Transcriptional Response of the Sulfur Chemolithoautotroph *Thiomicrospira crunogena* to Dissolved Inorganic Carbon Limitation

Kimberly P. Dobrinski,Steven A. Enkemann,Sean J. Yoder,Edward Haller, and Kathleen M. Scott

Department of Integrative Biology, University of South Florida, Tampa, Florida, USA,* and Microarray Core Laboratory, H. Lee Moffitt Cancer Center and Research Institute, University of South Florida, Tampa, Florida, USA

The hydrothermal vent gammaproteobacterium *Thiomicrospira crunogena* inhabits an unstable environment and must endure dramatic changes in habitat chemistry. This sulfur chemolithoautotroph responds to changes in dissolved inorganic carbon (DIC) (DIC = CO₂ + HCO₃⁻ + CO₃⁻²) availability with a carbon-concentrating mechanism (CCM) in which whole-cell affinity for DIC, as well as the intracellular DIC concentration, increases substantially under DIC limitation. To determine whether this CCM is regulated at the level of transcription, we resuspended cells that were cultivated under high-DIC conditions in chemostats in growth medium with low concentrations of DIC and tracked CCM development in the presence and absence of the RNA polymerase inhibitor rifampin. Induction of the CCM, as measured by silicone oil centrifugation, was hindered in the presence of rifampin. Similar results were observed for carboxysome gene transcription and assembly, as assayed by quantitative reverse transcription-PCR (qRT-PCR) and transmission electron microscopy, respectively. Genome-wide transcription patterns for cells grown under DIC limitation and those grown under ammonia limitation were assayed via microarrays and compared. In addition to carboxysome genes, two novel genes (Tcr_1019 and Tcr_1315) present in other organisms, including chemolithoautotrophs, but whose function(s) has not been elucidated in any organism were found to be upregulated under low-DIC conditions. Likewise, under ammonia limitation, in addition to the expected enhancement of ammonia transporter and PII gene transcription, the transcription of two novel genes (Tcr_0466 and Tcr_2018) was measurably enhanced. Upregulation of all four genes (Tcr_1019, 4-fold; Tcr_1315, ~7-fold; Tcr_0466, >200-fold; Tcr_2018, 7-fold), which suggests that novel components are part of the response to nutrient limitation by this organism, was verified via qRT-PCR.

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drothermal fluid emitted from cracks in the earth’s crust provides reduced chemicals (e.g., H₂S, H₂, CH₄, and Fe³⁺) utilized by chemolithoautotrophic microbes to fuel carbon fixation (6, 12, 22). The deep-sea hydrothermal vent environment, while being an extremely productive ecosystem (15, 20), presents challenges to which vent organisms must adapt. In this habitat, turbulent eddies of dilute hydrothermal fluid (30°C), which have a low pH and carry H₂S, mix with bottom seawater (2°C), which has alkaline pH and carries O₂, causing wide fluctuations in habitat chemistry over time (16). Concentrations of dissolved inorganic carbon (DIC) (DIC = CO₂ + HCO₃⁻ + CO₃⁻²; 2 mM to 7 mM) and pH values (~5 to 8) vary considerably, presenting very divergent concentrations of CO₂ (20 to 2,000 μM) and HCO₃⁻ to the autotrophs growing there (13, 17), which may necessitate adaptations to maintain a steady supply of DIC. One such adaptation is a carbon-concentrating mechanism (CCM) (4). Ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO), the carboxylase of the Calvin-Benson-Bassham cycle, has a low affinity for CO₂ and can use both CO₂ and O₂ as substrates. Two components of CCMs compensate for these catalytic limitations. First, active HCO₃⁻ transport generates a high intracellular concentration of this compound (26). Next, intracellular HCO₃⁻ enters carboxysomes, which are protein-bound inclusions where RubisCO is sequestered, with a trace of carbonic anhydrase activity. The carbonic anhydrase catalyzes the conversion of HCO₃⁻ to CO₂ which is then fixed by RubisCO before it can escape the carboxysome (7). CCMs have been well studied in cyanobacteria and facilitate the growth of these cells under low-CO₂ conditions, and they vary among species (4). Thus far, three evolutionarily distinct HCO₃⁻ transporters have been discovered: BCT₁, an ABC transporter which is induced under carbon limitation (23); a Na⁺-dependent transporter (29); and SulP, which is evolutionarily related to sulfate transporters (24). Regulation of DIC uptake occurs both at the level of transcription and posttranslationally (26).

The hydrothermal vent gammaproteobacterial chemolithoautotroph *Thiomicrospira crunogena* is the first chemolithoautotroph in which elevated intracellular DIC concentrations, consistent with the presence of a CCM, which may enable it to grow steadily despite environmental CO₂ fluctuations, have been measured (10). *T. crunogena* utilizes the Calvin-Benson-Bassham cycle for CO₂ fixation and grows rapidly even when the culture DIC is below 20 μM. *T. crunogena* cells cultivated under low-DIC conditions are capable of generating intracellular DIC concentrations 100-fold higher than extracellular concentrations (10) and can utilize extracellular CO₂ and HCO₃⁻ for carbon fixation. Whole-cell affinities for DIC respond to the DIC concentration (K_{DIC}) present during growth; when DIC concentrations are low, cell affinities for this substrate are substantially higher (K_{DIC} = 26 μM) than when cells are cultivated under elevated DIC concentrations (K_{DIC} = 660 μM) (10). Some components of a typical CCM are apparent in the ge-
ome of *T. crunogena*, but key components are not. A carboxysome operon, which encodes the shell proteins, carboxylic anhydrase (*csoSCA*), and carboxysomal form I Rubisco, is present. Elsewhere in the genome, two other RubiscoCs are encoded (one form I and one form II), as well as an alpha carboxylic anhydrase (α-CA) and two β-CA genes (including *csoSCA*). However, *CsoSCA* is the only carboxylic anhydrase that appears to play a role in DIC uptake and fixation (9), and no orthologs to the bicarbonate transporters found in cyanobacteria are apparent in the *T. crunogena* genome (28).

The objectives of this study were to determine whether CCM induction in *T. crunogena* occurs at the level of transcription and to compare the transcriptomes of *T. crunogena* cultivated under low- and high-DIC conditions to identify the genes whose expression is stimulated by growth under low-DIC conditions as a step in resolving all of the components of this proteobacterial CCM.

**MATERIALS AND METHODS**

**Cultivation.** To prepare cells for experiments to monitor changes in DIC uptake and fixation in response to exposure to low-DIC conditions and to determine whether these changes were dependent on transcription, we cultivated *T. crunogena* XCL-2 cells (DSM 25203) (1) overnight under ammonia-limited conditions in two chemostats (dilution rate = 0.1 h⁻¹) in artificial seawater (ASW) supplemented with thiosulfate (40 mM) and a high concentration of DIC (50 mM, as quantified with a gas chromatograph) (9). The pH (8) and oxygen concentration (10 to 100 μM) were monitored with electrodes and maintained with 10 N potassium hydroxide (KOH) and sparging with 5% CO₂, balance O₂ (9). The following day, cells were aseptically harvested via centrifugation (10,000 g, 10 min, 4°C) and resuspended to their original volume (2 ml/liter). Both cultures were then run as bioreactors (dilution rate = 0.1) in thiosulfate-supplemented ASW with a low DIC concentration of 50 mM (10) of DIC-free ASW. DIC fixation and resuspended the cell pellets from one 30-ml sample of each culture in ASW supplemented with glutaraldehyde (2.5%, vol/vol) and stored them overnight at 4°C. The following day, cells in these samples were harvested via centrifugation and washed three times with ASW (4°C) before treatment for 1 h with 1% osmium tetroxide in ASW. Cells were harvested and rinsed three times with distilled water, after which they were embedded in 3% agar. After trimming to 1-mm³ pieces, agar blocks were dehydrated with an ethanol series (50%, 70%, 95%, and 100%, twice) and ethanol/propylene oxide (50:50; 0:100, twice). Spurr’s resin was infused into the samples (propylene oxide/Spurr’s, 50:50, 25:75, 0:100; four times; first incubation was overnight; all incubations were agitated with a rotator). Spurr’s resin was polymerized overnight at 70°C, and 80-μm sections were cut and stained for 10 min with 8% aqueous uranyl acetate, followed by a 5-min staining with Reynolds’s lead citrate stain. Electron micrographs were produced with an FEI Morgagni 268D transmission electron microscope at 60 kV, and images were captured with an Olympus SIS MegaView III side-mounted camera with 1.4 megapixel image capture. Approximately 50 images were collected from each time point, and carboxysomes were tallied from at least 150 cell cross-sections per sample. Cell cross-section areas were estimated by measuring their long (L) and short (S) axes in Photoshop (CS3; Adobe), modeling them as rectangles with a half circle on each end, and calculating areas as π × (S²/2) − (SL − S²).

**csoSCA transcription quantification.** In preparation for RNA extraction, cell pellets from one 30-ml sample from both cultures were flash frozen with liquid nitrogen and stored at −80°C. RNA was purified and reverse transcribed using the RiboPure (Ambion) and Improm-II RT (Promega) systems. Transcription of *csoSCA* (locus tag Tcr_0841) was tracked via quantitative reverse transcription-PCR (qRT-PCR) using TaqMan primers and probes (ABI) as described in reference 9.

**Transcriptional profiling.** Oligonucleotide arrays were fabricated with probes designed to represent all genes within the *T. crunogena* genome, with two or three probes per gene and a probe length of 35 nucleotides (CombiMatrix, Mukilteo, WA). RNA was isolated from cells grown in six chemostats (three low-DIC and three high-DIC; ammonia-limited) as described in reference 9. RNA was purified further with an RNAeasy MinElute cleanup kit (Qiagen, Germantown, MD), which also served to remove EDTA remaining from the RiboPure system, and was eluted in RNase-free water. One microgram of total RNA was directly labeled for 1 h with a LabelIT Cy3 labeling reaction (Mirus Bio, Madison, WI). After labeling, the RNA underwent ethanol (EtOH) precipitation (5 min at −80°C and 30 min centrifugation). The RNA pellet was resuspended in 16 μl of water and 4 μl of fragmentation buffer (final concentration, 40 μM Tris-acetate [pH 8.1], 100 mM potassium acetate [KOAe], 30 mM MgOAc).

Prior to hybridization, arrays were rehydrated at 65°C with water for 10 min and then incubated at 45°C for 2 h in prehybridization solution (final concentration, 6× SSPE [1× SSPE is 0.18 M NaCl, 10 mM NaH2PO4, and 1 mM EDTA; pH 7.7]; Lonza Accugene), 0.05% Tween 20, 20 mM EDTA, 5× Denhardt’s solution, 100 ng/μl of herring sperm DNA, 0.05% SDS). Labeled RNA was then added to hybridization solution (final concentration, 6× SSPE, 0.05% Tween 20, 20 mM EDTA, 25% distilled formamide,100 ng/μl of herring sperm DNA, 0.04% SDS), and the array was incubated overnight at 45°C. Arrays were washed and imaged per the hybridization and imaging protocol for the CombiMatrix CustomArray 12K microarray. All arrays used were stripped and rehybridized a maximum of three times each, as subsequent stripping and hybridization resulted in substantial loss of signal (data not shown). Global normalization was used for all six arrays (three low-DIC and three high-DIC) to compensate for between-array variation in overall signal strength. For normalization, the average signal strength for each array was calculated using the fluorescence intensities for all spots on each array. Spot intensities on all arrays were normalized so that each array would have the same average signal. For a particular probe, fold changes in transcription were calculated by dividing the average spot intensity for microarrays hybridized with labeled miRNA purified from low-DIC cells by the spot intensity for...
those hybridized with labeled mRNA from high-DIC cells (low-DIC/high-DIC) or vice-versa (high-DIC/low-DIC). The Comparative Marker Selection module of GenePattern (14, 27) was used to generate asymptotic P values associated with gene expression levels. Asymptotic P values were calculated from P values from a standard independent two-sample Student t test. Rather than create an empirical distribution of the scores, we computed the P values with the assumption that test statistic scores follow Student’s t test distribution. Changes in transcription levels for genes were considered noteworthy if average spot intensities for more than one probe per gene were at least enhanced by 2-fold.

Quantification of gene transcription via qRT-PCR. RNA was isolated from low- and high-DIC cells (as well as low-PO4- cells) as described above; the primers used for cDNA synthesis are listed in Table 1. Verification of amplification efficiencies was carried out with primer/probe sets directed against 16S (calibrator) and target genes (Tcr_1019, Tcr_1315, Tcr_0466, Tcr_2018) as described in reference 9. Fold enhancement of transcription in low-DIC cells relative to that in high-DIC cells was calculated according to the 2−ΔΔCt method, where ΔΔCt = [(Ct target − Ct calibrator) − (Ct targetcalibrator − Ct calibrator)] / Ct calibrator and Ct target and Ct calibrator are the threshold cycle (Ct) values for target and 16S gene amplification in low-DIC cells, and Ct targetcalibrator − Ct calibrator is the corresponding value from high-DIC cells (19). Fold enhancement in high-DIC cells was calculated by using low-DIC cells as the reference.

Table 1: Primers and probes used to target T. crunogena conserved hypothetical genes

<table>
<thead>
<tr>
<th>Locus tag</th>
<th>Purpose</th>
<th>Function</th>
<th>Sequence (5′→3′)nt</th>
<th>Location on geneab (nt)</th>
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<td>Tcr_1019</td>
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<td>R primer</td>
<td>AGCCGGTTAGATCCCTATTG</td>
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<td></td>
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<td>cDNA synthesis</td>
<td>R primer</td>
<td>GGTATACGCCAGGTCATTGG</td>
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</tr>
<tr>
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<td>qRT-PCR</td>
<td>F primer</td>
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</tr>
<tr>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Probe</td>
<td>FAM CTGGCGACAGATTTA NFQ</td>
<td>368</td>
</tr>
<tr>
<td>Tcr_0466</td>
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<td>R primer</td>
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<tr>
<td></td>
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<td>GATCGTAACGCGACCCGAAAC</td>
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<td>Probe</td>
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<tr>
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<td>F primer</td>
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<td></td>
<td></td>
<td>Probe</td>
<td>FAM CCGGTTGCGCAAAGCAG NFQ</td>
<td>150</td>
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a Number of nucleotides at the 3′ end of the start codon of the gene.

b Location on gene.

Microarray data accession number. Microarray data are available at Gene Expression Omnibus (GEO) under accession number GSE36254.

RESULTS

DIC uptake and fixation. After resuspension and cultivation for 1 h in growth medium containing a low concentration of DIC (1 mM), T. crunogena cells expressed an ability to generate elevated intracellular DIC concentrations in short-term (30-s) incubations in the presence of 0.1 mM DIC (Fig. 1A). Carbon fixation lagged behind intracellular DIC accumulation, being substantially elevated after ∼1.5 h of cultivation (Fig. 1B). Cells incubated in the presence of rifampin did not develop an ability to accumulate intracellular DIC or to fix carbon under low-DIC conditions (Fig. 1).

Carboxysome presence and csoSCA transcription quantification. During cultivation under low-DIC conditions, carboxysomes became increasingly abundant in cells over the time course sampled here (Fig. 2), paralleling carbon fixation rates (Fig. 1). Cultures to which rifampin had been added did not have any visible carboxysomes 3 h after resuspension in low-DIC growth medium (Fig. 2). Carboxysome gene transcript abundance (csoSCA) was somewhat elevated in cells resuspended in low-DIC growth medium containing rifampin after 30 min and 1 h, which may indicate that the action of this antibiotic on RNA polymerase is not instantaneous. However, levels in cells that had not been exposed to rifampin were always substantially elevated relative to those in cells cultivated in the presence of this compound (Fig. 3).
those encoding RNA and DNA polymerase, ribosomal proteins, ATP synthase, and components of the electron transport chain (NADH dehydrogenase, bc1 complex, and the cbb3 complex), had similar transcript levels in low- and high-DIC cells (Fig. 4).

When cells were cultivated under low-DIC conditions, transcripts from the gene cluster encoding the carboxysomal shell proteins and enzymes (RubisCO and carbonic anhydrase; Tcr_0838 to Tcr_0848) were more abundant (Fig. 5). Transcripts from two other genes (Tcr_1019 and Tcr_1315, also verified by qRT-PCR) (Table 2) were more abundant under low-DIC conditions and may be novel components of the CCM of *T. crunogena*. Confirming previous results, transcripts from the \( /H9251 \)-CA gene (Tcr_1545) and the noncarboxysomal \( /H9252 \)-CA gene (Tcr_0421) were similar in abundance under both low- and high-DIC conditions (9).

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Under low-NH4⁺ (high-DIC) growth conditions, genes associated with a nitrogen starvation response had increased transcript levels (Fig. 5). This included two Amt family ammonium transporters and the regulatory protein PII, which stimulates transcription of the gene encoding glutamine synthetase as well as posttranslational modification of this enzyme via adenylation/deadenylation (3). Genes encoding two conserved hypothetical proteins, Tcr_0466, and Tcr_2018, were found to have increased transcription under low-NH4⁺ conditions (Fig. 5, confirmed by qRT-PCR; Table 2). Transcription levels of these genes were not enhanced under high-DIC, low-phosphate conditions, consistent with a response to ammonia limitation, not to high-DIC conditions.

FIG 4 Housekeeping genes that do not have a measureable change in transcription under low-versus high-DIC conditions, as determined with microarrays. Shown here listed by subunits are genes involved in DNA, RNA, and protein synthesis, as well as ATP synthesis and electron transport (NADH dehydrogenase, bc₁ complex, cbb complex). Two or three probes were designed to target each gene, and results from all are averaged per gene. Error bars represent standard deviations as propagated from normalized spot intensities from three low-DIC and three high-DIC chemostats.

Taxonomic distribution and phylogenetic analysis of homologs to Tcr_1019 and Tcr_1315. For Tcr_1019, only two genes were returned from BLAST queries of the IMG database (Table 3). The organisms from which these two genes derive are both capable of growing chemolithoautotrophically and phylogenetically disparate: Thioalkalivibrio sp. K90, a member of the Gammaproteobacteria, and Nitrobacter winogradskyi, an alphaproteobacterium. Many apparent orthologs of Tcr_1315 are present in the IMG database and have substantial sequence similarity (Fig. 6). Tcr_1315 falls within a well-supported clade with homologs from other marine proteobacteria.

DISCUSSION

Given the novel nature of a CCM in a member of the Proteobacteria (10), we were interested in determining the levels at which its components are induced in T. crunogena in response to low-DIC conditions. Unlike cyanobacterial DIC uptake, which is regulated both at the level of transcription and at posttranslational modification (26), it appears that DIC uptake by T. crunogena is regulated at the level of transcription, as the RNA polymerase inhibitor rifampin prevented development of the ability to generate elevated intracellular concentrations of DIC upon exposure to low-DIC conditions (Fig. 1). Like that of the betaproteobacterium Halothiobacillus neapolitanus (5), carboxysome synthesis is regulated at the level of transcription (Fig. 2 and 3). Interestingly, it appears that the lag in DIC uptake ability (−1 h) (Fig. 1A) is shorter than the lag in carbon fixation/carboxysome development (−1.5 to 2 h) (Fig. 1B and 2). Given that carboxysome genes and transporter genes are not cotranscribed (DIC transporter genes are not apparent in the carboxysome gene cluster), differences in timing for the development of these two components of the CCM
might be expected. Furthermore, given the size and structural complexity of carboxysomes, it seems feasible that their synthesis and assembly would take more time than insertion of structurally simpler transporter proteins into the cell membrane. However, the observation that csoSCA transcript abundance is high after 30 min suggests that CsoSCA protein should be more abundant as well. If CsoSCA is active before carboxysome assembly, it should be reflected in lower intracellular DIC concentrations, as free cytoplasmic carbonic anhydrase should convert DIC to CO₂, which would diffuse from the cell (25). The high intracellular DIC concentration at 30 min despite elevated levels of csoSCA transcripts is puzzling. Perhaps translation of csoSCA transcripts is delayed or CsoSCA protein is somehow less active until packaged into a carboxysome.

As this is the first genome-wide assay of transcriptional response to DIC and ammonia abundance in an obligate chemolithoautotroph, we anticipated that novel genes/patterns of transcription would be apparent. Indeed, transcripts from two conserved hypothetical genes (*Tcr_1019* and *Tcr_1315*) were more abundant under low-DIC conditions (Fig. 5A; Table 2). The proteins encoded by both genes are predicted to have an amino-terminal signal peptide without downstream transmembrane alpha helices. The protein encoded by *Tcr_1019* is likely to be localized in the periplasm, while the protein encoded by *Tcr_1315* falls within protein family IPR011250 (InterPro), whose members typically have a beta-barrel structure, which indicates that it may be present in the outer membrane.

Intriguingly, orthologs to both genes are present in other chemolithoautotrophs (Table 3; Fig. 6). While *Tcr_1019* homologs are restricted to two other bacteria, *Tcr_1315* homologs are present in many marine bacteria, many of which are also autotrophs. The function of this group of homologous genes in any organism has not been elucidated, and it will be of great interest to determine whether it has a uniform function (e.g., DIC acquisition or more generally facilitating growth at seawater pH and salinity) in all of these organisms. Gene disruption experiments to determine if either protein plays a role in the *T. crunogena* CCM are under way.

Novel genes (*Tcr_0466* and *Tcr_2018*) were discovered in the ammonia-limited (high-DIC) cells as well (Fig. 5B; Table 2). The function of these conserved hypothetical proteins could not be discerned based on their amino acid sequences. However, both these postulated genes have predicted amino-terminal signal peptides. Both also lack downstream transmembrane helices, suggesting a periplasmic location. Genes homologous to *Tcr_0466* and *Tcr_1315* are widespread among the Proteobacteria; this is the first report of differential transcription of these genes and it will be of
interest to see whether these proteins all play a role in nitrogen metabolism among their different host organisms.

These observations of novel gene transcription under DIC- and ammonia-limited conditions are strengthened both by consistency in the transcription of housekeeping genes (Fig. 4) and by transcription patterns consistent with previous work. For example, transcript levels from RubisCO genes varied with the DIC concentration available during growth; the carboxysomal form I had higher transcript levels under low-DIC conditions, while the other two (noncarboxysomal form I RubisCO and form II RubisCO) had increased transcript levels under high-DIC conditions (Fig. 5), which is consistent with what has been observed in the close relative Hydrogenovibrio marinus, a gammaproteobacterial hydrogen-oxidizing autotroph with similar RubisCO operon structure (33). Further, elevated transcript levels of Amt family ammonia transporters were observed in response to ammonium limitation, also consistent with observations of these proteins in other organisms (30).

It is surprising that relatively few genes appeared to have significant changes in transcript abundance under these growth conditions. This is likely the result of both the consistency of the growth conditions in chemostats, where only a single nutrient concentration differs between treatments and growth rates can be regulated very precisely, and the conservative nature of the criteria used for deciding which genes were represented with higher transcript levels. Still, it might be anticipated that more genes would have been highlighted; for example, it seems reasonable to expect that molecular chaperones might be more abundant when cells are grown under low-DIC conditions, due to greatly enhanced carboxysome assembly. The observation that these genes do not appear to respond this way suggests either that carboxysome assembly does not require these proteins or that the basal level present in the cell suffices.

A full understanding of how the CCM works in chemolithoautotrophs will be instrumental in gaining insight into how these organisms succeed in environments like the hydrothermal vents. Furthermore, identifying proteobacterial CCM components in T. crunogena will make it possible to identify them in other microorganisms. Until then, understanding of DIC uptake and fixation in

### TABLE 3 Orthologs of Tcr_1019

<table>
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<tr>
<th>Organism</th>
<th>Locus tag</th>
<th>Length of amino acid sequence</th>
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<th>% similarity of portion aligning to Tcr_1019</th>
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*a* Sequences were collected from the Integrated Microbial Genomes system ([http://img.jgi.doe.gov/cgi-bin/w/main.cgi](http://img.jgi.doe.gov/cgi-bin/w/main.cgi) [21]).

*b* Predicted from the nucleic acid sequence.


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**FIG 6** Phylogenetic analysis of amino acid sequences predicted from genes orthologous to Tcr_1315. The maximum likelihood method was implemented in MEGA5 to create an unrooted tree (32). Bootstrap values of $>70\%$ are displayed above the branches. Taxa are labeled with dots as follows: blue, marine; red, autotroph; purple, marine autotroph; black, methyloautroph or methanotroph.

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the many noncyanobacterial autotrophs that fix carbon dioxide in their challenging habitats is quite limited.

ACKNOWLEDGMENTS

We are deeply thankful to the National Science Foundation for their support of this project (NSF-MCB-0643713 to K.M.S.). We are also thankful to anonymous reviewers for suggestions that substantially improved the quality of the manuscript.

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