Functional Analysis of Three Sulfide:Quinone Oxidoreductase Homologs in Chlorobaculum tepidum

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Sulfide:quinone oxidoreductase (SQR) catalyzes sulfide oxidation during sulfide-dependent chemo- and phototrophic growth in bacteria. The green sulfur bacterium Chlorobaculum tepidum (formerly Chlorobium tepidum) can grow on sulfide as the sole electron donor and sulfur source. C. tepidum contains genes encoding three SQR homologs: CT0117, CT0876, and CT1087. This study examined which, if any, of the SQR homologs possess sulfide-dependent ubiquinone reduction activity and are required for growth on sulfide. In contrast to CT0117 and CT0876, transcripts of CT1087 were detected only when cells actively oxidized sulfide. Mutation of CT0117 or CT1087 in C. tepidum decreased SQR activity in membrane fractions, and the CT1087 mutant could not grow with ≥6 mM sulfide. Mutation of both CT0117 and CT1087 in C. tepidum completely abolished SQR activity, and the double mutant failed to grow with ≥4 mM sulfide. A C-terminal His6-tagged CT1087 protein was membrane localized, as was SQR activity. Epitope-tagged CT1087 was detected only when sulfide was actively consumed by cells. Recombinantly produced CT1087 and CT0117 proteins had SQR activity, while CT0876 did not. In summary, we conclude that, under the conditions tested, both CT0117 and CT1087 function as SQR proteins in C. tepidum. CT0876 may support the growth of C. tepidum at low sulfide concentrations, but no evidence was found for SQR activity associated with this protein.

Many bacteria can utilize sulfide at micro- to millimolar concentrations as an electron donor. Sulfide oxidation can be catalyzed by the enzyme sulfide:quinone oxidoreductase (SQR) (20, 52, 56) or flavocytochrome c (FCC; also known as flavocytochrome c sulfide dehydrogenase) (9, 43). Many phototrophic bacteria contain genes that encode both enzymes, and the most recent models of sulfur oxidation in both the green sulfur and purple sulfur bacteria indicate that these enzymes are alternate routes that result in the production of either polysulfide (green sulfur) or protein-encapsulated electrons (purple sulfur) in the periplasm (10, 16, 17). SQR donates electrons from sulfide to the electron transport chain at the level of quinone, upstream of the FCC complex (menaquinol:cytochrome c oxidoreductase), while FCC donates electrons at the level of cytochrome c (41). Theoretically, the energy yield should be greater for organisms utilizing SQR than for those utilizing FCC, because proton motive force is generated when electrons are passed through the b/c1 complex en route to the reaction center (41). Oxidation of sulfur/poly sulfides produced by sulfide oxidation in purple sulfur bacteria requires the action of gene products encoded by the dissimilatory sulfite oxidoreductase (Dsr) gene cluster (10–12). In green sulfur bacteria, the Dsr system has been proposed to be involved in elemental sulfur oxidation, but this has not been experimentally demonstrated as yet (16, 17).

For bacteria, evidence suggests that SQR is more important than FCC for chemo- and phototrophic sulfur oxidation. First, sequence analyses indicate that homologs of FCC seem to be confined to autotrophs that can utilize thiosulfate in addition to sulfide as an energy source (17, 20, 56). In these organisms, in vitro FCC activity is found in the SoxF protein (42), whose gene is part of a large cluster encoding the entire sulfur-oxidizing (Sox) enzyme system (14, 15). SoxF enhances thiosulfate and sulfide oxidation activity in vivo (3) but inhibits in vitro sulfide oxidation via the reconstituted Sox complex (44). In contrast, homologs of SQR are widely distributed in bacteria, and similar proteins have been identified in the archaea (17, 20, 56) and eukaryotes (21, 33, 48). Second, mutation of the fceAB genes in the phototrophic purple sulfur bacterium Allochromatium visnomis did not inhibit its ability to oxidize sulfide and grow photolithoautotrophically, though the specific growth rates and biomass yields were not reported (43). A. vinosum also contains SQR and the Dsr and Sox systems. Third, in the chemolithothrophic sulfur oxidizer Acidithiobacillus ferrooxidans NASF-1, sqr transcripts were threefold more abundant in sulfide-grown than in iron-grown cells (60). Finally, sulfide oxidation activities directly linked to energy production or detoxification have been demonstrated with the purified SQR proteins from the proteobacterium Rhodobacter capsulatus (46, 47, 51) and the cyanobacteria Oscillatoria limnetica and Aphanothece halophytica (1, 4, 49). R. capsulatus contains neither the Dsr nor the Sox sulfur oxidation system, and while genomic information is not available for O. limnetica and A. halophytica, other cyanobacterial genome sequences (28, 35, 39, 40) do not contain the Dsr or Sox systems.

Here we report the functional analysis of multiple SQR
homologs encoded by CT0117, CT0876, and CT1087 from the green sulfur phototrophic bacterium *Chlorobaculum tepidum* (27) (formerly *Chlorobium tepidum* [59]). With the exception of *Chlorobium ferrooxidans* (25), all green sulfur bacteria can utilize sulfide as an electron donor to support growth, and all green sulfur bacterial genome sequences encode at least one SQR homolog, including *C. ferrooxidans* (16, 17). Biochemically, *Chlorobium limicola* forms thiolsulfatophilum membranes have been shown to catalyze electron transfer from sulfide to plastoquinone in the dark (50). On the basis of sequence comparisons, CT0117 has been proposed to be the bona fide SQR in *C. tepidum*, while CT0876 and CT1087 have been labeled SQR-like-proteins (SQRLPs) (16), and it is unclear whether these gene products contribute to SQR activity in *C. tepidum*.

Recently, we reported that a *C. tepidum* mutant strain (CS, ΔCT0876-CT0876::TnOgm) in which the 5’ half of the SQR homolog, carrying the CT0876 gene, was replaced with a transposon insertion oxidized sulfide normally and grew well with sulfide as the sole electron donor (8). In the experiments reported here, we sought to define the roles of the three SQR homologs more precisely. The results indicate that either CT0117 or CT0876 is required for sulfide-dependent growth at >2 mM sulfide, while CT1087 is required for growth above 4 mM sulfide. Both proteins displayed SQR activity in *C. tepidum* and when produced recombinantly with a standard activity assay, clearly indicating that CT1087 is not a SQRLP but a bona fide SQR. Our results did not rule out a requirement for CT0876 for sulfide-dependent growth at concentrations less than or equal to 2 mM, but no evidence was found that CT0876 contributes to SQR activity in *C. tepidum* or that the recombinant protein possesses SQR activity, suggesting that CT0876 may indeed be a SQRLP.

### MATERIALS AND METHODS

**Bacterial growth conditions and media.** Bacterial strains, plasmids, and antibiotics are listed in Table 1. The *Escherichia coli* cloning strain TOP10 (Invitrogen, Carlsbad, CA) was grown in Luria-Bertani (LB) medium at 37°C (2). Strain BL21ΔDE3/pLysS (Novagen, San Diego, CA), used for the recombinant expression of SQR homologs, was grown anaerobically at 37°C in LB medium supplemented with 0.4% (wt/vol) glucose, 20 mM morpholinepropanesulfonic acid (MOPS), and 25 mM NaNO₃ (pH 7.8). For anaerobic culture, stopper-sealed 25-ml serum vials were filled with 10 ml of medium and were pressurized under a 95% N₂-5% CO₂ atmosphere. For larger volumes, 150-ml bottles filled with 100 ml medium were used. For anaerobic growth on plates, LB plates inoculated with *E. coli* cells were incubated at 37°C in sealed anaerobic jars. Growth of *E. coli* in liquid medium was routinely monitored by measuring the optical density at 680 nm (OD₆₈₀).

*C. tepidum* was grown in liquid P7-BTP, a mineral medium with no supplements, or on CP solid medium as previously described (8, 24, 59). Stationary-phase cultures (∼30 h of growth; ∼120 μg of protein ml⁻¹ and ∼40 μg of bacteriochlorophyll c ml⁻¹) of the wild-type and mutant strains were pelleted by centrifugation, washed with sulfur-free P7-BTP medium, and incubated overnight in sulfur-free medium before being inoculated to a density of 0.5 μg of bacteriochlorophyll c ml⁻¹, which corresponds to ∼4 μg of protein ml⁻¹, in a medium with specific electron donors for physiological and growth experiments (8). Since the antibiotic resistance conferred by the transposon TnOgm is temperature sensitive (6), all growth results described here are from cultures grown on plates incubated at 5°C in sealed anaerobic jars. Growth of *C. tepidum* was monitored by measuring total cellular protein with the Bradford microassay (38).

**Nucleic acid purification, PCR, and sequencing.** Primers for all PCR and RT-PCR amplification and sequencing reactions are listed in Table S1 in the
supplemental material. DNA was purified using the genomic DNA extraction kit from either Fermentas (Glen Burnie, MD) or Qiagen (Valencia, CA). PCR was conducted with the FailSafe PCR system (Epicentre, Madison, WI) or SuperMix high-fidelity PCR mix (Invitrogen) under standard conditions. Plasmid DNA was isolated with the Qiaprep miniprep kit (Qiagen). Reagents and T4 ligase for DNA ligation were from the LigFast rapid DNA ligation system (Promega, Madison, WI). RNA was purified with the NucleoSpin RNA purification kit from Macherey-Nagel (Bethyllem PA), and trace DNA contamination was removed by treating samples with the Turbo DNA-free kit (Ambion, Austin, TX). RT-PCR was performed with Epicentre's high-fidelity RT-PCR kit according to the manufacturer's instructions. When needed, PCRs or products were sequenced by standard procedures at the College of Agriculture and Natural Resources DNA Sequencing Facility at the University of Delaware, Newark.

IVTM of SQR-encoding genes in C. tepidum. In vitro transposition mutagenesis (IVTM) of C. tepidum was performed as previously described (8). PCR products carrying CT1017, CT0876, and CT1087 were amplified from C. tepidum wild-type genomic DNA with target-specific primers (see Table S1 in the supplemental material) and inserted into the pCR2.1 vector (Invitrogen), which was used to transform E. coli TOP10 electrocompetent cells (Invitrogen). Positive transformants were selected with 50 µg kanamycin ml⁻¹ on LB plates. Purified plasmids were used for transposition reactions with the EZ-TN transposase (Epitectene) and transposon TnOgM, generated by PCR from pTnMODoGm (13) using primers GmMoEori-F and GmMoEori-R (see Table S1 in the supplemental material). TnOgM-mutagenized plasmids were recovered in E. coli by selection with 25 µg kanamycin ml⁻¹ and 20 µg gentamicin ml⁻¹ on LB plates. Plasmids carrying the mutagenized SQR-encoding genes were identified by colony PCR. Purified plasmid DNA was used to transform C. tepidum as described previously, with the use of a male strain carrying the pRL21 plasmid at the aacC1 gene (7, 57). When needed, C. tepidum chlorosome-depleted membranes were prepared by sonicating the cell pellets in hypotonic density ultracentrifugation (7, 57). The membrane fraction was further treated with the surfactant Nonidet P-40 and centrifuged at 126,000 × g for 60 min (7, 53). The Nonidet P-40 pellet is enriched in outer membrane and peripherally bound inner membrane proteins, whereas the Nonidet P-40-soluble fraction is enriched in integral membrane proteins (7).

SDS-PAGE and immunoblotting. Equal amounts of protein from purified subcellular fractions or proteins extracted from intact cells by boiling in 1× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer were separated by SDS-PAGE on acrylamide gels (15% resolving, 4% stacking; 6 mM urea) according to the method of Laemmli (31). When needed, proteins were visualized by staining with BioSafe Coomassie dye (Bio-Rad, Hercules, CA). For immunoblotting, proteins separated on the acrylamide gel were transferred to polycrylamide membranes (Bio-Rad) with 25 mM Tris base, 192 mM glycine, 20% (vol/vol) methanol, and 0.5% (wt/vol) SDS at 100 V for 2 h at 4°C with continuous buffer stirring. Polycrylamide electrophoresis blots were incubated in the primary antibody (mouse anti-penta-His [penta-His]; Qiagen) and secondary antibody (alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G; Bio-Rad) in Tris-buffered saline and 5% (wt/vol) nonfat milk (1× Tris-buffered saline is 0.2 M Tris-Cl [pH 7.5] and 1.5 M NaCl). Blots were incubated with ECF chemiluminescence substrate (GE Healthcare Life Sciences, Piscataway, NJ) for 5 min in the dark in order to detect bound alkaline phosphatase-conjugated immunoglobulin G on a Typhoon 8600 scanner (GE Healthcare Life Sciences) with 532-nm excitation and 526-nm emission wavelengths. Band intensities from SDS-PAGE or immunoblots were quantified with Scion Image (Scion, Frederick, MD) according to the manufacturer's instructions.

SQR activity assay. Sulfide-dependent decyl-ubiquinone (dUQ) reduction activity was measured under anaerobic conditions in Na₂-flushed quartz cuvettes with septum screw caps (Starna Cells, Atascadero, CA) as previously described (49–51) with minor modifications. The typical assay mixture was prepared in the anaerobic chamber and consisted of 10 mM Tris-HCl (pH 7.4), 20 µg microgram protein, and 100 µM dUQ (Sigma, St. Louis, MO). Assays were performed at 47°C (for C. tepidum) or 37°C and 47°C (for E. coli) in a Peltier temperature-controlled cuvette holder attached to a DU7400 spectrophotometer (Beckman Coulter, Fullerton, CA). SQR activity was determined by calculating the difference in absorbances measured between ΔA284 and A284, which represent the absorbance of oxidized and reduced dUQ, respectively. The absorbance of the assay mixture was measured immediately after dUQ addition, and the reaction was initiated by adding sulfide to a final concentration of 1 mM or 0.5 mM from a neutralized, anoxic stock solution of Na₂S · H₂O₂. All C. tepidum SQR activities were controlled with lacking cDNA was performed for each primer set, and all reactions were performed in triplicate with RNA samples from independent cultures. Amplified products were confirmed by melting-curve analysis and gel electrophoresis. The expression levels of each gene were analyzed by the ΔΔCT method (32) using sigA (CT1551) as the reference gene. The threshold cycles (C_t) for sigA were found to be similar throughout the growth of C. tepidum cultures and whether the wild type or double mutant were compared, indicating that sigA was expressed at a constant level.

Recombinant expression of C. tepidum SQRs in E. coli. C. tepidum genes encoding SQR homologs were produced recombinantly by expression from pET-based plasmids in E. coli BL21DE3/pLysS (Novagen). PCR products containing genes with unique restriction sites were amplified from C. tepidum wild-type genomic DNA, digested with restriction endonucleases, and ligated to the T7 expression vector pET16b or pET28b (Novagen). Clones were recovered in E. coli TOP10 cells, and the insert was completely sequenced prior to the introduction of the clones into E. coli BL21DE3/pLysS. All recombinant SQR clones carried His tags, which were provided by the pET vectors. For induction of the recombinant proteins, E. coli cultures were inoculated to an OD₆₀₀ of 0.01 and grown under anaerobic conditions at 37°C for 4 h (OD₆₀₀ ~ 0.3); then 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added, and 1 h later, sulfide was added to a final concentration of 5 mM. Cells were harvested 20 h after sulfide addition.

Subcellular fractionation. E. coli expressing recombinant SQR proteins and C. tepidum strain CT1087-His₂aacC1 were pelleted, resuspended in anaerobic 100 mM Tris-HCl (pH 7.0), and lysed by sonication using a model 450 Sonifier equipped with a microtip probe (Branson Ultrasonics, Danbury, CT), housed in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI). The lysates were centrifuged at 20,000 × g at 4°C for 30 min, and the supernatant was used for two periods of 2 min each, with cooling on ice between each sonication. Unbroken cells and inclusion bodies in E. coli strains were removed by low-speed (~17,900 × g) centrifugation. Membrane and cytosolic fractions were separated by ultracentrifugation at 126,000 × g for 60 min. In C. tepidum, the membrane pellet also contains the chlorosome, the unique light-harvesting complex (19, 26). When needed, C. tepidum chlorosome-depleted membranes were prepared by sonicating the cell pellets in hypotonic density ultracentrifugation (7, 57). The membrane fraction was further treated with the surfactant Nonidet P-40 and centrifuged at 126,000 × g for 60 min (7, 53). The Nonidet P-40 pellet is enriched in outer membrane and peripherally bound inner membrane proteins, whereas the Nonidet P-40-soluble fraction is enriched in integral membrane proteins (7).
were corrected for nonenzymatic dUQ reduction, measured with an appropriate boiled membrane fraction. Recombinant SQR activities were corrected with membrane fractions prepared from empty-vector control strains. An extinction coefficient for dUQ of 15 (mmol cm)$^{-1}$, measured by Morton (37), was used to convert absorbance to molar concentrations.

**RESULTS**

**RT-PCR analysis of** *C. tepidum* **sqr homologs.** To investigate if *C. tepidum* might regulate the SQR homologs encoded by CT0117, CT0876, and CT1087, RT-PCR was performed on total RNAs isolated at different times during growth in medium containing both 0.7 mM sulfide and 9.2 mM thiosulfate (Fig. 1a). As previously described in detail (7, 8), *C. tepidum* oxidizes sulfide during early growth ($\leq$15 h) and produces extracellularly deposited elemental sulfur. After sulfide is depleted, elemental sulfur is consumed (15 to 24 h), followed by the oxidation of thiosulfate (24 to 48 h). Sulfate is produced as the end product of sulfur metabolism. While the transcripts of both CT0117 and CT0876 were found at all times, the transcript of CT1087 was observed only at early time points during sulfide oxidation and elemental sulfur production (Fig. 1a).

RT-PCR was also performed on total RNA harvested at $\sim$15 h from wild-type *C. tepidum* grown in medium supplemented with 4, 6, or 8 mM sulfide or 10, 20, 30, or 50 mM thiosulfate as the sole electron donor (Fig. 1b). Transcripts of CT0117 and CT0876 were observed at all concentrations of sulfide and thiosulfate (Fig. 1b). The CT0878 transcript was readily detected in sulfide-grown cultures but was much lower in abundance in cultures supplemented with thiosulfate alone (Fig. 1b). The mRNA of *sigA* (CT1551), encoding the major housekeeping sigma factor of RNA polymerase (22, 23), which is not expected to be regulated by sulfide, was observed under all growth conditions (Fig. 1a and b).

**Genetics and physiology of** *C. tepidum* **mutant strains lacking SQR homologs.** To determine their physiological roles in *C. tepidum*, the SQR-encoding homologs CT0117, CT0876, and CT1087 were individually inactivated by IVTM with the transposon TnOGm. A double-mutant strain carrying TnOGm and TnEm insertions in CT0117 and CT1087, respectively, was also constructed. Transcripts of each gene were assayed in the wild-type and mutant strains by RT-PCR using total RNA isolated at $\sim$15 h from cultures grown in medium containing both sulfide and thiosulfate (Fig. 2a). As expected, transcripts for each SQR homolog were found in the wild type, while the correct mRNA was lacking in each mutant strain, without any obvious effects on the mRNAs of the other putative SQR-encoding genes (Fig. 2a).

All *C. tepidum* strains were analyzed for their abilities to grow with sulfide as the sole electron donor at concentrations ranging from 2 to 10 mM by determining the growth yield at 48 h after inoculation. Wild-type *C. tepidum* had a maximum growth yield with 6 to 8 mM sulfide, but growth was strongly inhibited with 10 mM sulfide (Fig. 2b). In the range of 2 to 8 mM sulfide, the wild type displayed a linear increase in the growth yield of 9 g protein (mol sulfide)$^{-1}$ (linear regression $R^2 = 0.96$). This yield for *C. tepidum* is somewhat lower than the yield of 14 g protein (mol sulfide)$^{-1}$ calculated from sulfide-dependent cell production data for the green sulfur bac-
TABLE 2. SQR activities of wild-type and mutant C. tepidum strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sp act (µmol of dUQ reduced [mg of protein]⁻¹ min⁻¹) with:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1.0 mM Na₂S</td>
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<tr>
<td>Wild type</td>
<td>87.0 ± 6.9</td>
</tr>
<tr>
<td>CT0117::TnOGm</td>
<td>31.5 ± 4.5²</td>
</tr>
<tr>
<td>CT0876::TnOGm</td>
<td>82.5 ± 2.6²</td>
</tr>
<tr>
<td>CT0876::TnOGm</td>
<td>52.5 ± 5.2²</td>
</tr>
<tr>
<td>CT0117::TnOGm, CT0876::TnEm</td>
<td>ND³</td>
</tr>
</tbody>
</table>

* P < 0.05 (n = 3) by the t test for comparison to the wild-type strain assayed under the same conditions.

³ ND, not detected.

arginal Prosthecochloris aestuarii (54) and the yield of 13 g protein (mol sulfide)⁻¹ calculated from C. tepidum growth on sulfide (38). This decrease likely reflects differences between the simple batch tube culture system employed here and the elaborate bioreactor systems utilized in the other studies.

The growth yields of mutant strains CT0117::TnOGm and CT0876::TnOGm were similar to that of the wild type, except that the former strains had a ~40% yield increase relative to the wild type with 4 mM sulfide (Fig. 2b). These mutant strains also displayed maximal growth yield with 6 to 8 mM sulfide (Fig. 2b). In contrast, strain CT0876::TnOGm failed to grow at ≥6 mM sulfide and displayed a 25% growth yield reduction with 4 mM sulfide (Fig. 2b). The double-mutant strain CT0117::TnOGm, CT0876::TnEm failed to grow with ≥4 mM sulfide, and its growth yield was 35% lower than that of the wild type with 2 mM sulfide (Fig. 2b).

Between 2 and 8 mM sulfide, the wild type and mutants had equivalent doubling times of about 2 h, in agreement with previously published studies (8, 24, 59). These data indicate that in this range, the sulfide concentration affects the growth yield, but not the growth rate, of C. tepidum. In addition, wild-type C. tepidum and all mutant strains displayed similar growth yields of 99 to 110 µg of protein ml⁻¹ after 48 h of growth with 10 mM thiosulfate, indicating that the yield defect observed for the mutant strains are limited to growth on sulfide alone.

SQR activity in wild-type and mutant C. tepidum strains.

Prior studies demonstrated that the membrane fraction of C. limicola forma thiosulfatophilum catalyzed sulfide oxidation with ubiquinone as the electron acceptor (50), so a similar assay was used to determine if mutations in the putative SQR-encoding genes affected this activity in C. tepidum.

Under anaerobic conditions, freshly prepared C. tepidum membranes readily catalyzed sulfide oxidation with dUQ as an electron acceptor, but this activity was not found in the cytosolic protein fraction. SQR activity could be eliminated by boiling or exposing the membrane fraction to air and was also decreased >30% if membrane fractions were frozen prior to the assay. SQR activity measured with 20 µg of freshly prepared C. tepidum wild-type membranes and 100 µM dUQ saturated at a concentration of 1 mM sulfide.

With 1 mM sulfide, wild-type C. tepidum membranes displayed an SQR activity of 87 ± 7 µmol of dUQ reduced (mg of protein)⁻¹ min⁻¹ (Table 2). Mutant strains CT0117::TnOGm and CT0876::TnOGm displayed 64% ± 5% and 39% ± 7% reductions in SQR activity from that of the wild type (Table 2). With 0.5 mM sulfide, the SQR activities of strains CT0117::TnOGm and CT0876::TnOGm were reduced by 88% ± 4% and 28% ± 13%, respectively, from that of the wild type (Table 2). Strain CT0876::TnOGm displayed no significant reduction in SQR activity from that of the wild type at either sulfide concentration (Table 2).

SQR activity could not be detected in the double-mutant strain CT0117::TnOGm, CT0876::TnEm under any conditions tested (Table 2). This is consistent with the observation that strain CT0876::TnOGm displayed no change in SQR activity from that of the wild type. Furthermore, if the residual activities observed for strains CT0876::TnOGm and CT0117::TnOGm are added, the combined activities are identical to that observed for the wild type at either sulfide concentration (Table 2). Therefore, one would expect to see no SQR activity in double-mutant strain CT0117::TnOGm, CT0876::TnEm, which expresses only CT0876.

Growth of strain CT0117::TnOGm, CT0876::TnEm at low sulfide concentrations. The mechanism by which the double-mutant strain (CT0117::TnOGm, CT0876::TnEm), which lacks detectable SQR activity, grows at low sulfide concentrations was investigated. To determine if the FCC sulfide dehydrogenase of C. tepidum can support growth at low sulfide concentrations, RT-PCR was carried out for both genes encoding homologs of the FccB subunit: CT1015 (fccB1) and CT2081 (fccB2). Transcripts of both CT1015 and CT2081 were detected at all stages of growth on sulfide (Fig. 3a). However, we failed to detect any FCC sulfide dehydrogenase activity in C. tepidum soluble or membrane fractions prepared from cultures that were actively oxidizing sulfide. The assays utilized horse

FIG. 3. RT-PCR and Q-RT-PCR analyses of transcripts encoding CT0876 and FccB homologs. (a) RT-PCR to detect transcripts of CT1015 (fccB1) and CT2081 (fccB2) in total RNA harvested from wild-type C. tepidum at the indicated times during growth in a medium containing 0.7 mM sulfide and 9.2 mM thiosulfate. (b) Q-RT-PCR analysis of the abundances of CT0876, CT1015, and CT2081 transcripts relative to that of CT1551 (sig4) after 15 h of growth in medium with both thiosulfate and sulfide in the wild type (open bars) and strain CT0117::TnOGm, CT0876::TnEm (filled bars). An expression ratio of 1 indicates that an equal number of copies of the target gene and CT1551 were present in the sample. The P value above the CT1015 data indicates a slight, but statistically significant, increase in transcript abundance in strain CT0117::TnOGm, CT0876::TnEm relative to the wild type. Data are means ± standard errors for three independent experiments.
heart cytochrome c as an electron acceptor, which was successful for measuring this activity in extracts of *A. vinosum* (43). However, this experiment may simply indicate that horse heart cytochrome *c* is not a suitable acceptor for *C. tepidum* FCC sulfide dehydrogenase.

If either a cryptic SQR activity in CT0876 or FCC sulfide dehydrogenase is responsible for the growth of the double mutant, one might expect compensatory positive regulation of the relevant gene in strain CT0117::TnOgms, CT0117::TnEm. This was tested by Q-RT-PCR analysis of the CT0876- and FccB homolog-encoding genes CT0105 and CT2081 in the wild type and the double-mutant strain. The results indicated that the abundance of the CT0876 transcript relative to that of a control gene (*sigA* [CT1551]) was not statistically different between the wild-type and double-mutant strains (Fig. 3b). The abundance of CT0105 transcripts, encoding FccB1, was statistically significantly increased by ~10% in the double mutant, while CT2081 transcripts, encoding FccB2, were not significantly different. Transcripts of all three genes were present at levels similar to those of *sigA*. Thus, either CT0876 or FCC sulfide dehydrogenase may support growth at low sulfide concentrations, but these activities clearly cannot support growth at higher sulfide concentrations in the absence of the other SQR homologs.

**Epitope tagging and localization of CT0107.** To test if a specific *C. tepidum* SQR homolog is membrane associated, as would be predicted from the SQR activity measurements, we constructed a strain harboring a C-terminal His6 epitope by modifying CT0107 in its native location in the *C. tepidum* genome to produce strain CT0107-His6::aacC1. The epitope-tagged version of CT0107, CT0107-His6, should therefore reflect any regulation of CT0107 gene expression. The growth yield of strain CT0107-His6::aacC1 with 6 mM sulfide and 10 mM thiosulfate was similar to that of the wild type, suggesting that CT0107-His6 does not cause any physiological defects.

*C. tepidum* strain CT0107-His6::aacC1 was grown on 6 mM sulfide, and cells were harvested at 10 to 15 h after inoculation. For localization, immunoblotting targeting the His6 epitope was performed on subcellular fractions equally loaded with 30 μg of cytoplasmic, chlorosome, and Nonidet-P40-soluble and -insoluble membrane proteins. The Nonidet-P40-soluble fraction enriches integral membrane proteins, whereas outer membrane proteins and peripherally associated inner membrane proteins tend to partition to the Nonidet-P40-insoluble fraction (7). CT0107-His6 was found primarily in the Nonidet-P40-insoluble membrane fraction (Fig. 4a), suggesting that CT0107-His6 is membrane localized and is likely a peripherally associated inner membrane protein. The observed mass of CT0107-His6 is ~44 kDa, which closely matches the size of 43.9 kDa predicted from the *C. tepidum* genome sequence.

To corroborate the apparent regulation of CT0107 mRNA levels by sulfide, CT0107-His6 was monitored in membrane fractions throughout the growth of *C. tepidum* on both sulfide and thiosulfate by immunoblot analysis (Fig. 4b). CT0107-His6 was abundant during early growth (~15 h) in *C. tepidum* (Fig. 4b), coinciding with active sulfide oxidation and elemental sulfur production. While CT0107-His6 was readily detectable in sulfide-grown cultures, it was present at a much lower abundance in thiosulfate-grown cultures (Fig. 4c).

**Analysis of recombinant SQRs.** To further analyze the SQR activities of *C. tepidum* SQR homologs, CT0876 and CT1087 were recombinantly expressed from pET16b in *E. coli*, while CT0117 was expressed from pET28b due to the presence of an NdeI site in the native coding sequence. The recombinant proteins all contained His tags and so were detectable by immunoblotting with an α-His antibody. In these experiments, all *E. coli* strains were cultured under anaerobic conditions in LB medium with glucose plus nitrate, and expression was initiated by the addition of 0.4 mM IPTG and 5 mM sulfide. Subcellular fractionation and immunoblotting indicated that most of the recombinant proteins were found as inclusion bodies, with a small portion of each protein found in the membrane fraction (data not shown). The observed masses of the recombinant proteins in immunoblots were similar to the sizes predicted from the sequence of each expression construct.

Because some recombinant CT0117-His, CT0876-His, and CT0107-His protein was found in the membrane fractions of *E. coli*, these were assayed for SQR activity at both 37°C and 47°C. SQR activity was detected only in CT0117-His- and CT0876-His-containing membrane fractions. At 37°C, the activities of CT0117-His and CT0107-His were ~4.5 and 31.5 ± 8.4 μmol of dUQ reduced (mg of protein)⁻¹ min⁻¹, respectively. At 47°C, the activities for these strains were 5.6 ± 3.0 and 79.5 ± 9.4 μmol of dUQ reduced (mg of protein)⁻¹ min⁻¹, respectively. SQR activity was never detected in membranes containing recombinant CT0876-His or in the empty-vector controls.

**Phylogenetic analysis of *C. tepidum* SQR homologs.** Because our data indicated that *C. tepidum* possesses two active SQRs and one apparently inactive homolog, we sought to use phylo-
Type I SQR consists of sequences from the cyanobacteria phylogeny (Fig. 5) that had been defined previously (20, 48, 56). Type II SQRs are more similar to FCCs and other disulfide oxidoreductases than the other SQR types. Type III SQRs include CT0117 and the SQRs of most other green sulfur bacteria, including the second SQR homolog from *C. phaeobacteroides* BS-1 (*Cphamm1_2428*), *C. phaeobacteroides* DSM 266, *Chlorobium chlorochromatii*, and *C. ferrooxidans*, even though the latter strain cannot grow on sulfide (25). CT0187 clustered with the AO0788 protein of *A. aeolicus*, and these proteins together form a cluster that branches as deeply as the division between type I and type III SQRs. Similarly, CT0876, the apparently inactive SQR homolog, appears to be affiliated with archaean proteins annotated as SQR in a deeply branching cluster (Fig. 5). To the best of our knowledge, SQR activity has never been demonstrated in any archaean, which may suggest that this cluster represents a branch of the SQR family with altered catalytic properties.

**DISCUSSION**

This paper reports the first analysis of multiple SQR homologs found in a single microbe, the green sulfur phototrophic bacterium *Chlorobaculum tepidum*. The data reported above led us to conclude that the *C. tepidum* genes CT0117 and CT0876 encode functional SQRs and that either can support sulfide-dependent growth at sulfide concentrations of $\geq 2\, \text{mM}$. While the results support the annotation of CT0117 and CT0876 as SQRs, they are not absolutely definitive, due to the lack of a complementation system in *C. tepidum*. While *C. tepidum* will apparently accept and maintain conjugally transferred plasmids (58), there has been no report of plasmid-based complementation to date, and efforts to develop this in our laboratory have been unsuccessful. The recent report of heterologous gene expression from the chromosomal integration of a carotenoid biosynthetic gene (34) will hopefully allow us to address this issue in the near future by integration of the wild-type SQR genes at an alternative chromosomal location in the mutant strains.

The data were not able to resolve whether or not CT0876 encodes an active SQR. While CT0876 is expressed in the double-mutant strain and this strain grows at 2 mM sulfide, no SQR activity was found in this strain or in recombinantly produced CT0876 protein. If the CT0876 protein does possess SQR activity, it must not be able to use dUQ as an electron acceptor in vitro. We are in the process of purifying native quinones from *C. tepidum* in order to test whether any of these may serve as an electron acceptor for CT0876. Transcripts of genes encoding both homologs of the FccB subunit of FCC sulfide dehydrogenase were also found in the mutant strain, suggesting that this activity may support growth at low sulfide levels. Alternatively, the growth of the double-mutant strain with 2 mM sulfide might be due to a low level of thiosulfate consistently observed when sulfide is added to Pf-7-BTP medium, as has been previously documented (8). Experiments in which strain CT0117::TnOGm, CT0876::TnEm was grown at the levels of thiosulfate ($\sim 0.75\, \text{mM}$) observed in medium with 2 mM sulfide as the sole electron donor indicate that this strain

![Image](http://jb.asm.org)
achieves a density (20 to 30 μg of protein ml\(^{-1}\)) similar to that reported in Fig. 2b.

This study has expanded the range of sulfide concentrations known to be utilized for growth by \textit{C. tepidum}. \textit{C. tepidum} was initially reported to have an upper limit for sulfide tolerance of 4 mM (59), while our data indicate a maximal yield at 8 mM sulfide for the wild type. The observations that CT1087 appears to be expressed only during active sulfide oxidation and that it is required for growth at high levels of sulfide suggest that this protein may be adapted to function at high concentrations of sulfide. This suggestion is supported by the observation that a higher SQR-specific activity was observed in strain CT0117:TnOGm than in CT087:TnOGm when both were assayed at a saturating sulfide concentration. Both CT0117 and CT1087 could be recombinantly expressed in an active, His-tagged form in \textit{E. coli} in an active, His-tagged form in \textit{E. coli} in an active, His-tagged form. The preliminary data reported here indicate that the SQR activities of both CT0117 and CT1087 are adapted to high temperatures, as would be expected for an enzyme from a moderate thermophile such as \textit{C. tepidum}.

CT1087-His\(_6\) can be produced in \textit{C. tepidum} by epitope tagging, the first demonstration of this technique in this organism. Unfortunately, attempts to produce a His\(_{12}\)-tagged version of CT0117 in \textit{C. tepidum} have failed. Our results indicate that CT1087-His\(_6\) is membrane associated, and Nonidet-P40 treatment suggests that it is a peripherally associated inner membrane protein. Previous analysis indicates that FmoA, another peripherally associated inner membrane protein, was, like CT1087-His\(_6\), observed in both the total-membrane and Nonidet P-40-insoluble fractions, while the integral membrane protein PscD, a component of the photosynthetic reaction center, was not found in the Nonidet P-40-insoluble fraction (7). A CT1087::phoA fusion protein should help to confirm the precise location of CT1087 in \textit{C. tepidum}. This technique was used to suggest that the \textit{R. capsulatus} SQR is associated with the periplasmic face of the cytoplasmic membrane (46).

While this study has significantly improved our understanding of sulfide oxidation in \textit{C. tepidum}, intriguing questions remain. Quinone specificity, noted above as a possible reason for the failure to observe activity in CT0876, may provide a rationale for multiple SQRs in \textit{C. tepidum}, which possesses both menaquinone and chlorobiumquinone (18, 30, 37). Chlorobiumquinone is found in membranes and the chlorosome, where it is thought to help mediate the quenching of excited states of bacteriochlorophyll and prevent the photodegradation of chlorosomes (29). Menaquinone in \textit{C. tepidum} is found associated with the photosynthetic reaction center (30) and in the cytoplasmic membrane. A larger question is the mechanism of growth inhibition by sulfide in \textit{C. tepidum}, which is poorly understood. Since the strains lacking SQRs displayed differing levels of resistance to sulfide, they may help to provide insights into sulfide tolerance specifically in \textit{C. tepidum} and in the green sulfur bacteria generally, especially when these studies are coupled to proteomic and genome-wide expression analyses.

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**References**


