrate consumption rates presumably allow the *dsr* mutants to obtain reducing equivalents at a rate similar to that of the wild-type.

Table 3. Sulfide and thiosulfate oxidation rates in *C. tepidum* wild-type and mutant strains

The measurements were performed on suspensions of whole cells ($100\text{--}200\ \mu\text{g protein ml}^{-1}$) in CL3 medium with 2 mM sulfide or 2 mM thiosulfate for about 2 h. The mean $\pm$ SD are shown ($n=4$ for sulfide oxidation measurements in wild-type and all *sqr* mutants; $n=2$ for all other measurements).

Strain	Oxidation rate [$\mu\text{mol h}^{-1}$ (mg protein) $^{-1}$]	
	Sulfide	Thiosulfate
Wild-type	12.0 ± 2.8	9.6 ± 0.3
<i>sqrD sqrF</i>	5.7 ± 1.3	9.9 ± 2.8
<i>sqrD</i>	6.7 ± 1.0	8.5 ± 0.7
<i>sqrF</i>	11.0 ± 1.5	8.6 ± 2.9
<i>dsrM</i>	17.9 ± 2.1	25.2 ± 3.1
<i>dsrT</i>	17.2 ± 1.8	21.9 ± 0.8
<i>dsrCABL1/2</i>	20.3 ± 0.1	25.6 ± 6.2

Growth rates and growth yield

Growth rates were determined by measuring BChl *c* concentrations in the cultures, when 3 mM sulfide or 3 mM thiosulfate as sole sulfur source was supplied. The ratio of BChl *c* to protein was determined to be constant during the experiment. The wild-type grew at a rate of $0.34 \pm 0.02\ \text{h}^{-1}$ ($n=2$) when sulfide was supplied. This agrees with the published doubling time of *C. tepidum* of 2 h (Wahlund *et al.*, 1991). The *dsrCABL1/2* and *sqrD sqrF* mutants displayed growth rates of about 70 % ($n=2$) and 82 % ($n=2$) of that of the wild-type, respectively, when grown on sulfide. A similar difference in the growth rates of the wild-type ($0.38 \pm 0.01\ \text{h}^{-1}$, $n=2$) and *dsrCABL1/2* ($0.30 \pm 0.02\ \text{h}^{-1}$, $n=2$) was observed when thiosulfate was the sulfur source supplied.

Although the growth rates of the wild-type and *dsrCABL1/2* mutants showed little variation, the growth yields were significantly different. To determine the growth yields, cells were inoculated in CL3 medium with a limiting amount of thiosulfate (3.5 mM). The increase in cellular protein was determined when the culture had consumed all of this thiosulfate and ceased to grow. The wild-type produced $37 \pm 6\ \text{g protein (mol thiosulfate)}^{-1}$ (two biological replicates), which was twice as much as the *dsrCABL1/2* mutant, $18 \pm 0\ \text{g protein (mol thiosulfate)}^{-1}$ (two biological replicates). This observation supports the idea that the *dsr* mutant obtains a growth rate similar to that of the wild-type by increasing the oxidation rate of its substrates, but reaches a much lower growth yield due to the incomplete oxidation of thiosulfate. The *sqrD sqrF* mutant, due to its reduced sulfide oxidation rate (Table 3), has less reducing equivalents available for growth, so a reduced growth rate is expected. The growth yield of the *sqrD sqrF* mutant grown on sulfide at low concentrations has been reported to be similar to that of the wild-type (Chan *et al.*, 2009), in agreement with the observation that this mutant oxidizes sulfide completely to sulfate.

DISCUSSION

Function and genetic makeup of the DSR system in GSB

The phenotype of the *dsr* mutants showed that the DSR system in *C. tepidum* is essential for the oxidation of sulfur globules. The same observation has been made in studies of *dsr* mutants of *A. vinosum* (Dahl *et al.*, 2005). All sequenced GSB contain the same *dsr* genes (*dsrNCABLUEFHTMKJO-PVW*), suggesting that the DSR system functions in the same manner in all GSB. The only exception is *Chlorobaculum parvum*, which lacks *dsrEFH* (http://www.ncbi.nlm.nih.gov/nuccore/NC_011027) and it is not obvious what, if anything, substitutes for the function of DsrEFH in this strain.

C. tepidum has two *dsrCABL* clusters (CT0851–CT0854 and CT2250–CT2247), which are 99.4 % identical at the

nucleotide sequence level, although the upstream regions containing putative promoters are different (Fig. 2) (Eisen *et al.*, 2002). The frameshift mutation detected in *dsrB2* (Eisen *et al.*, 2002) has been restored in our wild-type strain such that the sequences of *dsrB1* and *dsrB2* are identical. It is possible that the frameshift restoration and/or *dsrCABL* duplication is selected for by an increased content of the DsrCABL enzymes, which may be rate-limiting for growth under sulfur globule-oxidizing conditions.

Thiosulfate formation

Transient formation of thiosulfate during sulfide oxidation has been observed in certain GSB and PSB (Brune, 1989; Chan *et al.*, 2008b; Steinmetz & Fischer, 1982; Steudel *et al.*, 1990; Trüper *et al.*, 1988), although the mechanism is not known. Thiosulfate oxidation in *C. tepidum* is performed by the SOX system (Azai *et al.*, 2009; Ogawa *et al.*, 2008), but is inhibited in the presence of sulfide (Chan *et al.*, 2008b). In our *C. tepidum* wild-type cultures, thiosulfate accumulated under sulfide-oxidizing conditions and was subsequently consumed when sulfide was depleted (Fig. 3b). This formation of thiosulfate was not observed in the *dsr* mutants (Fig. 3d and Supplementary Fig. S1b), suggesting that the DSR system is involved in thiosulfate formation. We propose that a fraction of the reduced sulfur is oxidized by the DSR system to sulfite prior to sulfide depletion, which is then further converted to thiosulfate by an abiotic reaction. Several abiotic mechanisms for thiosulfate formation from sulfite have been reported. Sulfite can react with sulfide, forming elemental sulfur as an intermediate, which then subsequently reacts with sulfite to form thiosulfate and water (Heunisch, 1977). A second possible abiotic reaction forming thiosulfate is a sulfite molecule attacking an oligosulfide chain according to the following reaction scheme (Roy & Trudinger, 1970):

Sulfite could accumulate in the cells if sulfite oxidation is inhibited or is not as rapid as the sulfite formation at high sulfide concentrations. Subsequent formation of thiosulfate from sulfite could take place either in the cytoplasm or in the periplasm, depending on where the sulfite accumulates. Excretion of sulfite or thiosulfate from the cytoplasm could be mediated by the putative sulfate transporter in GSB (CT0714; Eisen *et al.*, 2002; Fig. 1), since some sulfate transporters allow transfer of related anions such as sulfite and thiosulfate (Kredich, 1996).

Sulfide oxidation

We conclude that sulfide is oxidized by several enzymes in *C. tepidum*. Table 3 shows that a mutant lacking the two SQR homologues, SqrD and SqrF, has sulfide oxidation rates about 50 % of those of the wild-type. Chan and co-workers could not detect any sulfide-dependent quinone

reduction activity in membrane preparations from an *sqrD sqrF* mutant of *C. tepidum* (Chan *et al.*, 2009). Thus, sulfide oxidation activity in *C. tepidum* cells other than that due to SQR enzymes could be due to periplasmic enzymes or to membrane-bound enzymes that either do not retain their activity when membranes are prepared or use an electron acceptor different from the ones tested by Chan *et al.* (2009). The *sqrD sqrF* double mutant transiently accumulates sulfur globules. Thus, at least one of the non-SQR sulfide-oxidizing enzymes must be producing precursors of sulfur globule formation, possibly oligosulfides. The observation that the *sqrD sqrF* mutant was not able to increase the sulfide oxidation rate, as observed in the *dsr* mutants (Table 3), even though sulfide oxidation appeared to be limiting for growth, indicates that it is the SQR-catalysed sulfide oxidation which is enhanced in the *dsr* mutants.

Ecophysiological significance of the DSR system

Chloroherpeton is an early branching GSB that lacks *dsr* genes (Frigaard & Bryant, 2008). Sulfide is required for growth of *Chloroherpeton* and sulfur globules accumulate outside the cells upon sulfide oxidation. These sulfur globules ‘only very slowly disappear’ in culture (Gibson *et al.*, 1984), although it is not clear what the degradation product is. The DSR system that occurs in other GSB is an evolutionary chimera, since the membrane-bound proteins DsrTMKJOP are related to those in sulfate-reducing chemotrophs, and the soluble proteins DsrABCLEFH are related to those in PSB (Sander *et al.*, 2006). These and additional phylogenetic analyses suggest that the DSR system may have been acquired by the Chlorobi lineage after the divergence of *Chloroherpeton* (Frigaard & Bryant, 2008). If so, the Chlorobi lineage may have been unable to completely oxidize sulfide to sulfate prior to the horizontal transfer of *dsr* genes. However, the absence of a DSR system is not necessarily a significant disadvantage, because the growth rates of the *dsr* mutants observed in the present work suggest that GSB are able to sustain efficient growth by oxidizing sulfide exclusively to elemental sulfur provided that sulfide is not limiting. In natural environments, sulfur-reducing prokaryotes could maintain a non-limiting supply of sulfide. Examples of such stable symbiotic associations of GSB and a sulfur reducer, *Desulfuromonas acetoxidans*, have been described (Biebl & Pfennig, 1978). *Chloroherpeton* also grows very well in co-culture with *D. acetoxidans* (Gibson *et al.*, 1984). In these co-cultures, complete oxidation of sulfide to sulfate is unfavourable to the GSB partner because the sulfur-reducing partner cannot reduce sulfate. In addition, the availability of sulfur globules may be advantageous to GSB, because they can use the sulfur globules (and not sulfate) as electron acceptor in the dark, and thereby presumably increase the energy gained from fermentation of intracellular storage polyglucose (Brune, 1989). The mechanism that causes release of elemental sulfur in GSB, as opposed to the intracellular sulfur globules in PSB, could thus be

illustrating a lifestyle in which complete oxidation is not beneficial. However, once GSB grow in environments with very low concentrations of sulfide, such as at the edge of the sulfide gradient in stratified water columns or sediments (Overmann, 2008), complete oxidation of sulfur and a wider substrate range (e.g. thiosulfate) could become a competitive advantage. In fact, studies with continuous cultures of GSB show that under sulfide limitation, the organisms will produce both sulfur globules and sulfate (Van Gemerden, 1986).

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