A genomic region required for phototrophic thiosulfate oxidation in the green sulfur bacterium *Chlorobium tepidum* (syn. *Chlorobaculum tepidum*)

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The specific enzymes employed by *Chlorobium tepidum* for the anaerobic oxidation of thiosulfate, sulfide and elemental sulfur during anoxygenic photosynthesis are not well defined. In particular, it is unclear how *C. tepidum* completely oxidizes thiosulfate. A *C. tepidum* genomic region, encoding a putative quinone-interacting membrane-bound oxidoreductase (Qmo) complex (CT0866–0868), hypothetical proteins (CT0869–0875) and a sulfide : quinone oxidoreductase (SQR) homologue (CT0876), was analysed for its role in anaerobic sulfur oxidation. Transcripts of genes encoding the Qmo complex, which is similar to archaeal heterodisulfide reductases, were detected by RT-PCR only while sulfide or elemental sulfur were being oxidized, whereas the SQR homologue and CT0872 were expressed during thiosulfate oxidation and into early stationary phase. A mutant of *C. tepidum* was obtained in which the region between CT0868 and CT0876 was replaced by a transposon insertion resulting in the truncation or deletion of nine genes. This strain, C5, was completely defective for growth on thiosulfate as the sole electron donor in *C. tepidum*, but only slightly defective for growth on sulfide or thiosulfate plus sulfide. Strain C5 did not oxidize thiosulfate and also displayed a defect in acetate assimilation under all growth conditions. A gene of unknown function, CT0872, deleted in strain C5 that is conserved in chemolithotrophic sulfur-oxidizing bacteria and archaea is the most likely candidate for the thiosulfate oxidation phenotype observed in this strain. The defect in acetate assimilation may be explained by deletion of CT0874, which encodes a homologue of 3-oxoacyl acyl carrier protein synthase.

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INTRODUCTION

The green sulfur bacteria (GSB; *Chlorobiaceae*) are obligate anoxygenic phototrophs that occur in high densities in diverse aquatic environments (Castenholz *et al.*, 1990; Jung *et al.*, 2000; Wahlund *et al.*, 1991) containing sources of light and reductant, usually reduced sulfur compounds. Although the *Chlorobiaceae* are thought to be important players in anaerobic sulfur oxidation in aquatic environments, the specific mechanisms and enzymes involved in this process have not been precisely determined. In this work, *Chlorobium tepidum* was employed as a model

system to study sulfur oxidation in the *Chlorobiaceae*. *C. tepidum* can be genetically manipulated to produce single mutations (Frigaard & Bryant, 2001; Hanson & Tabita, 2001) but no transposon mutagenesis system has to our knowledge been previously described for this organism. There is also no complementation system yet in place.

C. tepidum can use elemental sulfur (S^0), sulfide (H_2S/HS^-) and thiosulfate ($S_2O_3^{2-}$) as electron donors to support phototrophic growth. While some *Chlorobiaceae* strains can utilize alternative electron donors such as H_2 (Overmann, 2000) and Fe^{2+} (Heising *et al.*, 1999), growth of *C. tepidum* is dependent on reduced sulfur compounds. Under phototrophic growth conditions, most *Chlorobiaceae* are known to oxidize sulfide to elemental sulfur, which accumulates extracellularly (Brune, 1995). After sulfide is depleted, most strains oxidize elemental sulfur to sulfate. In addition, many *Chlorobiaceae* strains, including *C. tepidum*, can oxidize thiosulfate to sulfate (Brune, 1995). Little is known about what capacity the *Chlorobiaceae* have for regulating and

Abbreviations: bChl, bacteriochlorophyll; BTP, 1,3-bis(tris(hydroxymethyl)methylamino)propane; Gm, gentamicin; GSB, green sulfur bacteria; IVTM, *in vitro* transposition mutagenesis; Qmo, quinone-interacting membrane-bound oxidoreductase; SQR, sulfide, quinone oxidoreductase.

Two supplementary tables are available with the online version of this paper.

integrating the interdependent processes of electron donor oxidation, light harvesting and CO_2 fixation.

The complete annotated *C. tepidum* genome has been used to propose models of sulfur oxidation pathways (Eisen *et al.*, 2002; Hanson & Tabita, 2003). Many of the predicted sulfur oxidation genes in *C. tepidum* are clustered in groups, which we have termed sulfur islands (Chan *et al.*, 2007). Within these sulfur islands, putative sulfur oxidation genes are interspersed with genes encoding hypothetical and conserved hypothetical proteins of unknown physiological relevance. Sulfur island I is a 32 kb region spanning *CT0841* to *CT0876* that encodes homologues of the dissimilatory sulfite oxidoreductase complex (Dsr), sulfate adenosyltransferase (Sat), adenosylphosphosulfate reductase (Aps), quinone-interacting membrane oxidoreductase complex (Qmo), thioredoxin reductase, a rhodanese-like protein, and a number of hypothetical and conserved hypothetical proteins. Both the Dsr and Qmo systems have been previously implicated in photosynthetic sulfur oxidation (Pott & Dahl, 1998) and sulfate reduction (Pires *et al.*, 2003), respectively.

How *C. tepidum* oxidizes thiosulfate remains enigmatic. The genome contains a partial sulfur oxidation (Sox) gene cluster related to that of *Paracoccus pantotrophus* GB17, whose role in thiosulfate oxidation has been well documented (Friedrich *et al.*, 2001). However, conspicuously absent from the *C. tepidum* genome are copies of the *soxCD* genes encoding a sulfur dehydrogenase activity. In fact, *soxCD* is lacking from all GSB genome sequences collected to date (see Table 4) and from the *Chlorobium limicola* Pond Mud isolate Sox gene cluster (Verté *et al.*, 2002).

In *P. pantotrophus* GB17, SoxCD oxidizes a SoxY-bound sulfur atom transferring six electrons to a cytochrome *c* acceptor (Friedrich *et al.*, 2000). These six electrons are 75 % of the total reducing equivalents available from the oxidation of thiosulfate. Two models have been proposed for how *C. tepidum* harvests these reducing equivalents. That proposed with the genome sequence (Eisen *et al.*, 2002) involves the cleavage of SoxY-bound sulfur to free sulfide followed by periplasmic oxidation of the resulting sulfide via the SoxF flavocytochrome *c* or SQR. The second, proposed by Hanson & Tabita (2003), invokes transfer of the SoxY-bound sulfur to an unknown low-molecular-mass thiol for subsequent transport and cytoplasmic oxidation.

Here, we report the characterization of the first *C. tepidum* mutant with a specific defect in thiosulfate-dependent growth, strain C5 ($\Delta CT0867-CT0876::TnOGm$). The mutation responsible for this phenotype is not in the Sox gene cluster, but in a section of sulfur island I, termed SI-I-3, that primarily encodes hypothetical proteins (Fig. 1a) (Chan *et al.*, 2007; Eisen *et al.*, 2002). The phenotype of strain C5 indicates that gene(s) between those encoding the Qmo complex and the SQR homologue in sulfur island I are required for growth on thiosulfate and play a role in acetate assimilation. Cross-genome comparisons with GSB and other bacteria and archaea suggest that the loss of

CT0872 is responsible for the thiosulfate oxidation defect while the loss of *CT0874* may cause the acetate oxidation defect observed in strain C5. The 256 amino acid polypeptide encoded by *CT0872* (30.2 kDa) is annotated as a putative lipoprotein whereas the 159 amino acid product of *CT0874* (16.9 kDa) is not currently functionally annotated, suggesting that a novel gene required for anaerobic thiosulfate oxidation is included in this region.

METHODS

Organisms and growth conditions. All strains, plasmids and antibiotic selections used in this study are listed in Table 1. *Escherichia coli* strains were routinely grown in Luria–Bertani (LB) medium at 37 °C (Ausubel *et al.*, 1987).

C. tepidum was grown in Pf-7 medium with the addition of 1,3-bis(tris(hydroxymethyl)methylamino)propane (BTP) as an additional buffering reagent. To make 1 litre of Pf-7-BTP medium with both thiosulfate and sulfide, three components were prepared. All components were prepared with water from a Nanopure Diamond purification system (Barnstead). Component 1 contained 280 ml double-distilled (dd) H_2O to which was added 20 ml 50 × Pf-7 salts, 1 ml trace elements (Wahlund *et al.*, 1991), 100 µl vitamin B₁₂ stock (250 µg cyanocobalamin ml⁻¹), 0.5 g KH_2PO_4 , 0.5 g CH_3COONH_4 , 0.4 g NH_4Cl and 2.3 g $Na_2S_2O_3 \cdot 5H_2O$. Component 2 contained 2 g $NaHCO_3$ and 600 ml dd H_2O in separate containers. Component 3 contained 0.65 g $Na_2S \cdot 9H_2O$ surface-sterilized with ethanol and 100 ml dd H_2O in a small screw-cap bottle.

After sterilization at 121 °C for 20 min, components 1 and 2 were cooled under a 5 % CO_2 /95 % N_2 atmosphere that had been passed through hot copper filings. Component 3 ingredients were mixed while the dd H_2O was still hot and sealed. Component 2 was assembled by dissolving the $NaHCO_3$ in the cooled, anoxic dd H_2O while bubbling with N_2/CO_2 , followed by bubbling with 100 % CO_2 for 20 min. Components 1, 2 and 3 were then combined aseptically followed by the addition of 10 ml sterile, anoxic 1 M BTP pH 7.0 stock. The pH was checked aseptically and, if needed, adjusted to a value of 6.95 with filter-sterilized 2 M Na_2CO_3 or HCl. Medium was dispensed into sterile 125 ml serum bottles, which were sealed and flushed with the scrubbed 5 % CO_2 /95 % N_2 atmosphere for several minutes. Pf-7-BTP liquid medium was stored at room temperature until use. When made by this method, dissolved sulfide concentrations in the final medium are routinely observed to be 0.6–0.8 mM, which reflects losses by volatilization during medium preparation. Pf-7-BTP was also made with thiosulfate omitted from component 1 and without component 3. This sulfur-free medium was amended with sterile, neutralized, anoxic stock solutions of thiosulfate or sulfide (Overmann, 2000) as needed.

C. tepidum cultures were routinely grown at 47 °C with 20 µmol photons m⁻² s⁻¹ of irradiance supplied by 40 W neodymium full-spectrum bulbs (GE Lighting). Irradiance was measured with a quantum PAR sensor attached to a radiometer (Li-COR Biosciences). All cultures were pressurized to 69 kPa with 5 % CO_2 /95 % N_2 , which was maintained throughout growth by addition of scrubbed gas as culture aliquots were removed for biomass and sulfur compound determination.

Growth of *C. tepidum* on CP plates was previously described (Hanson & Tabita, 2001). For all sulfur compound tracking experiments, cells from starter cultures were washed, incubated overnight at 42 °C in sulfur-free Pf-7-BTP medium, and inoculated to a density of 0.5 µg bChl c ml⁻¹. Bacteriochlorophyll (bChl) *c* and protein concentrations were determined using methanol extraction and the Bradford microassay as previously described (Mukhopadhyay *et al.*, 1999).

Fig. 1. (a) Schematic map of *C. tepidum* sulfur island region SI-I-3 in the wild-type and mutant strain C5. Black arrows, genes encoding predicted sulfur oxidation activities; white arrows, hypothetical and conserved hypothetical ORFs; grey arrows, TnOGm features: origin of replication (Rep) and Gm-resistance marker (*aacC1*). The end-points of the deletion in the two maps are connected by dashed lines. (b) RT-PCR analysis of SI-I-3 gene transcripts in RNA samples harvested from *C. tepidum* after the indicated period of growth in thiosulfate + sulfide medium. (c) RT-PCR analysis of SI-I-3 gene transcripts in RNA samples harvested from mutant strain C5 and the wild-type after 15 h growth in thiosulfate + sulfide medium. Negative control reactions omitted reverse transcriptase (RT, –). The lengths of all cDNA products are indicated in (b).

Nucleic acid preparation and PCR amplification conditions. Genomic DNA from *C. tepidum* was purified either by caesium chloride density-gradient centrifugation or by a commercial

kit (Fermentas). Plasmid DNA was harvested from *E. coli* cultures by a commercial kit (Qiaprep Spin Miniprep kit, Qiagen) or by the boiling lysis DNA extraction method (Ausubel *et al.*, 1987). RNA

Table 1. Strains and plasmids used in this study

Strain or plasmid	Genotype	Antibiotic selection	Source or reference
<i>C. tepidum</i> strains			
WT2321	Wild-type	NA	Wahlund <i>et al.</i> (1991)
C5	Δ CT0867–CT0876::TnOGm	Gm*	This study
<i>E. coli</i> strain			
TOP10	F [–] <i>mcrA</i> Δ (<i>mrr</i> – <i>hsdRMS</i> – <i>mcrBC</i>) ϕ 80 <i>lacZAM15</i> Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>araD139</i> Δ (<i>ara</i> – <i>leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Str ^R) <i>endA1</i> <i>nupG</i>	NA	Invitrogen
<i>E. coli</i> plasmids			
pCR-XL-TOPO	Cloning vector	Km [†] , Amp [‡]	Invitrogen
pTnModOGm	Plasposon vector	Gm*, Amp [‡]	Dennis & Zylstra (1998)

NA, Not applicable.
*Gm: 4 µg ml^{–1} in Pf-7-BTP medium, 8 µg ml^{–1} in CP plates, 10 µg ml^{–1} in LB medium.
†50 µg kanamycin ml^{–1} in LB medium.
‡100 µg ampicillin ml^{–1} in LB medium.

Table 2. Oligonucleotide primers used in this study

Primer	Sequence (5'–3')	Target
PCR		
SI-I-3 F	CAGGAGTGTGATTTTTGAGAGTTT	SI-I-3 fragment
SI-I-3-R	ATGAGAGGACACTATGACCGTTTAG	
Gm-ME-ori-F	GTCAGTATCTGTCTCTTATACACATCTCATGTGCGACGGTACCAGGA	pTnModOGm for producing TnOGm with mosaic ends (underlined)
Gm-ME-ori-R	GTATAGGCCTGTCTCTTATACACATCTTGC GGCCGCACTAGTCTAGAT	
Sequencing		
TnOGm-Seq-L	CTGGCCTTTTGCTCACATGTTC	Sequence at site of TnOGm insertion
RT-PCR		
CT0866-F-RT	CAATCCGAAAATCACCGTCT	<i>qmoA</i> (CT0866)
CT0866-R-RT	ACTTTCTTGACCTCCTTGTATCC	
CT0868-F-RT	CAGACTCGCCTCACCAATG	<i>qmoC</i> (CT0868)
CT0868-R-RT	CCTGATGAGAAAATAGAAGTCGTC	
CT0872-5'-F-RT	CATTCTCGACAAACTCGGCATA	CT0872
CT0872-R-RT	CCTGATGAGAAAATAGAAGTCGTC	
CT0872-3'-R-RT	TGCTTGAGGTTTCATCGTCGG	
CT0876-F-RT	ACCTGCTCTTTATTCCCAACATC	<i>sqr</i> -like homologue (CT0876)
CT0876-R-RT	CTTGCCTTTCGTTCCCTGA	

was purified with a column-based kit purchased from Macherey-Nagel. Trace DNA contamination was removed from RNA samples with TURBO DNA-free (Ambion).

Oligonucleotide primers (Table 2) were purchased from MWG-Biotech. All PCR amplification reactions utilized the FailSafe system (Epicentre). The SI-I-3 PCR product (Fig. 1a) was amplified in FailSafe buffer G, cloned into pCR-XL-TOPO vector (Invitrogen), and transformed into *E. coli* TOP10 (Invitrogen) by electroporation. Transposon TnOGm was constructed by amplifying the Gm-resistance gene (*aacC1*) and the conditional origin of replication (*rep*) from pTnMod-OGm (Dennis & Zylstra, 1998) using FailSafe buffer C. A 19 bp palindromic mosaic end (ME) sequence (Table 2), recognized by the EZ::TN transposase, was incorporated into the primers.

Reverse transcription polymerase chain reactions (RT-PCRs) were carried out using Epicentre's High Fidelity kit according to the

manufacturer's instructions. For RT-PCR of transcripts containing CT0872, two distinct reverse primers were used to prevent a non-specific amplification product. Primer CT0872-3'-R-RT was used to generate cDNAs, which were used as the template for product amplification with CT0872-F-RT and CT0872-R-RT. The identity of the CT0872 product was verified by direct sequencing. The size of all amplification products was determined by agarose gel electrophoresis and compared to predicted fragment sizes derived from sequence data using the Vector NTI software suite (Invitrogen).

In vitro transposition mutagenesis (IVTM) reactions. IVTM of TnOGm into the cloned SI-I-3 fragment (see Fig. 1) utilized commercial EZ::TN transposase (Epicentre). For each reaction, 0.2–0.6 µg of plasmid DNA containing SI-I-3 was used. The reaction was allowed to proceed overnight at 37 °C and the mutagenized plasmid was transformed into *E. coli* TOP10 (Invitrogen) by

Table 3. Growth parameters of *C. tepidum* wild-type and mutant strain C5 under standard conditions with the electron donors indicated

The genotype of strain C5 is Δ CT0867-CT0876::TnOGm. The data are means of three or four independent replicates.

Strain	Electron donor	Doubling time* (h) ± sd	Yield† (µg ml ⁻¹) ± sd		Ratio bChl c: protein
			µg bChl c ml ⁻¹	µg protein ml ⁻¹	
Wild-type	0.7 mM HS ⁻ + 9.2 mM S ₂ O ₃ ²⁻	2.1 ± 0.0	11.7 ± 1.1	195.9 ± 45.6	0.06
	2.5 mM HS ⁻	2.3 ± 0.9	11.0 ± 3.7	39.1 ± 13.9‡	0.28
	12.5 mM S ₂ O ₃ ²⁻	3.3 ± 0.6‡	44.7 ± 2.7‡	77.1 ± 29.4‡	0.58
C5	0.7 mM HS ⁻ + 9.2 mM S ₂ O ₃ ²⁻	3.2 ± 0.1§	6.8 ± 1.0§	92.6 ± 29.1§	0.07
	2.5 mM HS ⁻	3.8 ± 1.3	6.7 ± 0.7	29.9 ± 16.9‡	0.22
	12.5 mM S ₂ O ₃ ²⁻	NA	NA	NA	NA

*Doubling time was the maximum rate observed between 4 and 15 h.

†Yields were obtained from the 80 h time point.

‡P < 0.10, for comparison to the same strain growing in full media.

§P < 0.10, for comparison to wild-type within the same growth condition.

NA, Not applicable; no growth was observed.

Table 4. Relationship between SI-I-3 genes, Sox genes and thiosulfate oxidation activity in genomes of GSB strains, *T. denitrificans* and *S. fumaroxidans*

The *qmoC* homologue in *T. denitrificans* (*Tbd1646*) noted in parentheses is not strictly orthologous, but is the best *T. denitrificans* homologue of the *C. tepidum* CT0868 gene.

Genome	Status	Orthologue (bidirectional BLASTP best homologue)								<i>qmoC</i> –CT0872 colocalized	S ₂ O ₃ ^{2−} oxidation
		<i>sat</i>	<i>apsBA</i>	<i>qmoA</i>	<i>qmoB</i>	<i>qmoC</i>	CT0869	CT0872	<i>soxY</i>	<i>soxCD</i>	
<i>Chlorobium tepidum</i>	Complete	+	+	+	+	+	+	+	+	−	+
<i>Pelodictyon</i>	Draft	+	+	+	+	+	+	+	+	−	+
<i>phaeoclathratiforme</i> BU-1											
<i>Thiobacillus denitrificans</i> ATCC 25259	Complete	+	+	+	+	(+)	−	+	+	−	+
<i>Syntrophobacter fumaroxidans</i> MPOB	Complete	+	+	+	+	+	+	+	−	−	?
<i>Chlorobium chlorochromatii</i> CaD3	Complete	+	+	+	+	+	+	+	+	−	−
<i>Chlorobium ferrooxidans</i> DSM 13031	Draft	−	−	−	−	−	−	+	−	−	−
<i>Chlorobium limicola</i> DSM 245	Draft	−	−	−	−	−	−	+	+	−	−
<i>Chlorobium phaeobacteroides</i> BS-1	Draft	+	−	−	+	+	+	+	−	−	−
<i>Chlorobium phaeobacteroides</i> DSM 266	Complete	−	−	−	−	−	−	+	+	−	−
<i>Prosthecochloris aestuarii</i> DSM 271	Draft	−	−	−	−	−	−	−	+	−	−
<i>Pelodictyon luteolum</i> DSM 273	Complete	−	−	−	−	−	−	−	−	−	−
<i>Prosthecochloris vibrioformis</i> DSM 265	Draft	−	−	−	−	−	−	−	+	−	−

electroporation. *E. coli* clones containing TnOGm were selected on LB plates containing 50 µg kanamycin ml^{−1} and 10 µg Gm ml^{−1}. Individual colonies were pooled to form an IVTM library, which was stored as a glycerol stock at −70 °C.

Natural transformation of *C. tepidum*. Conditions for chromosomal gene inactivation in *C. tepidum* via natural transformation have been described (Frigaard & Bryant, 2001; Hanson & Tabita, 2001). Modifications for transformation with IVTM libraries follow. Plasmid DNA carrying a mutagenized *C. tepidum* SI-I-3 fragment was purified from the *E. coli* library containing IVTM-mutagenized clones. The DNA was linearized, mixed with *C. tepidum* cells, and incubated on CP plates at 42 °C overnight. Dilutions to 1 × 10^{−7} were plated on both nonselective and selective CP plates containing 8 µg Gm ml^{−1} and incubated for up to 7 days. Colonies were restreaked onto freshly prepared CP-Gm plates as dense patches for secondary selection. After growth, half of the cells in a patch were used to inoculate Pf-7-BTP medium containing 4 µg Gm ml^{−1}. The remaining cells were used to confirm the TnOGm insertion site by PCR. Precise localization of the TnOGm insertion site was determined by sequencing PCR products from mutant strains with a primer directed outward from the left end of TnOGm (Table 2). DNA sequencing was performed at the University of Delaware, College of Agriculture and Natural Resources DNA sequencing core facility.

Quantification of sulfur compounds and acetate by HPLC. Sulfur compounds were quantified by HPLC as described by Rethmeier *et al.* (1997) on a class VP HPLC system (Shimadzu Scientific Instruments) equipped with a column oven and UV/visible and fluorescence detectors with the following modifications. Elemental sulfur and monobromobimane-derivatized sulfur compounds (sulfide, thiosulfate, sulfite) were

separated on a Prevail C18 5 µm column. Sulfate was determined with an IC AN-1 column using indirect UV detection as previously described (Rethmeier *et al.*, 1997). Thiosulfate and acetate were quantified using a Prevail Organic Acids 5 µm column eluted with 25 mM potassium phosphate buffer, pH 2.5, with UV detection at 210 nm. All columns were purchased from Alltech Associates and the identity of all compounds was confirmed by co-elution with authentic standards.

Data analysis and comparison. Growth yield data and stoichiometries of sulfur compound consumption and production were calculated from the data in Figs 2, 3 and 4. Values for each of three or four independent cultures were compared between growth conditions or strains by homoscedastic single-tailed *t*-tests assuming no difference between means and that variance was not constant between sets of values. Tests were conducted in Excel (Microsoft). *P*-values <0.10 were considered to indicate a significant difference between strains or conditions using this method.

Sequence retrieval and analysis. Whole-genome comparisons and *C. tepidum* orthologue analyses were performed using tools of the Integrated Microbial Genomes server located at <http://img.jgi.doe.gov/> (Markowitz *et al.*, 2006).

RESULTS

Optimization of *C. tepidum* culture conditions

At 47 °C, *C. tepidum* exhibited poorly reproducible growth in Pf-7 medium buffered solely with CO₂ and HCO₃[−]

Fig. 2. Sulfur compound concentrations vs time post-inoculation in *C. tepidum* batch cultures supplied with 0.7 mM sulfide and 9.2 mM thiosulfate. (a) Concentrations of sulfide (HS^-) and elemental sulfur (S^0). (b) Concentrations of thiosulfate ($S_2O_3^{2-}$) and sulfate (SO_4^{2-}). The data are means and standard errors of three or four independent replicates.

(Wahlund *et al.*, 1991). When cultures were incubated under a pressurized headspace of N_2/CO_2 , the pH of the medium routinely rose above 7.2, inhibiting the growth of

C. tepidum. Therefore, Pf-7 medium was buffered with BTP (Pf-7-BTP), which maintained a stable pH of 6.9–7.0, improving the reproducibility of *C. tepidum* growth. BTP

Fig. 3. Sulfur compound concentrations in batch cultures of *C. tepidum* wild-type (▲) and SI-I-3 mutant strain C5 (●) grown in Pf-7-BTP with 12.5 mM thiosulfate. Concentrations of thiosulfate (a), elemental sulfur (b) and sulfate (c) were measured as described in Methods and the total sulfur recovery (d) was expressed as a percentage of the total of all sulfur compounds detected at time 0. The data are means and standard errors of three or four independent replicates.

Fig. 4. Acetate utilization of *C. tepidum* wild-type (▲) and SI-I-3 mutant strain C5 (●) grown in Pf-7-BTP with 0.7 mM sulfide + 9.2 mM thiosulfate (a), 2.5 mM sulfide (b), or 12.5 mM thiosulfate (c). The data are means and standard errors of three or four independent replicates.

does not support the growth of *C. tepidum* when added to $\text{CO}_2/\text{HCO}_3^-$ -free Pf-7 as the sole carbon source (data not shown).

Application of IVTM to *C. tepidum*

IVTM with a transposon (TnOGm) derived from pTnModOGm was applied to one subsection of sulfur island I, SI-I-3 (Fig. 1a), encoding homologues of the *Desulfovibrio desulfuricans* ATCC 27774 QmoABC complex (CT0866–CT0868), a SQR homologue (CT0876), and a number of hypothetical and conserved hypothetical

proteins (CT0869–CT0875). TnOGm was utilized in conjunction with a commercial Tn5 transposase to generate transposon insertions in a cloned copy of SI-I-3, generating a library in *E. coli* containing randomly distributed TnOGm insertions throughout the cloned SI-I-3 fragment. This library was then used to transform *C. tepidum*, producing a collection of strains carrying TnOGm insertions in SI-I-3, including strains carrying single TnOGm insertions in genes encoding the *C. tepidum* homologues of QmoB (CT0867::TnOGm), QmoC (CT0868::TnOGm), and strain C5 ($\Delta\text{CT0867-CT0876}$::TnOGm, Fig. 1a). Both CT0867::TnOGm and CT0868::TnOGm mutant strains display very minor phenotypes compared to strain C5 and will be described in detail elsewhere. A *C. tepidum* strain carrying a single TnOGm insertion in the CT0876 gene was also constructed. The detailed properties of this strain will be described as part of the analysis of the three SQR homologues encoded by the *C. tepidum* genome (L. K. Chan & T. E. Hanson, unpublished results).

Expression of SI-I-3 genes in wild-type and mutant strain C5

To verify that genes within SI-I-3 were indeed expressed and therefore contribute to *C. tepidum*'s physiology, RT-PCR was used to monitor the expression of *qmoA* (CT0866), *qmoC* (CT0868), CT0872 and the *sqr*-like orthologue (CT0876) in wild-type and mutant strain C5 throughout growth in standard Pf-7-BTP, which contains 9.2 mM thiosulfate and 0.7 mM sulfide as electron donors. In the wild-type strain, transcripts of the *qmoA* and *qmoC* homologues were found up to 32 h after inoculation (Fig. 1b), which corresponds to the period of active sulfur oxidation in these cultures (Fig. 2). Sulfide was completely consumed 15 h post-inoculation and elemental sulfur produced from sulfide oxidation was consumed by 24 h (Fig. 2a). Thiosulfate consumption commenced at 15 h and continued until about 40 h post-inoculation, concomitant with sulfate production (Fig. 2b). In contrast, transcripts of CT0872 and the *sqr*-like orthologue (CT0876) were found in all stages of growth sampled (Fig. 1b), with no obvious connection to sulfur compound dynamics.

RT-PCR was also used to confirm that the gene deletion in strain C5 eliminated the expression of SI-I-3 genes affected by this rearrangement. As expected from the genotype (Fig. 1a), transcripts of CT0868, CT0872 and CT0876 were not detected in strain C5 (Fig. 1c). However, transcripts of *qmoA* (CT0866) were detected in strain C5, indicating that the partial deletion of *qmoB* (CT0867) and complete deletion of *qmoC* (CT0868) did not directly affect the expression of the *C. tepidum* *qmoA* homologue.

Growth of wild-type and mutant strain C5

When first isolated, mutant strain C5 exhibited a severe growth rate defect in the presence or absence of Gm

selection at 47 °C in Pf-7 medium with no additional buffer (Chan *et al.*, 2007). In contrast, the CT0867::TnOGm and CT0868::TnOGm mutant strains only displayed strong growth defects in the presence of Gm, suggesting that the Gm resistance marker carried by TnOGm is temperature sensitive (Chan *et al.*, 2007). Therefore, all physiological measurements of C5 were conducted at 47 °C in the absence of Gm selection in well-buffered Pf-7-BTP. The genotype of strains carrying TnOGm insertions examined to date is stable in the absence of Gm selection (L. K. Chan & T. E. Hanson, unpublished results).

In PF-7-BTP with both sulfide and thiosulfate as sulfur and electron donors, *C. tepidum* wild-type grew with an average doubling time of 2.1 h (Table 3). Mutant strain C5 grew 1.5-fold more slowly, with an average doubling time of 3.2 h (Table 3). When 2.5 mM sulfide was used as the sole sulfur and electron donor, *C. tepidum* wild-type had a doubling time of 2.3 h, similar to the sulfide + thiosulfate medium, and about 1.7-fold faster than strain C5 growing under the same conditions (Table 3). The total amount of bChl *c* accumulated by each strain was similar when grown on thiosulfate + sulfide medium versus sulfide alone (Table 3). However, the final yield of biomass (protein) was significantly decreased in sulfide-grown cultures compared to sulfide + thiosulfate, resulting in an increased bChl *c*:protein ratio (Table 3) for both the wild-type and strain C5. With 12.5 mM thiosulfate as the sole electron donor, *C. tepidum* wild-type displayed a doubling time of 3.3 h, 1.4-fold slower than cultures grown with sulfide or sulfide + thiosulfate (Table 3). Interestingly, the wild-type accumulated about fourfold higher bChl *c* when grown on thiosulfate, resulting in the highest bChl *c*:protein ratio of any growth condition examined (Table 3). Strain C5 was incapable of growth on thiosulfate beyond an initial doubling of biomass (Table 3).

To narrow down gene candidates for this phenotype, the thiosulfate-dependent growth yield (at 72 h) of the wild-type was compared with single TnOGm insertion mutants. The yields were 102 µg protein ml⁻¹ for wild-type, 111 µg protein ml⁻¹ for CT0867::TnOGm, 82 µg protein ml⁻¹ for CT0868::TnOGm, and 105 µg protein ml⁻¹ for CT0876::TnOGm. In contrast, the yield for strain C5 was ≤1 µg protein ml⁻¹. Clearly, TnOGm insertions in genes flanking each side of the deletion in strain C5 have no effect on thiosulfate-dependent growth, suggesting that the loss of genes internal to the deleted region causes the phenotype.

Strain C5 oxidizes sulfide and elemental sulfur, but is deficient for thiosulfate oxidation

When sulfide was provided as the sole reductant for growth, *C. tepidum* mutant strain C5 oxidized it as well as the wild-type, transiently producing elemental sulfur and eventually producing stoichiometric amounts of sulfate (data not shown). Low, but detectable, amounts of thiosulfate were detected in sulfide-only cultures of both

strains, but this was oxidized quickly by the wild-type whereas it persisted in mutant strain C5 (data not shown).

When thiosulfate was provided as the sole reductant, *C. tepidum* consumed it completely within 48 h (Fig. 3a). No free sulfide was detected during growth of *C. tepidum* on thiosulfate (data not shown). Elemental sulfur was transiently observed in cultures of the wild-type, and sulfate was the final product of thiosulfate oxidation (Fig. 3b, c). In the wild-type, the maximum concentration of elemental sulfur was 1.2 mM at 25 h, about 5 % of the total sulfur pool originally present in the medium. Extracellular sulfur globules were clearly visible in thiosulfate-oxidizing cultures by microscopy (data not shown). The wild-type produced 1.9 mol sulfate for each mole of thiosulfate oxidized, close to the expected stoichiometry. In contrast to the wild-type, mutant strain C5 did not oxidize thiosulfate (Fig. 3a), nor did it produce elemental sulfur or sulfate (Fig. 3b, c). Sulfur mass balance averaged 91 % ± 9 % for the wild-type and 96 % ± 3 % for strain C5 (Fig. 3d). However, wild-type cultures displayed a poor mass balance at the 25 and 48 h time points (88 % and 77 %, respectively). This may indicate that a significant internal pool of sulfur compounds was not detected by the current assay scheme.

Acetate consumption by wild-type and mutant strain C5

Pf-7-BTP contains acetate at a concentration of 6.5 mM and therefore supports mixotrophic growth. Acetate concentrations are routinely measured during analysis of thiosulfate, which led to the serendipitous observation of an acetate assimilation phenotype in strain C5. *C. tepidum* assimilated acetate in the presence of sulfide + thiosulfate with no noticeable lag, consuming ~75 % during the first 40 h of growth (Fig. 4a). The wild-type also assimilated acetate when grown with either sulfide (Fig. 4b) or thiosulfate (Fig. 4c). In contrast, strain C5 accumulated an additional 1.5 mM acetate during the early stages of growth with thiosulfate + sulfide (Fig. 4a). This accumulation was statistically significant from 10 to 46 h of growth ($P < 0.10$). From a peak of 8 mM acetate at 15 h, strain C5 consumed only about 30 %, reaching a final concentration of 5.6 mM after 60 h of growth (Fig. 4a). When cultures were grown with sulfide, statistically significant differences ($P < 0.10$) in acetate concentrations between the wild-type and strain C5 were observed after 10 h (Fig. 4b), indicating that the mutant did not consume acetate as well as the wild-type under this growth condition. Strain C5 also did not consume acetate when incubated in thiosulfate-containing medium (Fig. 4c), as it did not grow (Fig. 2c).

Analysis of the genomic region deleted in strain C5

The deletion in strain C5 completely eliminates seven ORFs from the genome (Fig. 1a). When compared with other

GSB genomes, a similar region was found in *Pelodictyon phaeoclathratiforme* BU-1 and *Chlorobium phaeobacteroides* BS-1 (Fig. 5). In the BS-1 strain, this region was found at the end of a scaffold in the draft genome sequence and so the upstream section is implied by light grey shading in Fig. 5, but has not yet been described. The presence of this genomic region is strongly correlated with the ability to oxidize thiosulfate in GSB (Table 4).

Similar genomic regions were also found in *Thiobacillus denitrificans* ATCC 25259 (*Betaproteobacteria*) and *Syntrophobacter fumaroxidans* MPOB (*Deltaproteobacteria*) (Fig. 5, Table 4). The conserved features of this region are genes encoding sulfate adenylyltransferase, adenosine-5'-phosphosulfate reductase, and orthologues of the *C. tepidum* genes *CT0869* and *CT0872*, which are deleted in strain C5 (Fig. 5). Orthologues of *CT0869* and *CT0872* are also found amongst sulfate-reducing bacteria and archaea (see Supplementary Table S1, available with the online version of this paper). Any genome that contained an orthologue of *CT0869* also contained an orthologue of *CT0872*, though the converse was not true (Table S1). Genomes encoding an orthologue of *CT0872* were searched by TBLASTN with the *C. tepidum* *CT0869* amino acid sequence, but no additional homologues of *CT0869* were detected. No obvious functional motifs were detected in *CT0869*, but the product of *CT0872* was weakly similar to PFAM family PF03692 (unknown protein family 0153) and COG0727 (predicted [Fe-S] cluster oxidoreductase). Both families are defined by eight conserved cysteine residues that are also conserved in *CT0872*.

Also deleted in strain C5 is *CT0874*, which encodes a protein similar to 3-oxoacyl-acyl-carrier-protein (ACP) synthase IIIs (EC 2.3.1.41, COG0332 and PFAM08545). However, *CT0874* encodes only the middle section of a

typical FabH enzyme (Davies *et al.*, 2000), and lacks a C-terminal domain (PFAM08541: 2-oxoacyl-ACP synthase III C-terminal) present in bona fide FabH enzymes. A total of 18 proteins that possess a single ACP-synthase III domain in the absence of the C-terminal domain were found to be present in bacterial and eukaryotic genomes (see Supplementary Table S2, available with the online version of this paper). No homologues of *CT0874* were detected in other GSB by TBLASTN searches.

The remaining predicted ORFs in this region (*CT0870*, *CT0871* and *CT0875*) have no significant homology to any other predicted or known ORFs or proteins in current databases. When the SI-I-3 DNA sequence was used in a BLASTX search against the GenBank non-redundant database, an ORF encoding a fragment of anthranilate phosphoribosyltransferase (TrpD, EC 2.4.2.18) was found between *CT0872* and *CT0874*. Presumably a gene model named *CT0873* was rejected from this region during annotation because of the presence of *CT1609*, which encodes a full-length version of TrpD (Eisen *et al.*, 2002).

DISCUSSION

This paper describes the identification of a genomic region apparently required for phototrophic growth with thiosulfate as an electron donor in *C. tepidum* as deduced from the phenotypic analysis of mutant strain C5 (Δ *CT0867*–*CT0876*::TnOGm). The region deleted in strain C5 is part of a larger cluster termed sulfur island I (Chan *et al.*, 2007) and is not co-localized with the Sox gene cluster (*CT1009*–*CT1027*) that is associated with thiosulfate oxidation in other bacteria (Friedrich *et al.*, 2001). Similarly organized regions are found in some GSB and other bacteria, and many of the individual ORFs in this region were found in a

Fig. 5. Genomic regions similar to that deleted in *C. tepidum* strain C5 are found in *P. phaeoclathratiforme* BU-1, *C. phaeobacteroides* BS-1, *S. fumaroxidans* MPOB and *T. denitrificans* ATCC 25259. The genome regions are centred on orthologues of *CT0872*. Vertical and diagonal lines indicate an orthology between gene products (best bidirectional BLASTP homologue). The region deleted and replaced with TnOGm in *C. tepidum* strain C5 is indicated with a double arrow.

number of sulfur-oxidizing and -reducing prokaryote genomes even though they are not co-localized as they are in *C. tepidum*.

In addition to this observation, this work has provided important basic data on sulfur compound oxidation in *C. tepidum*. Our data show conclusively that *C. tepidum* can grow in the absence of sulfide, contradicting reports that sulfide was required for growth of this organism (Wahlund *et al.*, 1991). Furthermore, this report demonstrates the formation of elemental sulfur as a transient intermediate in thiosulfate oxidation in GSB. Elemental sulfur produced by the oxidation of thiosulfate and sulfide was consumed concomitantly with these electron donors.

Two distinct phenotypes were exhibited by mutant strain C5: a specific defect in thiosulfate oxidation that prevented it from growing with thiosulfate as the sole photosynthetic electron donor and a more general defect in acetate assimilation that was found under all growth conditions tested. Two lines of evidence lead us to propose that the thiosulfate oxidation defect of strain C5 is due to the loss of either CT0869 or CT0872, or both genes, in strain C5. First, mutations in genes encoding QmoB (CT0867), QmoC (CT0868) and the SQR homologue (CT0876) in SI-I-3 did not cause a thiosulfate-dependent growth phenotype. Second, homologues of CT0869 and CT0872 are present in the genomes of other thiosulfate-oxidizing GSB. Both genes are also present in two GSB incapable of thiosulfate oxidation, *Chlorobium chlorochromatii* CaD3 and *Chlorobium phaeobacteroides* BS-1. However, in these two genomes, these genes are not colocalized in the same genomic region with genes encoding the Qmo complex, suggesting that the overall organization of this gene region may be important to confer thiosulfate oxidation (Table 4). There may also be other genes missing from the CaD3 and BS-1 genomes, like *soxY* in the draft BS-1 genome, that prevent these strains from oxidizing thiosulfate.

Evidence for the expression of both CT0872 and CT0869 has been obtained. RT-PCR data reported here indicate that transcripts containing CT0872 are present under standard growth conditions in *C. tepidum*, and additional RT-PCR experiments indicate that CT0870, CT0871 and CT0872 are present on a single transcript (data not shown). While CT0869 transcription was not assayed here, the CT0869 protein was detected in a proteomic profiling experiment on cytoplasmic extracts of *C. tepidum* whereas the CT0872 protein was not (Zhou *et al.*, 2007). However, the observed inefficiency of the cytoplasmic profiling experiment for proteins >30 kDa may have prevented detection of the 30.2 kDa CT0872 protein even though it is predicted to have a cytoplasmic localization when examined by pSORTb (<http://www.psort.org/psortb/index.html>). Alternatively, the failure to observe CT0872 protein may indicate that it is post-translationally modified.

CT0872 seems the most likely candidate for a direct role in thiosulfate oxidation based on its wider distribution in other thiosulfate-oxidizing bacteria, like *T. denitrificans* (Table 4),

and other sulfur-oxidizing and -reducing bacteria and archaea (Table S1). The weak homology of the CT0872 protein to predicted [Fe-S] oxidoreductases (PF03692 and COG0727), based on eight conserved cysteines, may indicate that it participates in redox reactions via bound metals or [Fe-S] clusters, but this remains to be experimentally determined. The *T. denitrificans* orthologue of CT0872, Tbd0871, was found to be highly expressed during both aerobic and anaerobic growth with thiosulfate as the electron donor (Beller *et al.*, 2006), along with the genes encoding sulfate adenylyltransferase and adenosine-5'-phosphosulfate reductase. Like *C. tepidum* and all other GSB, *T. denitrificans* lacks genes encoding a SoxCD complex, which is required for complete thiosulfate oxidation in *P. pantotrophus* GB17.

In the purple sulfur bacterium *Allochromatium vinosum*, genes of the *dsr* cluster have been implicated in the oxidation of periplasmic elemental sulfur, formed as an intermediate during the oxidation of thiosulfate. *A. vinosum*, like *C. tepidum*, lacks genes encoding the SoxCD sulfur dehydrogenase, and it has been suggested that elemental sulfur is an intermediate of thiosulfate oxidation in strains lacking SoxCD. *C. tepidum* was shown here to produce limited quantities of elemental sulfur during thiosulfate oxidation, in agreement with the lack of SoxCD. However, it is clear that the *dsr* genes are not required for photolithotrophic growth on either sulfide or thiosulfate in *A. vinosum* (Pott & Dahl, 1998). This strongly contrasts with the phenotype of strain C5, which is completely incapable of thiosulfate-dependent growth. Further experiments on growth-independent thiosulfate turnover in this strain will reveal whether or not C5 accumulates intermediates. The existence of additional intermediates in thiosulfate oxidation in *C. tepidum* is suggested by the observation that *C. tepidum* does not display a good sulfur mass balance in the late stages of growth on thiosulfate as the sole electron donor (Fig. 3d).

Another key difference between *A. vinosum* and *C. tepidum* is the formation of periplasmic elemental sulfur globules in *A. vinosum*, which is dependent on the sulfur globule proteins encoded by *sgpA*, *sgpB* and *sgpC* (Prange *et al.*, 2004). The formation of these globules appears to be required for photolithotrophic growth, as indicated by the inability of an *A. vinosum* *sgpBC* double mutant to grow on sulfide or thiosulfate (Prange *et al.*, 2004). *C. tepidum* accumulates elemental sulfur extracellularly, does not have *sgp* gene homologues, and yet grows perfectly well on thiosulfate and sulfide. This indicates that key differences must exist between these strains in how elemental sulfur is formed and oxidized. Strain C5 appears to consume elemental sulfur produced from sulfide oxidation normally, so we conclude that the mutation in strain C5 has not affected any putative *dsr*-dependent elemental sulfur oxidation capacity, but rather the entry of thiosulfate-derived sulfur into such a pathway.

Regarding the acetate assimilation defect, we propose that CT0874 provides a mechanism for *C. tepidum* to buffer or

retain acetate intracellularly. This is consistent with the observation that strain C5 accumulates acetate in culture supernatants during growth with thiosulfate + sulfide under both mixotrophic (Fig. 4a) and autotrophic (data not shown) conditions.

While it is a formal possibility that one or more of the genes deleted in strain C5 is a regulatory factor that controls thiosulfate oxidation or acetate oxidation, we feel this is unlikely. None of the genes in this region display any similarity to known regulatory genes or contain recognized regulatory motifs such as DNA-binding domains or protein–protein interaction domains (data not shown). To further examine this point, we are developing RT-PCR primers for Sox genes and those encoding key steps in acetate assimilation to determine if these genes are expressed normally in strain C5. When compared to the wild-type strain by SDS-PAGE, strain C5 displays no obvious protein profile differences (data not shown) that might suggest severe alterations in global regulation as observed in the $\Omega::R$ LP mutant strain (Hanson & Tabita, 2001, 2003).

While our results are suggestive of the function proposed for specific gene products above, they are obviously not conclusive due to the lack of a plasmid-based complementation system in *C. tepidum*. Experiments are under way to directly test the proposed functions for CT0872 and CT0874 by the construction and phenotypic characterization of *C. tepidum* strains carrying single TnOGm insertions in these genes. Together with our earlier report (Chan *et al.*, 2007), the data reported here indicate that IVTM is an extremely useful technique for characterizing genomic regions of interest in *C. tepidum*.

Finally, the data suggest that *C. tepidum* dynamically regulates light harvesting in response to the electron donor provided. Cells grown on sulfide + thiosulfate displayed the lowest specific bChl *c* content, with sulfide-grown and thiosulfate-grown cells displaying 4-fold and 10-fold increases, respectively. Temperature and light intensity also affect photosynthetic antenna structure and function in wild-type cells while strain C5 appears chronically affected, as will be detailed elsewhere (R. M. Morgan-Kiss, L. K. Chan, T. S. Weber & T. E. Hanson, unpublished).

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