Functional Analysis of Three Sulfide:Quinone Oxidoreductase Homologs in Chlorobaculum tepidum\textsuperscript{\textdagger}†

Leong-Keat Chan, Rachael M. Morgan-Kiss,§ and Thomas E. Hanson*

College of Marine and Earth Studies and Delaware Biotechnology Institute, University of Delaware, 15 Innovation Way, Newark, Delaware 19711

Received 15 August 2008/Accepted 12 November 2008

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 \( \geq 6 \) mM sulfide. Mutation of both CT0117 and CT1087 in C. tepidum completely abolished SQR activity, and the double mutant failed to grow with \( \geq 4 \) mM sulfide. A C-terminal His\textsubscript{6}-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 elemental sulfur globules (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 cytochrome b/c\textsubscript{1} 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/c\textsubscript{1} complex en route to the reaction center (41). Oxidation of sulfur/polsulfides 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 sulfide 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 fccAB genes in the phototrophic purple sulfur bacterium Allochromatium vinosum 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 chemolithoautotrophic 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 in C. tepidum.
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 ferroxidans (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 CT0117 (formerly Chlorobium tepidum [59]). With the exception of Chlorobium ferroxidans (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 CT0117 (formerly Chlorobium tepidum [59]). With the exception of Chlorobium ferroxidans (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 CT0117 (formerly Chlorobium tepidum [59]). With the exception of Chlorobium ferroxidans (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 CT0117 (formerly Chlorobium tepidum [59]). With the exception of Chlorobium ferroxidans (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 CT0117 (formerly Chlorobium tepidum [59]). With the exception of Chlorobium ferroxidans (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 CT0117 (formerly Chlorobium tepidum [59]). With the exception of Chlorobium ferroxidans (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 CT0117 (formerly Chlorobium tepidum [59]). With the exception of Chlorobium ferroxidans (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 CT0117 (formerly Chlorobium tepidum [59]).
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 (Epitope, 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 NucleaseSpin RNA purification kit from Macherey-Nagel (Bethlehem, PA), and trace DNA contamination was removed by treating samples with the Turbo DNA-free kit (Ambion, Austin, TX). RT-PCR was performed with Epitope’s high-fidelity RT-PCR kit according to the manufacturer’s instructions. When needed, PCR products or plasmids were sequenced by standard procedures at the College of Agriculture and Natural Resources DNA Sequencing Facility at the University of Delaware, Newark.

**ITVM of SQR-encoding genes in C. tepidum.** In vitro transposition mutagenesis (IVTM) of *C. tepidum* was performed as previously described (8). PCR products carrying CT0117, CT0876, and CT0187 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 on LB plates with 50 μg kanamycin ml⁻¹ and 20 μg gentamicin ml⁻¹. Plasmids purified from these transposons were treated with restriction endonucleases EcoRI and Acc65I and sequenced by standard procedures at the College of Agriculture and Natural Resources DNA Sequencing Facility at the University of Delaware, Newark.

**Phenotypic characterization of mutants.** Strains—CT0117::TnOGm, CT0876::TnOGm, and CT1087::TnOGm—were constructed by this method. A transposon (TnEm) carrying the erythromycin resistance marker from plasmid pRL21 (5) was PCR amplified with primers Em-ME-F and Em-ME-R (see Table S1 in the supplemental material). Three *C. tepidum* strains—CT0117::TnOGm, CT0876::TnOgm, and CT0187::TnOgm—were constructed by this method.

**Codon usage analysis.** 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 polyvinylidene difluoride membranes (Bio-Rad) for 2 h at 4 °C with continuous buffer stirring. Polyvinylidene difluoride blot membranes were then incubated with the primary antibody (mouse anti-β- His; Sigma-Aldrich) and secondary antibody (alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G; Bio-Rad) in Tris-buffered saline and 5% (wt/vol) nonfat milk for 60 min. The membranes were washed three times in 2× Tris-buffered saline and 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 (dQ) reduction activity was measured under anaerobic conditions in N₂-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 protein, and 100 μM dQ (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 between the rate of coupled oxidation of dQ and dQ reduction by the SQR per minute. To normalize the assay, the amount of oxidized and reduced dQ, respectively. The absorbance of the assay mixture was measured immediately after dQ addition, and the reaction was initiated by adding sulfide to a final concentration of 1 mM or 0.5 mM from a sterile, neutralized, anoxic stock solution of Na₂S·9H₂O. All *C. tepidum* SQR activities were 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 (Cₜ) for sigA were found to be similar throughout the growth of *C. tepidum* cultures and when the wild type and 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 O₄D₀ of 0.1 and grown under anaerobic conditions at 37°C for 4 h (O₄D₀ ~ 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* cells expressing recombinant SQR proteins and *C. tepidum* strain CT0187-His₅::aacC1 were pelleted, resuspended in anaerobic 100 mM Tris-HCl (pH 7.0), and lysed by sonication using a model 450 Sonifier with a microtip probe (Branson Ultrasonics, Danbury, CT), housed in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI). The membrane fraction was isolated by centrifugation at 30,000 × g for 30 min, the supernatant was further centrifuged for two periods of 2 min each, with cooling on ice between each sonication. Unbroken cell 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 an equivalent dilution 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 polyvinylidene difluoride membranes (Bio-Rad) with 20 μg ml⁻¹ methanol and 20% (v/v) methanol for 2 h at 4 °C with continuous buffer stirring. Polyvinylidene difluoride blot membranes were incubated in the primary antibody (mouse anti-β- His; Sigma-Aldrich) 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-HCl [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.
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 (∼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 ∼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). The CT0117 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 ∼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-

![Fig. 1](http://jb.asm.org/)

![Fig. 2](http://jb.asm.org/)

---

**Amino acid sequence analysis and comparison.** SQR and FCC sequences were identified by BLASTP (http://www.ncbi.nlm.gov/BLAST) with _C. tepidum_ squirrel sequences as queries. A neighbor-joining (45) phylogram of selected sequences was constructed with tools in MEGA 4 (55). SQR and FCC sequences were classified as previously described (20, 48, 56).

**Fig. 1.** RT-PCR analysis of gene transcripts with total RNA harvested at ∼15 h from wild-type _C. tepidum_ either at the indicated time when the organism was grown in medium containing 0.7 mM sulfide and 9.2 mM thiosulfate (a) or at 15 h in medium with sulfide or thiosulfate as the sole electron donor at the indicated concentration (b). The gene encoding the major housekeeping sigma factor (CT1551, or _sigA_) was used as a control gene that was not expected to be regulated under these conditions. Negative-control reactions omitted reverse transcriptase (RT$^{-}$).

**Fig. 2.** Genetics and physiology of wild-type and mutant _C. tepidum_ strains. (a) RT-PCR analysis of gene transcripts using total RNA prepared at ∼15 h from cultures grown with 0.7 mM sulfide and 9.2 mM thiosulfate. Negative-control reactions omitted reverse transcriptase (RT$^{-}$). (b) Protein yield measured at 48 h postinoculation in cultures grown with sulfide at varying concentrations as the sole electron donor. Data are averages and standard deviations (error bars) for three or more independent replicates. Symbols: ●, wild type; ■, CT0117::TnOGm; △, CT0876::TnOGm; ○, CT1087::TnOGm; ★, CT0117::TnOGm, CT0876::TnEm.
terium 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 $\sim$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 CT1087::TnOGm failed to grow at $\geq$6 mM sulfide and displayed a 25% growth yield reduction with 4 mM sulfide (Fig. 2b). The double-mutant strain CT0117::TnOGm, CT0876::TnOGm, CT1087::TnEm failed to grow with $\geq$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 $\mu$g of protein ml$^{-1}$ after 48 h of growth with 10 mM thiosulfate, indicating that the growth yield defects 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 ability in C. tepidum.

Under anaerobic conditions, freshly prepared C. tepidum membrane fractions 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 $\mu$g of freshly prepared C. tepidum wild-type membranes and 100 $\mu$M dUQ saturated at a concentration of 1 mM sulfide.

With 1 mM sulfide, wild-type C. tepidum membranes displayed an SQR activity of 87 $\pm$ 7 $\mu$mol of dUQ reduced (mg of protein)$^{-1}$ min$^{-1}$ (Table 2). Mutant strains CT0117::TnOGm and CT1087::TnOGm displayed 64% $\pm$ 5% and 39% $\pm$ 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% $\pm$ 4% and 28% $\pm$ 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, CT1087::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 CT1087::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, CT1087::TnEm, which expresses only CT0876.

**Growth of strain CT0117::TnOGm, CT1087::TnEm at low sulfide concentrations.** The mechanism by which the double-mutant strain (CT0117::TnOGm, CT1087::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

### TABLE 2. SQR activities of wild-type and mutant C. tepidum strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sp act (mol of dUQ reduced [mg of protein]$^{-1}$ min$^{-1}$) with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.0 mM Na$_2$S</td>
</tr>
<tr>
<td>Wild type</td>
<td>87.0 $\pm$ 6.9</td>
</tr>
<tr>
<td>CT0117::TnOGm</td>
<td>31.5 $\pm$ 4.5*</td>
</tr>
<tr>
<td>CT0876::TnOGm</td>
<td>82.5 $\pm$ 2.6</td>
</tr>
<tr>
<td>CT1087::TnOGm</td>
<td>52.5 $\pm$ 5.2*</td>
</tr>
<tr>
<td>CT0117::TnOGm, CT1087::TnEm</td>
<td>ND</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.

$^b$ ND, not detected.
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::TnOGm, CT0107::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 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 CT1087.** 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 CT1087 in its native location in the _C. tepidum_ genome to produce strain CT1087-His6::aacC1. The epitope-tagged version of CT1087, CT1087-His6, should therefore reflect any regulation of CT1087 gene expression. The growth yield of strain CT1087-His6::aacC1 with 6 mM sulfide and 10 mM thiosulfate was similar to that of the wild type, suggesting that CT1087-His6 does not cause any physiological defects.

_C. tepidum_ strain CT1087-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-P-40-insoluble and -soluble membrane proteins. The Nonidet-P-40-soluble fraction enriches integral membrane proteins, whereas outer membrane proteins and peripherally associated inner membrane proteins tend to partition to the Nonidet-P-40-insoluble fraction (7). CT1087-His6 was found primarily in the Nonidet-P-40-insoluble membrane fraction (Fig. 4a), suggesting that CT1087-His6 is membrane localized and is likely a peripherally associated inner membrane protein. The observed mass of CT1087-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 CT1087 mRNA levels by sulfide, CT1087-His6 was monitored in membrane fractions throughout the growth of _C. tepidum_ on both sulfide and thiosulfate by immunoblot analysis (Fig. 4b). CT1087-His6 was abundant during early growth (~15 h) in _C. tepidum_ (Fig. 4b), coinciding with active sulfide oxidation and elemental sulfur production. While CT1087-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. All lanes were loaded with 30 µg of protein. (a) Cultures grown in the presence of 6 mM sulfide were harvested at ~15 h post inoculation. Lanes: cytosolic, ~126,000 × g supernatant; chlorosome, chlorosome-containing sucrose gradient fraction; Nonidet P-40 soluble, proteins solubilized from the sucrose gradient membrane pellet by NP-40; Nonidet P-40 insoluble, NP-40-insoluble proteins in the sucrose gradient membrane pellet. (b) Membrane proteins were harvested from cultures grown with 0.7 mM sulfide and 9.2 mM thiosulfate at the indicated times. (c) Membrane proteins were harvested at ~15 h from cultures grown in medium with sulfide or thiosulfate as the sole electron donor at the indicated concentrations.

**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, to III) of SQR and SQR-like homologs in a neighbor-joining fashion. Our analysis successfully reconstructed the three types (I-like, and FCC sequences to provide context for our observations (Fig. 5). 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 (Cphamn1_2428), C. phaeobacteroides DSM 266, Chlorobium chlorochromatii, and C. ferrooxidans, even though the latter strain cannot grow on sulfide (25). CT0878 clustered with the AQ0788 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 archaeon, 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 CT0878 encode functional SQRs and that either can support sulfide-dependent growth at sulfide concentrations of ≥2 mM. While the results support the annotation of CT0117 and CT0878 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 integra-
tion 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 (~0.75 mM) observed in medium with 2 mM sulfide as the sole electron donor indicate that this strain
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 C. tepidum. 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 CT1087:TnOgm when both were assayed at a saturating sulfide concentration. Both CT0117 and CT1087 could be recombiantly expressed in an active, His-tagged form in E. coli, which will facilitate protein purification to examine the kinetic differences between these proteins in greater detail. 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 C. tepidum.

CT1087-His₆ can be produced in C. tepidum by epitope tagging, the first demonstration of this technique in this organism. Unfortunately, attempts to produce a His₆-tagged version of CT0117 in C. tepidum have failed. Our results indicate that CT1087-His₆ 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-His₆, 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 C. tepidum. This technique was used to suggest that the 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 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 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 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 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 C. tepidum and in the green sulfur bacteria generally, especially when these studies are coupled to proteomic and genome-wide expression analyses.

ACKNOWLEDGMENTS

This project was supported by a National Science Foundation CAREER award (MCB-0447619, to T.E.H.) and utilized common instrumen- tation facilities provided in part by the National Institute of Health, P20-RR116472-04 from the IDEA Networks of Biomedical Research Excellence program of the National Center for Research Resources.

Bruce Kingham is acknowledged for providing DNA sequencing services. We also thank Nicole Donofrio and Kun Huang for their expertise in Q-RT-PCR analysis and David Kirchman for assistance in editing the manuscript.

This article is dedicated to the memory of Tom Wahlund (1948–2008), who isolated C. tepidum and facilitated the application of molecular genetics to the Chlorobiaceae. Without his efforts, this work and that of many other groups would not have been possible.

REFERENCES
