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by

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Abstract

Many autotrophic organisms living in environments with episodically low dissolved inorganic carbon (DIC) concentrations compensate for these fluctuations by employing a carbon concentrating mechanism (CCM). By utilizing a CCM, these organisms can generate intracellular DIC concentrations much higher than extracellular, thereby providing sufficient substrate for carbon fixation. Carbon concentrating mechanisms have been well-studied in cyanobacteria but studies are lacking in other autotrophs. The gammaproteobacterium *Thiomicrospira crunogena* XCL-2 is a hydrothermal vent chemolithoautotroph that has a CCM, which is functionally similar to that of cyanobacteria. At hydrothermal vents, DIC concentrations and pH values fluctuate over time, with CO_2 concentrations ranging from 20 μM to greater than 1 mM, therefore having a CCM would provide an advantage when CO_2 availability is very low. The CCM in *T. crunogena* includes α-carboxysomes (intracellular inclusions containing form IA RubisCO and carbonic anhydrase), and also presumably requires at least one active HCO_3^- transporter to generate the elevated intracellular concentrations of DIC previously measured in this organism. In this study, to determine whether RubisCO itself might be adapted to low CO_2 concentrations, the affinity (K_{CO2}) for purified carboxysomal RubisCO was measured, and found to be 250 μM (SD ± 40) which was much greater than that of whole cells (1.03 μm). This finding suggests that the primary adaptation by *T. crunogena* to low-DIC conditions has been to enhance DIC uptake, presumably by energy-dependent membrane transport systems that are either ATP-

dependent and/or dependent on membrane potential ($\Delta\Psi$). To determine the mechanism for active DIC uptake, cells were incubated in the presence of inhibitors targeting ATP synthesis and $\Delta\Psi$. After separate incubations with the ATP synthase inhibitor N, N'-dicyclohexylcarbodiimide (DCCD) and the protonophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP), intracellular ATP was diminished, as was the concentration of intracellular DIC and fixed carbon, despite an absence of an inhibitory effect on $\Delta\Psi$ in the DCCD-incubated cells. In some organisms, DCCD inhibits the NADH dehydrogenase (NDH-1) and bc1 complexes so it was necessary to verify that ATP synthase was the primary target of DCCD in *T. crunogena*. Both electron transport complex activities were assayed in the presence and absence of DCCD and there was no significant difference between inhibited (309.0 μmol/s for NDH-1 and 3.4 μmol/s for bc1) and uninhibited treatments (271.7 μmol/s for NDH-1 and 3.6 μmol/s for bc1). These data support the hypothesis that an ATP-dependent transporter is responsible for HCO_3^- transport in *T. crunogena*; however, the activity of a secondary transporter could not be ruled out. The gene encoding the solute-binding protein (cmpA), of the ATP-dependent bicarbonate transporter in *Synechococcus elongatus* PCC 7942, was used to perform a BLAST query and *Tcr_1153* was the closest match in the *T. crunogena* genome. Examination of the *Tcr_1153* gene neighborhood and the result of a maximum likelihood tree suggest that *Tcr_1153* is a nitrate transporter protein. Bacterial DIC uptake has been very well-studied only in cyanobacteria. Finding the genes that encode the transporter(s) responsible for DIC uptake in *T. crunogena* will aid in metagenomic studies of environmental samples to help determine how widely distributed these genes are, and will help to characterize CCMs in other cultivated organisms.

Chapter 1- Introduction

1.1 Hydrothermal Vent Habitat

Deep-sea hydrothermal vents occur at spreading centers between tectonic plates where superheated fluid rises through the Earth's crust (47). Hydrothermal vent fluid is hot (approximately 350˚C), acidic (pH 3-5), anoxic, and is enriched with reductants including H_2S (3-12 mM; 83). Carbon dioxide, HCO_3^-, and various transition metals are also enriched in the fluid (21, 29, 40).

Highest biomass densities at the vents are found where hydrothermal fluid has been diluted with bottom water before emission from the basalt; accordingly, this diluted hydrothermal fluid is cooler (2-21˚C) and has a chemical composition that is intermediate between hydrothermal vent fluid and bottom water. Dilute hydrothermal fluid generally has pH values ranging from 5.6 to 7.2 (29). Dissolved inorganic carbon ($DIC = CO_2 + HCO_3^- + CO_3^{-2}$) can fluctuate from 2.7 mM to 6.5 mM, while sulfide concentrations can range from undetectable levels to 63 µM (29). Oxygen concentrations have been measured as low as 30 µM to as high as 100 µM, with detectable levels dropping off if the temperature reaches or exceeds 7˚C (39, 54). As dilute vent fluid flows into the cold (1-4˚C), alkaline (approximately pH 8), oxic seawater, turbulent eddies form, causing erratic conditions in which the concentration of the molecules needed to support primary production oscillate broadly over time. Carbon dioxide concentration, for

example, can fluctuate between 20 μM to 1 mM in a matter of seconds to days (29, 40).

1.2 *Thiomicrospira crunogena* XCL-2

Chemolithoautotrophs are primary producers at these deep-sea hydrothermal vents and obtain energy and carbon from inorganic sources. Members of the gammaproteobacterial genus *Thiomicrospira* are chemolithoautotrophs that can be dominant members of microbial communities at hydrothermal vents (8), and are also present in marine Arctic sediments, intertidal mudflats, and hypersaline lake sediments (10, 11, 44, 45, 74, 77). Many *Thiomicrospira* spp. are motile, obligate chemolithoautotrophs, utilizing CO_2 as a carbon source, reduced sulfur compounds as electron donors, and oxygen as a terminal electron acceptor, with optimal growth at microaerophilic conditions. *Hydrogenovibrio marinus* is an exception in that it utilizes hydrogen as an electron donor. Some *Thiomicrospira* spp., including *T. pelophila*, *T. thermophila*, and *T. thyasirae*, are also able to grow heterotrophically. Morphologically, *Thiomicrospira* spp. are both spiral and rod-shaped (9, 10, 11, 12, 44, 45, 55, 74, 77, 90).

Thiomicrospira crunogena XCL-2 (hereafter referred to as *Thiomicrospira crunogena*) is a microaerophilic mesophile that grows well in seawater salt concentrations (16). *T. crunogena* has been isolated from deep-sea hydrothermal vents in both the Atlantic and Pacific Oceans (38). It is one of the fastest growing

chemolithoautotrophs known, which may be due in part to adaptations in DIC uptake and fixation (16).

1.3 Ribulose-1,5-bisphosphate Carboxylase Oxygenase (RubisCO)

RubisCO is present in *T. crunogena* and other microorganisms that use the Calvin-Benson-Bassham (Calvin) cycle. It catalyzes the first step of the Calvin cycle by carboxylating ribulose-1,5-bisphosphate which ultimately results in the production of phosphoglyceraldehyde that can be used for cellular biosynthesis. Two forms of RubisCO (I and II) are associated with the Calvin cycle, while form III RubisCO found in archaea, has been proposed to play a role primarily in purine recycling (24, 25, 26, 36, 64, 88).

Form I RubisCOs are hexadecamers with eight large and eight small subunits encoded by the *cbbL* and *cbbS* genes, respectively (76). Based on amino acid sequences, there are four variations of form I (IA, IB, IC, and ID). Form IA is present in oceanic cyanobacteria and some proteobacteria, while form IB occurs in many freshwater cyanobacteria and green plastids (76). Forms IC and ID are found in proteobacteria and non-green algae, respectively (5, 6). Form II RubisCO holoenzyme consists of two to eight large subunits that are encoded by the *cbbM* genes, which are evolutionarily related to *cbbL* genes, and is found in proteobacteria and some dinoflagellates (76). In addition to structural differences, form I and form II RubisCOs have differences in kinetic parameters as well.

RubisCO can use both CO_2 (Calvin cycle, carbon fixation) and O_2 (photorespiration) as a substrate. Since photorespiration results in a net loss of

fixed carbon, some RubisCOs have been selected for greater CO_2 specificity. Relative specificities for CO_2 versus O_2 are expressed as specificity factors ($\tau = V_C K_O / V_O K_{CO2}$, where V_C and V_O are the maximum velocities of carboxylation and oxygenation, respectively and K_O and K_{CO2} are the Michaelis-Menten constants for O_2 and CO_2; 27). Generally, low specificity factors and high K_{CO2} values indicate that the RubisCO is better adapted for a higher CO_2 and lower O_2 environment while high specificity factors and low K_{CO2} values are found in those organisms adapted for CO_2-limited environments (6). However, organisms that have carbon concentrating mechanisms (CCMs) can have lower specificity factors and higher K_{CO2} values presumably because the CCM compensates for the catalytic limitation of the enzyme by increasing intracellular DIC levels (6, 92).

Form II RubisCO has the lowest specificity factors, ranging from 9 to 15 in organisms such as *Rhodobacter sphaeroides* and *Rhodospirillum rubrum*, which are adapted for low O_2 environments (28, 75, 86). Form I RubisCO specificity factors in proteobacteria, cyanobacteria, and green algae can range from 30-83 (6). Specificity factors can also differ within a form. For example, the specificity range of non-carboxysomal form IA in proteobacteria is 30-53, while carboxysome-associated form IA in cyanobacteria and some proteobacteria has a more narrow range from 33 to 37 (6). Organisms that are well adapted to high O_2 environments have enzymes with much higher specificity factors, such as form IB in plants (78-90) and form ID in non-green algae (129-238; 6).

The affinities of the enzyme for CO_2 also vary greatly between and within forms of RubisCO, which is likely due to the great variation of CO_2

concentrations in different habitats. K_{CO2} values for form II RubisCO, which is generally suited for high CO_2 environments, range from 80 μM to 247 μM (6). Non-carboxysomal form I RubisCOs tend to have higher affinities for CO_2, from 6 to 138 μM (6), than those packaged into carboxysomes (72 μM to 250 μM; Chapter 2, this thesis; 6, 65, 69, 84, 92).

Some organisms have both form I and form II RubisCO and preferentially express one over the other under different growth conditions (79). *R. sphaeroides* has a form I RubisCO that is predominantly expressed under autotrophic growth conditions, while its form II RubisCO is expressed preferentially under heterotrophic growth conditions. The form I RubisCO acts to fix carbon to facilitate autotrophic growth; the form II RubisCO also facilitates flux through the Calvin cycle, but this metabolic pathway acts in this context primarily as an electron sink (79). In contrast, differential RubisCO expression in *Thiobacillus denitrificans* occurs in response to oxygen tension in the growth medium. Expression of form I RubisCO increased under aerobic conditions while form II expression increased under denitrifying conditions, which may be explained by the higher specificity factor ($\tau = 46$) of *T. denitrificans* form I compared to its form II ($\tau = 14$; 7). *H. marinus* alters expression of its three RubisCO genes in response to CO_2 concentrations. It has two form I RubisCO enzymes (*cbbLS-1* and *cbbLS-2*) and one form II RubisCO. One form I RubisCO, *cbbLS-2* ($\tau = 33.1$), is expressed under low CO_2 conditions while *cbbLS-1* ($\tau = 26.6$) and the form II RubisCO ($\tau = 14.8$) are expressed under intermediate and high CO_2 concentrations, respectively (30, 80).

1.4 Carbonic Anhydrase

Due to the rather low affinity of many RubisCOs for CO_2, it is beneficial for an organism that utilizes the Calvin cycle to have an adaptation that creates a CO_2-enriched environment in which RubisCO can function. Carbonic anhydrase, a metalloenzyme that catalyses the interconversion of HCO_3^- and protons to CO_2 and H_2O, as well as the reverse, can assist in elevating CO_2 concentrations around RubisCO.

Several evolutionarily independent forms of carbonic anhydrases are known to exist; forms found in bacteria include α-carbonic anhydrase, β-carbonic anhydrase, and γ-carbonic anhydrase. Many bacterial α-carbonic anhydrases are periplasmic. Many β-carbonic anhydrases, including the one present in *Escherichia coli*, are cytoplasmic (73). In some organisms with carboxysomes, β-carbonic anhydrase has been found in close association with the shell and is encoded by the *ccaA* gene for β-carboxysomes and *csoSCA* in α-carboxysomes (see below; 31, 92). γ-carbonic anhydrase (CcmM) is often cytoplasmic, and is also found within carboxysomes in β-cyanobacteria, such as *Themosynechococcus elongates* BP-1 (60).

1.5 Carboxysomes

Carboxysomes are icosahedral, proteinaceous microcompartments that are 800 to 1500 Å in diameter (92). The shell consists of hexameric subunits that form ~20 Å thick layers with pores 4 to 7 Å in diameter at the vertices, which

may control transport in and out of the compartment (82, 92). Carboxysomes contain tightly packed RubisCO and carbonic anhydrase molecules (4, 5).

There are currently two known types of carboxysomes, α-carboxysomes and β-carboxysomes. α-carboxysomes are present in oceanic cyanobacteria and in proteobacteria. They contain form IA RubisCO (*cbbLS*), and the genes encoding this enzyme as well as the carboxysome shell proteins (*csoS1-4*) and a β-carbonic anhydrase (*csoS3* or *csoSCA)* co-occur in a single operon (4, 5, 92). β-carboxysomes primarily occur in freshwater cyanobacteria and contain form IB RubisCO (*rbcLXS*), which is found in a gene cluster separate from the carboxysome shell protein operon (*ccmKLMNO*). Carbonic anhydrase activity in β-carboxysomes can result from the presence of β-carbonic anhydrase (CcaA), γ-carbonic anhydrase (CcmM), or a combination of both (60).

1.6 Carbon Concentrating Mechanisms

Carbon concentrating mechanisms allow autotrophic organisms to accumulate elevated intracellular DIC concentrations when environmental availability of DIC is very low (16, 41). Cyanobacterial CCMs have been well-studied and include RubisCO, carboxysomes, and bicarbonate transporters (4, 5, 13, 76, 92). Transporters play an important role in CCMs because in their absence, cells would have to rely on diffusion of CO_2 across the cell membrane. Under these circumstances intracellular CO_2 concentrations would be, at best, equal to the extracellular concentration. Given that environmental CO_2 concentrations can be quite low, this would result in low rates of carbon fixation

by RubisCO (48, 59, 61). There are several well-characterized primary and secondary bicarbonate transporters in cyanobacteria that help to elevate intracellular DIC.

Primary transporters couple an exergonic reaction, such as the hydrolysis of ATP, to uptake, such that primary transport does not rely directly on membrane potential ($\Delta\Psi$) or proton potential (Δp; 89). Some well-characterized primary transporters are those in the ABC transporter family, which are widespread among the three domains of life. ABC transporters typically have two highly conserved nucleotide binding subunits that bind and hydrolyze ATP in the cytoplasm (33). They also have less-conserved transmembrane subunits that act as a translocation pathway. The number of transmembrane helices ranges from 10 to 20 (33). Finally, ABC transporters have a solute-binding protein which can be specific or bind multiple solutes (e.g. more than one amino acid) in the periplasm (33). In contrast to primary transporters, secondary transporters utilize the electrochemical gradient generated by other cellular processes, such as the electron transport chain. Secondary transporters couple an energetically unfavorable solute translocation (e.g., HCO_3^-, when $\Delta\Psi$ is negative and intracellular HCO_3^- concentration is high) to energetically favorable solute translocation (e.g., Na^+ import, when $\Delta\Psi$ is negative).

Three primary and secondary transporters are currently known to contribute to CCMs in cyanobacteria. Under severe DIC limitation, the primary, ATP-hydrolyzing ABC transporter BCT1, encoded by *cmpABCD*, is expressed in freshwater cyanobacteria (57). The Na^+-dependent secondary transporter, BicA,

is a member of the SulP family, which has medium to low transport affinity for bicarbonate (61). BicA is inducible in *Synechococcus* sp. PCC 7002, but appears to be constitutively expressed in *Synechocystis* sp. PCC 6803 (22, 62). Another high-affinity, inducible Na^+/HCO_3^- symporter, SbtA, is expressed by cyanobacteria with α- and β-carboxysomes under low-DIC conditions (23, 71).

In addition to primary and secondary transporters, many cyanobacteria have CO_2 hydration complexes that have carbonic anhydrase-like CO_2-hydrating activity. By converting cytoplasmic CO_2 to HCO_3^-, these complexes prevent CO_2 diffusion out of the cell. NDH-I_3 (*ndhF3*, *ndhD3*, and *chpY*) is an inducible, high affinity CO_2 trap found primarily in β-cyanobacteria (63). NDH-I_4 is found in both β and α-cyanobacteria (*ndhF4*, *ndhD4*, and *chpX*) and is a low affinity complex that is constitutively expressed (49, 62, 70). Unlike a canonical carbonic anhydrase enzyme, which is bidirectional, NDH-$I_{3/4}$ couples CO_2 hydration to exergonic electron transport and cannot catalyze the reverse reaction (70).

While cyanobacterial CCMs have been well-characterized, CCMs in other organisms are not well-studied. The CCM of *T. crunogena* is the first non-cyanobacterial CCM to be characterized and has proven to be functionally, but not genetically, similar to those in cyanobacteria (68). Physiological evidence for a CCM in *T. crunogena* includes the ability to utilize both CO_2 and HCO_3^- for carbon fixation, rapid growth at very low-DIC concentrations, the ability to create intracellular DIC concentrations 100-fold higher than extracellular concentrations, and the presence of carboxysomes (16).

Genome data and microarray analysis have clarified some aspects of how *T. crunogena* copes with varying CO_2 availability. Encoded in its genome is a form II RubisCO as well as a non-carboxysomal form I RubisCO, which are transcribed under high-DIC conditions, and are presumably low-affinity with respect to CO_2. It also has a carboxysomal form I RubisCO that is induced at low-DIC conditions (17).

T. crunogena has several carbonic anhydrase genes (17). The α-carbonic anhydrase is likely periplasmic, since it has an amino-terminal signal peptide. Furthermore, its activity is inhibited in intact cells by relatively membrane-impermeable acetazolamide (17). It does not appear to facilitate DIC uptake in *T. crunogena* and its function is currently unknown (17). *T. crunogena* has two β-carbonic anhydrases. One β-carbonic anhydrase is carboxysomal and is encoded by *csoSCA*, which is found in an operon with genes encoding other carboxysome components. CsoSCA appears to facilitate carbon fixation but not DIC uptake (17). The second β-carbonic anhydrase is in an operon with the form II RubisCO, but it is not preferentially transcribed under low-DIC conditions (17). A γ-carbonic anhydrase homolog is also encoded in the *T. crunogena* genome, but its gene is not present in the carboxysome operon and its function is currently unclear. This carbonic anhydrase is evolutionarily distant from other γ-carbonic anhydrases and does not demonstrate carbonic anhydrase activity when expressed heterologously. It could have another catalytic function (17).

T. crunogena has α-carboxysomes whose genes are preferentially transcribed under low CO_2 conditions (18). The carboxysome operon in *T.*

crunogena includes genes encoding carboxysome shell proteins (*csoS1* and *csoS2*), carboxysomal carbonic anhydrase (*csoSCA*), and form IA RubisCO (*cbbL*, *cbbS*).

Although it was expected that genes encoding transporters in *T. crunogena* that are evolutionarily related to those found in cyanobacteria would be present, no orthologs to any of the cyanobacterial transporters are apparent in the genome of *T. crunogena* (68). The *T. crunogena* genome contains ten operons for ABC transporters that include genes for the ATP-hydrolyzing subunit, transmembrane subunit, and a solute-binding subunit. The remaining ATP-dependent transporters appear to be involved in protein secretion, H^+, Na^+, or other cation efflux (68). Fifty-eight secondary transporters representing 28 transporter families, including one SulP family transporter, are present in the *T. crunogena* genome.

The following physiological investigation aims to identify whether DIC uptake in *T. crunogena* is accomplished by primary and/or secondary transporters. *T. crunogena* cells were incubated with a protonophore and an ATP synthase inhibitor to investigate secondary and primary transport, respectively. To determine the degree to which a HCO_3^- transporter may be responsible for whole cell DIC affinity and carbon fixation, carboxysomal RubisCO was purified and its K_{CO2} was found and compared to the previously measured whole-cell affinity. Finally, a phylogenetic analysis of a candidate transporter gene was performed. Completion of this study will shed light on the phylogenetic distribution of CCMs and give insight into the ecophysiology of carbon fixation.

Chapter 2- Materials and Methods

2.1 Reagents

Tritiated water, $NaH^{14}CO_3$, ^{14}C-methylamine hydrochloride and ^{14}C-D-sorbitol were purchased from MP Biomedicals. The metabolic inhibitors carbonyl cyanide m-chlorophenylhydrazone (CCCP) and N, N'-dicyclohexylcarbodiimide (DCCD) were obtained from Sigma. ^{14}C-tetraphenylphosphonium bromide was purchased from American Radiolabeled Chemicals, Inc.

2.2 Carboxysomal RubisCO Purification

T. crunogena cells (8 L) were cultivated in thiosulfate-supplemented artificial seawater (TASW) as previously described (16), harvested and resuspended in 15 ml of TEMB buffer (10 mM TRIS, 1 mM EDTA, 10 mM $MgSO_4$, 20 mM $NaHCO_3^-$) containing 2 mg/ml lysozyme, and 0.2 µM PMSF. Carboxysomes were purified by B. Menon (53; Fig. 1). The cell suspension was inverted several times after addition of 15 ml of B-PER II reagent (Pierce Scientific). The consistency of the cell suspension became mucoid, which is an indication of cell lysis. The cell slurry was then sonicated using a disruption horn (9.5 mm diameter) connected to a Branson model 450 sonifier set at a constant duty cycle and a power output control of 7 for 10 s. A 150 µl portion of a 1 mg/ml bovine pancreatic DNase I solution in TEMB buffer was added. The

sonicated cell lysate was then agitated for 30 min at room temperature on a Reliable Scientific D55 shaker set at approximately 60 cycles per min, and centrifuged at 10,000 x g for 10 min. The resulting cell pellet was resuspended in 30 mL TEMB buffer (Fig. 1), which was then sonicated for four 30 s bursts with 1 min cooling intervals between each burst. A 1% v/v Nonidet P-40 was added to the sonicated lysate for solubilization of membranes. Contents were stirred for 1 h at room temperature. The sonicated lysate was centrifuged at 10,000 x g for 10 min. The resulting supernatant was centrifuged at 48,000 x g for 30 min. The pellet obtained from this high speed spin, also referred to as P_{20K} pellet, was resuspended in 3 ml TEMB buffer and centrifuged at 1000 x g for 3 min. This pre-clearing step yielded a supernatant fraction highly enriched in carboxysomes, which was then loaded onto a 36 ml 10-60% w/v continuous sucrose gradient in TEMB buffer prepared using a gradient former (Bethesda Research Laboratories). The tube was then centrifuged in a JS 28.38 swinging bucket rotor (Beckman) at 104,000 x g for 30 min at 4°C. Typically, carboxysomes migrated as a single milky white band towards the middle of the gradient. This band was harvested and transferred to a 25 x 89 mm polycarbonate centrifuge bottle. After adjusting the volume to ~36 ml with TEMB buffer, the tube was then centrifuged at 150,000 x g for 2 h at 4°C in a Type 70Ti rotor (Beckman). The resulting pellet was resuspended in 1 ml TEMB buffer and stored at 4°C until further use. To purify RubisCO from these carboxysome preparations, they were freeze-thawed multiple times, which disrupts the carboxysome shell and releases the RubisCO.

Ruptured carboxysome shells were removed by centrifugation (~30 min, 4˚C, 14,000 x g).

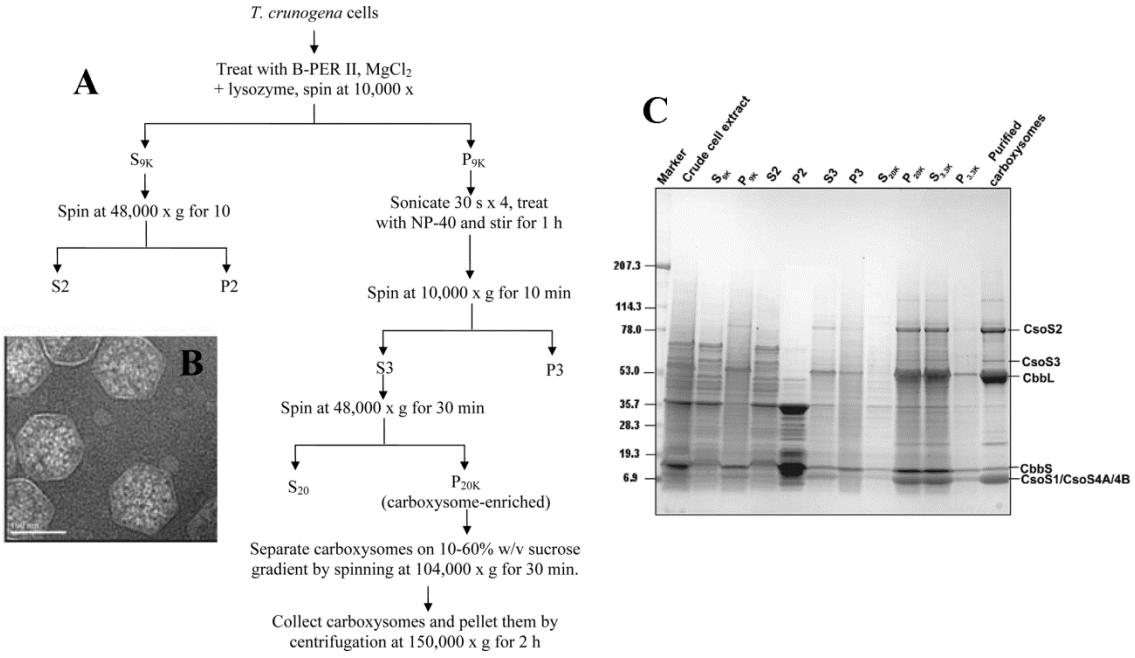

Figure 1. Carboxysomal RubisCO purification. (A) Flowchart for purification of carboxysomes. (B) Transmission electron micrograph of purified carboxysomes. Samples were stained with 1% w/v ammonium molybdate and observed under a transmission electron microscope at 30,000X magnification. The white scale bars represent 100 nm. (C) SDS-PAGE analysis of fractions obtained during the purification of *T. crunogena* carboxysomes. For purified carboxysomes, carboxysome shell proteins (CsoS1, CsoS2, CsoS3, CsoS4A/4B) and RubisCO subunits (CbbL, CbbS) are labeled accordingly. Protocol developed and performed by B. Menon (53).

RubisCO activity was assayed by adding 50 µl of purified RubisCO to vials containing 1 ml of buffer (50 mM Bicine, 25 mM $MgSO_4$; pH 7.5, 0.5 mg carbonic anhydrase, 1 mM dithiothreitol) and increasing concentrations of radiolabeled DIC (specific activity of ~50 mCi $mmol^{-1}$). Aliquots (100 µl) were added to scintillation vials containing 200 µl glacial acetic acid, in one minute intervals for four minutes. Samples were dried by gently sparging them with air, 3 ml of scintillation cocktail was added, and radioactivity was quantified via

scintillation counting. Four independent RubisCO assays were performed using the same RubisCO preparation.

2.3 Silicone Oil Centrifugation: Measuring DIC, Fixed Carbon, ΔΨ, and pH

To measure DIC uptake and fixation by cells grown in the presence of different DIC concentrations, cells were cultivated at room temperature in TASW in chemostats at a dilution rate of 0.1 h^{-1} under DIC limitation (0.1 mM DIC, sparged with O_2 as needed) or NH_3 limitation (50 mM DIC, sparged with 5% v/v CO_2 in O_2, intermittently; 16). A single chemostat was cultivated for each growth condition. Growth medium was supplemented with 0.33 M NaCl so that cells would be dense enough to travel through silicone oil during centrifugation (see below). Approximately 360 ml of the culture was aseptically removed from the chemostat and centrifuged (5,000 x g, 4˚C, 10 min). Thiosulfate was removed by washing the cell pellet three times with thiosulfate-free and DIC-free TASW medium. The cell pellet was resuspended in 4 ml of thiosulfate-free and DIC-free TASW, and bubbled with CO_2-free air for ~15-30 min until the DIC concentration was undetectable by gas chromatography (16).

This cell suspension was divided into three aliquots (Fig. 2): one was incubated with 1 mM of the ATP synthase inhibitor DCCD, the second aliquot was incubated with 10 μM of the protonophore CCCP, and the third portion was without an inhibitor, but was amended with DMSO to 0.1% v/v to act as a solvent control, as both the DCCD and CCCP solutions were prepared in this solvent. Inhibitor concentrations were determined by pilot experimentation on *T.*

crunogena using a range of inhibitor concentrations and time courses. Cell suspensions were incubated on ice for one hour before use in incubations.

Dissolved inorganic carbon uptake and fixation were measured under four conditions: 1) in the absence ('unenergized' cells) and 2) presence of thiosulfate (40 mM; 'energized' cells), as well as 3) 1 mM DCCD (plus thiosulfate) to infer whether DIC uptake was sensitive to intracellular ATP concentrations, and 4) 10 µM CCCP (plus thiosulfate), to determine whether DIC uptake was driven by $\Delta\Psi$. All incubations had a final concentration of 0.1% v/v DMSO to control for the solvent added with the DCCD or CCCP (Fig. 2).

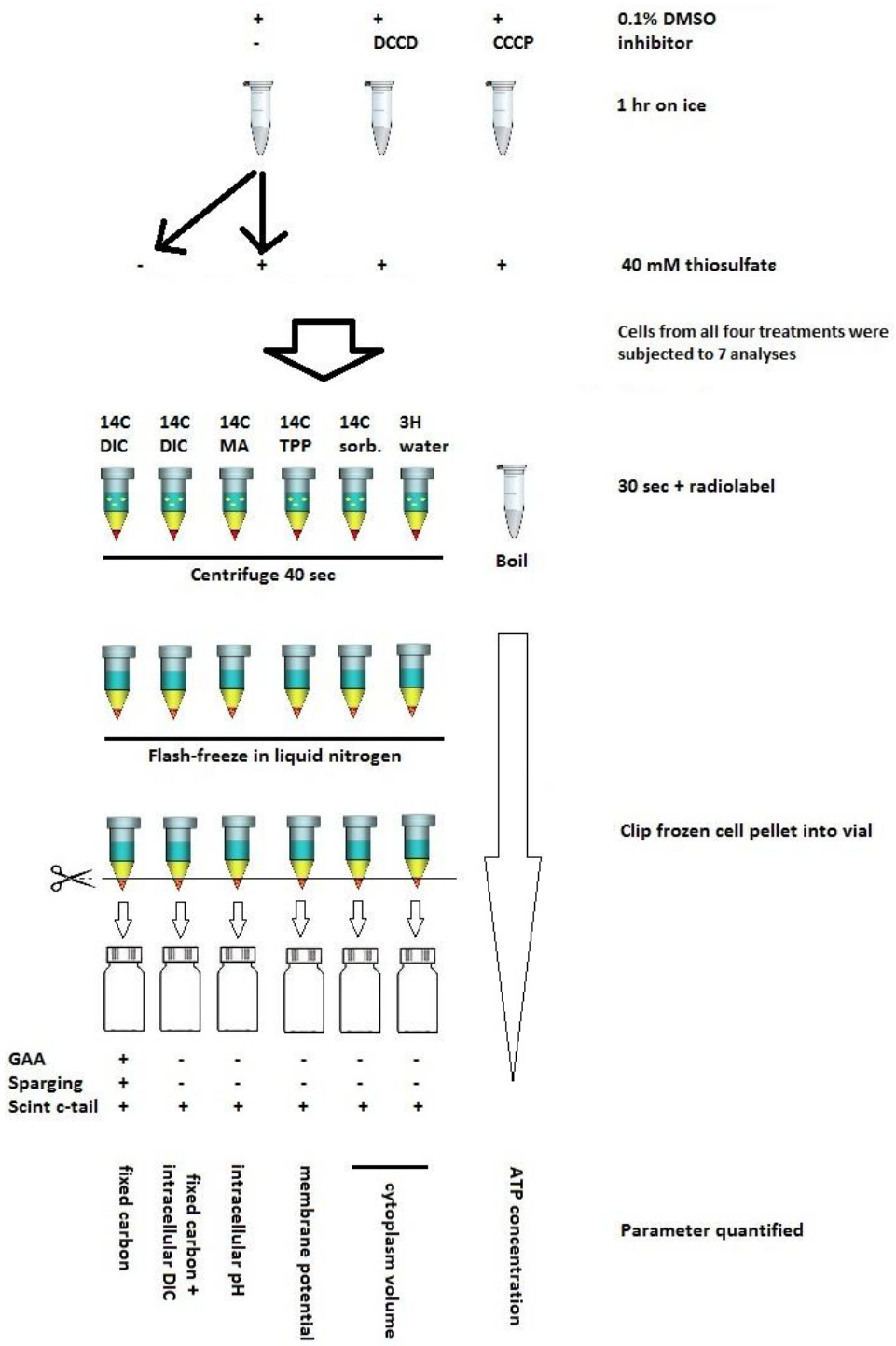

Figure 2. Silicone oil centrifugation. Abbreviations are as follows: CCCP, carbonyl cyanide m-chlorophenylhydrazone; DCCD, N, N'-dicyclohexylcarbodiimide; GAA, glacial acetic acid; MA, ^{14}C-methylamine hydrochloride; sorb., ^{14}C-D-sorbitol; TPP, ^{14}C-tetraphenylphosphonium bromide; 3H water, tritiated water.

To begin an incubation to quantify DIC uptake or fixation, 20 µl of appropriate cell suspension was added to 400 µl of a 200 µM ^{14}C-DIC buffer with a specific activity of ~50 mCi mmol^{-1}, with inhibitors and thiosulfate added as described above. A 200 ul aliquot of each incubation was layered on top of 65 µl of silicone oil (GE SF-1154) which was layered on top of 20 µl of a killing solution [2:1 (vol/vol) 1 M glycine, (pH 10), Triton, colored with 0.1% w/v phenol red]. Thirty seconds after cell suspensions were added to incubation buffer, the tubes were centrifuged for 40 s such that the cells were separated from the media, and traveled through the silicone oil into the killing solution. The tubes were immediately frozen in liquid nitrogen and the bottom layer containing the cells was clipped off into the appropriate scintillation vial. Each physiological parameter (intracellular DIC, fixed carbon, $\Delta\Psi$, and pH) was measured four times.

To measure fixed carbon plus intracellular DIC, cells were clipped into 3 ml of scintillation cocktail plus 50 µl of Scintiverse (Fisher Scientific #SX18-4). Cells were also clipped into 200 µl of glacial acetic acid, vortexed aggressively to dissipate the cell pellet, and sparged with air overnight to remove ^{14}C-DIC to measure fixed carbon only.

$\Delta\Psi$ was measured via silicone oil centrifugation, using tetraphenylphosphonium bromide, which diffuses across the cell membrane and accumulates in the cytoplasm in proportion to the $\Delta\Psi$ (56, 67). Incubation medium was identical as noted above, but ^{14}C-DIC was replaced with ^{12}C-DIC,

and all incubations were amended with ^{14}C-tetraphenylphosphonium bromide to a concentration of 20 mM with a specific activity of 5 mCi mmol^{-1}.

To measure intracellular pH via silicone oil centrifugation, cells were incubated with 2 mM (specific activity 56 mCi mmol^{-1}) ^{14}C-methylamine (16) which accumulates in the cell in proportion to pH. The intracellular pH was calculated from the intracellular versus extracellular concentration of methylamine by using the Henderson-Hasselback equation. To calculate Δp, the measured values of $\Delta\Psi$ and intracellular pH were incorporated into a modified version of the Nernst equation, in which $\Delta p = \Delta\Psi + (RT/F)\ln\{[H]_{in}/[H]_{out}\}$, where $[H]_{out}$ equals 8 as measured by an Accumet AR15 pH meter.

These experiments required that the intracellular volume was known so that the intracellular DIC and ATP concentrations, as well as the pH and $\Delta\Psi$, could be estimated. Incubations with 0.3 mM ^{14}C-sorbitol (289 mCi mmol^{-1}) and 1 mM $^{3}H_2O$ (100 mCi mmol^{-1}) were conducted using silicone oil centrifugation, and $^{3}H_2O$ or ^{14}C-sorbitol carried through the silicone and into the killing solution by intact cells was quantified by scintillation counting. $^{3}H_2O$ permeates both the periplasm and the cytoplasm while ^{14}C-sorbitol permeates only the periplasm; each volume was measured four times. Intracellular space was estimated by subtracting the volume of ^{14}C-sortibol-permiable space from the volume of $^{3}H_2O$-permeable space.

2.4 ATP Quantification

ATP was measured in cells incubated as above for silicone oil centrifugation. A 20 µl portion of each cell suspension was incubated in 400 µl of incubation buffer as described above for measuring DIC uptake and fixation, with ^{14}C-DIC replaced with ^{12}C-DIC. After 30 s the suspension was split into 50 ul aliquots and quenched in Eppendorf tubes at 95˚C for 2 min after which they were cooled on ice to be stored at -80˚C. ATP was quantified four times for each treatment using a commercially available bioluminescence kit (SIGMA #FL-AA) and luminometer (Promega, Glomax 20/20).

2.5 NADH Dehydrogenase (NDH-1) and Bc1 Complex Assays

DCCD has been shown to inhibit electron transport in the NDH-1 and bc1 complexes in some organisms (87, 91) so it was necessary to verify that the primary target for this inhibitor in *T. crunogena* cells was the intracellular ATP pool and not cellular reductant levels (e.g., NAD/NADH or ubiquinone/ubiquinol). Cell membranes were prepared according to Verkhovskaya (72), once for each assay, with modification. Approximately 100 mg of cells were suspended in 5 ml of buffer containing 50 mM HEPES at pH 7, 100 mM KCl, 0.5 mM EDTA, 0.5 mM PMSF. Cells were sonicated until lysed and centrifuged at 10,000 g for 20 min at 4˚C. The supernatant was centrifuged at 35,000 g for 30 min, the resulting pelleted cell membranes were resuspended in 500 µl of buffer containing 25 mM bis-tris-propane at pH 6 and 10 mM betaine, and were flash frozen using liquid nitrogen and stored at -80˚C.

NDH-1 activity was assayed, three times for each treatment, according to Verkhovskaya (72) with the following modified procedure. Thawed membrane suspension (20 µl) was added to 1 ml of buffer containing 25 mM HEPES (pH 7.5), 200 µM NADH, and 3.5 mM NaCN, with and without substrate (50 µM decylubiquinone). To test for inhibition by DCCD, 1 mM DCCD was added to the sample and incubated on ice for one hour. NADH oxidation was followed at 340 nm ($\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$).

Bc1 complex activity was assayed according to a modified version of Rotsaert (66) by the reduction of 50 µM cytochrome c in a buffer containing 50 mM KPO_4 (pH 6), 250 mM sucrose, 0.2 mM EDTA, 0.1% BSA and 50 µM decylubiquinol. Decylubiquinol was synthesized by adding decylubiquinone to a buffer (50 mM KPO_4 pH 6, 250 mM sucrose, 0.2 mM EDTA, 1 mM NaCN and 0.1% BSA) to a concentration of 620 µM (81). Sodium borohydride was added to a concentration of 5.3 mM and incubated for 1 hour until H_2 bubbles stopped forming. Cell membranes were incubated for one hour with and without 1 mM DCCD. Bc1 complex activity was monitored by tracking cytochrome c reduction ($\varepsilon = 21.5\ mM^{-1}\ cm^{-1}$ at 550nm) in the presence and absence of 50 µM decylubiquinol. Cytochrome c reduction was measured three times for each treatment (without substrate, uninhibited with substrate, and with substrate and inhibitor).

2.6 Statistical Analysis

For silicone oil centrifugation, NDH-1, and bc1 complex experiments, treatment groups were compared using a one-way ANOVA in SPSS Statistics 19, with a significance level of $\alpha \leq 0.05$.

2.7 Phylogenetic Analysis

The amino acid sequence predicted from the gene encoding the solute-binding protein from the BCT1 transporter (*cmpA*) in *Synechococcus elongatus* PCC 7942 (*Synpcc7942_1488*) was used to query the *T. crunogena* genome using BLAST (1, 37, 51). One gene (*Tcr_1153*) was retrieved. Other homologs of *cmpA*, including the *S. elongatus* PCC 7942 nitrate transporter solute-binding protein (*Synpcc7942_1239*) were collected from IMG via BLAST. Amino acid sequences were aligned using MUSCLE and GBLOCKS (15, 20). A maximum likelihood tree was created using MEGA 5 with 1,000 bootstrap replicates (78). The candidate operon containing *Tcr_1153*, visualized via the Integrated Microbial Genomes website (37, 51) was scrutinized for genes possibly involved in HCO_3^- uptake.

Chapter 3- Results

3.1 Carboxysomal RubisCO Michaelis-Menten Kinetics

Carboxysomes and carboxysomal RubisCO were successfully purified from *T. crunogena* (Fig. 1). The K_{CO2} of the purified carboxysomal RubisCO was 250 μM (SD ± 40) and the V_{max} was 251 nmol CO_2 min^{-1} mg $protein^{-1}$ (SD ± 22; Fig. 3).

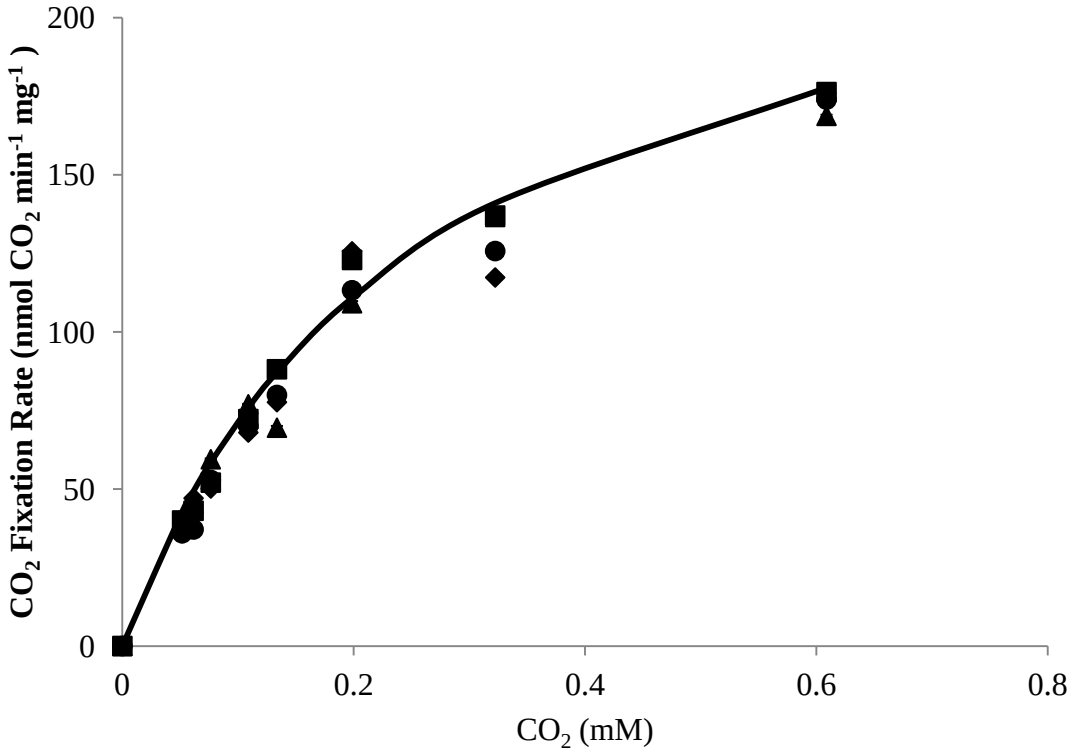

Figure 3. Carbon fixation by carboxysomal RubisCO. Standard error bars for the carbon fixation rates are not visible, as error was small enough to be obscured by the symbol on the graph. The curve is a rectangular hyperbola based on the Michaelis-Menten kinetics constants calculated from the data (K_{CO2} = 250 μM, V_{max} = 251 nmol min^{-1} mg $protein^{-1}$). Each symbol series is an individual trial using the same RubisCO preparation.

3.2 Effect of Metabolic Inhibitors on DIC Uptake and Fixation

For cells grown under DIC limitation, the concentration of intracellular DIC was substantially higher than extracellular (0.12 mM) when thiosulfate was present (Table 1). In the absence of thiosulfate, intracellular DIC levels were lower (Fig. 4A). Inclusion of metabolic inhibitors (DCCD or CCCP) with thiosulfate also resulted in diminished intracellular DIC and fixed carbon (Fig. 4A; $p < 0.05$). As anticipated, incubations with 10 μM CCCP resulted in a diminishment of $\Delta\Psi$, as well as significantly lower ATP concentrations ($p < 0.05$).

Table 1. Physiological parameters for energized DIC- and NH_3-limited cells

	DIC-limited Cells[a]	**NH_3-limited Cells**[a]
Intracellular DIC Pool	10.8 mM ± 0.5	1.0 mM ± 0.1
Carbon Fixed	4.6 mM ± 0.2	0.3 mM ± 0.0
Membrane Potential	-146.3 mV ± 2.1	-159.4 mV ± 0.4
Intracellular pH	6.9 ± 0.1	6.6 ± 0.1
[ATP]	0.4 mM ± 0.0	0.2 mM ± 0.0

[a] All values are for cells incubated with thiosulfate and standard deviation is provided. For all parameters n= 4.

DCCD also diminished the intracellular ATP concentrations. As expected, DCCD did not collapse the $\Delta\Psi$. In fact, $\Delta\Psi$ increased (Fig. 4A), which might be anticipated given the inhibition of the ATP synthase complex.

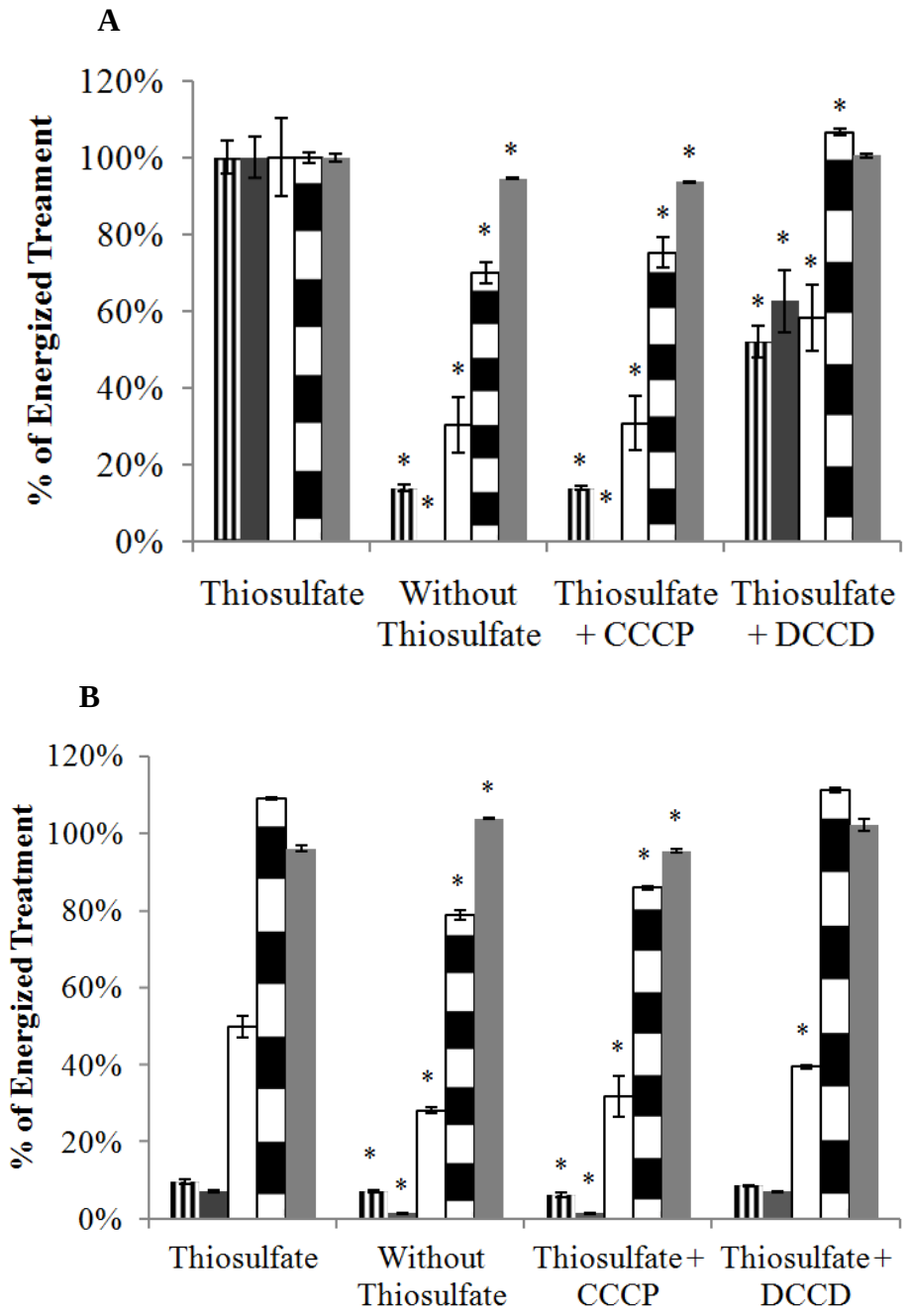

Figure 4. Influence of thiosulfate and inhibitors (10 µM CCCP, 1 mM DCCD) on physiological parameters of *Thiomicrospira crunogena* cells. Parameters include intracellular DIC (▥), fixed carbon (■), ATP (▭), ΔΨ (▤), and pH (▩) under DIC limitation (A) and NH_3 limitation (B). Data are shown as a percent of the values determined for cells incubated in the presence of 40 mM thiosulfate (Table 1). Values that were significantly different ($\alpha = 0.05$) from those cells incubated with 40 mM thiosulfate, and in the absence of inhibitors are labeled with asterisks.

Intracellular DIC concentrations were lower in cells grown under NH_3 limitation (and DIC abundance), even when provided with thiosulfate (Table 1), which is consistent with a CCM being repressed under these growth conditions. The overall impact of the inhibitors on intracellular DIC concentrations was less severe than that which was seen in the DIC-limited cells, likely because the CCM is not induced under high-DIC conditions (Fig. 4B). For cells treated with DCCD, the fixed carbon concentration was nearly identical to that of the energized control group (Fig. 4B).

DCCD did not significantly affect NDH-1 or bc1 complex activity (Table 2), suggesting that its impact on the CCM was not due to interaction with electron transport complexes.

Table 2. NDH-1 and bc1 complex activity in the absence and presence of DCCD.

	NDH-1 Rate (μmol/s)[b]	**Bc1 complex Rate (μmol/s)[b]**
- Substrate[a]	31.2 (n=1)	0.1 ± 0.4 (n=3)
+ Substrate[a]	271.7 ± 48.4 (n=3)	3.6 ± 0.3 (n=3)
+ Substrate[a] + DCCD	309.0 ± 41.4 (n=3)	3.4 ± 0.3 (n=3)

[a] Substrates are 50 μM decylubiquinone or decylubiquinol for NDH-1 and bc1 complexes, repectively.
[b] Rates are ±SD

A BLAST-based query of the *T. crunogena* genome to find a potential ATP-sensitive transporter that could be mediating intracellular DIC accumulation resulted in the identification of a gene (*Tcr_1153)* encoding the solute-binding component of an apparent ABC transporter system. *Tcr_1153* was the best match

to the solute-binding protein from the cyanobacterial BCT1 complex (42% sequence similarity), and is present in a possible operon with the other components of an ABC transporter (permease and ATP-binding proteins; Fig. 5).

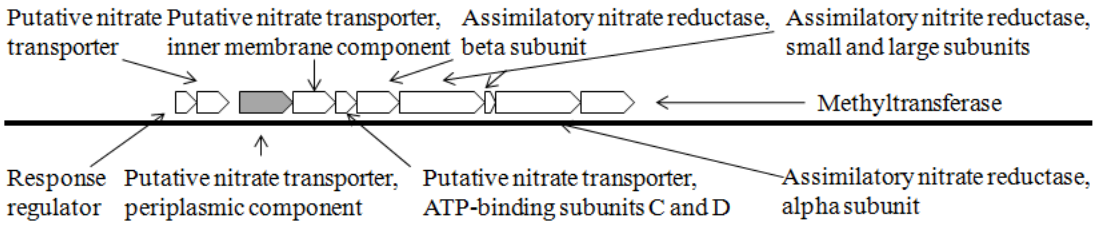

Figure 5. *Thiomicrospira crunogena* putative NO_2/NO_3 transporter gene cluster. The *cmpA* homolog (*Tcr_1153*) is in grey.

Phylogenetic analysis of this gene, however, placed it within a clade that is dominated by other likely nitrate transporter genes and void of any confirmed bicarbonate transporter genes (Fig. 6). Furthermore, the other genes present in the possible operon include nitrate and nitrite reductase (Fig. 5), further evidence that these genes actually encode a nitrate transporter.

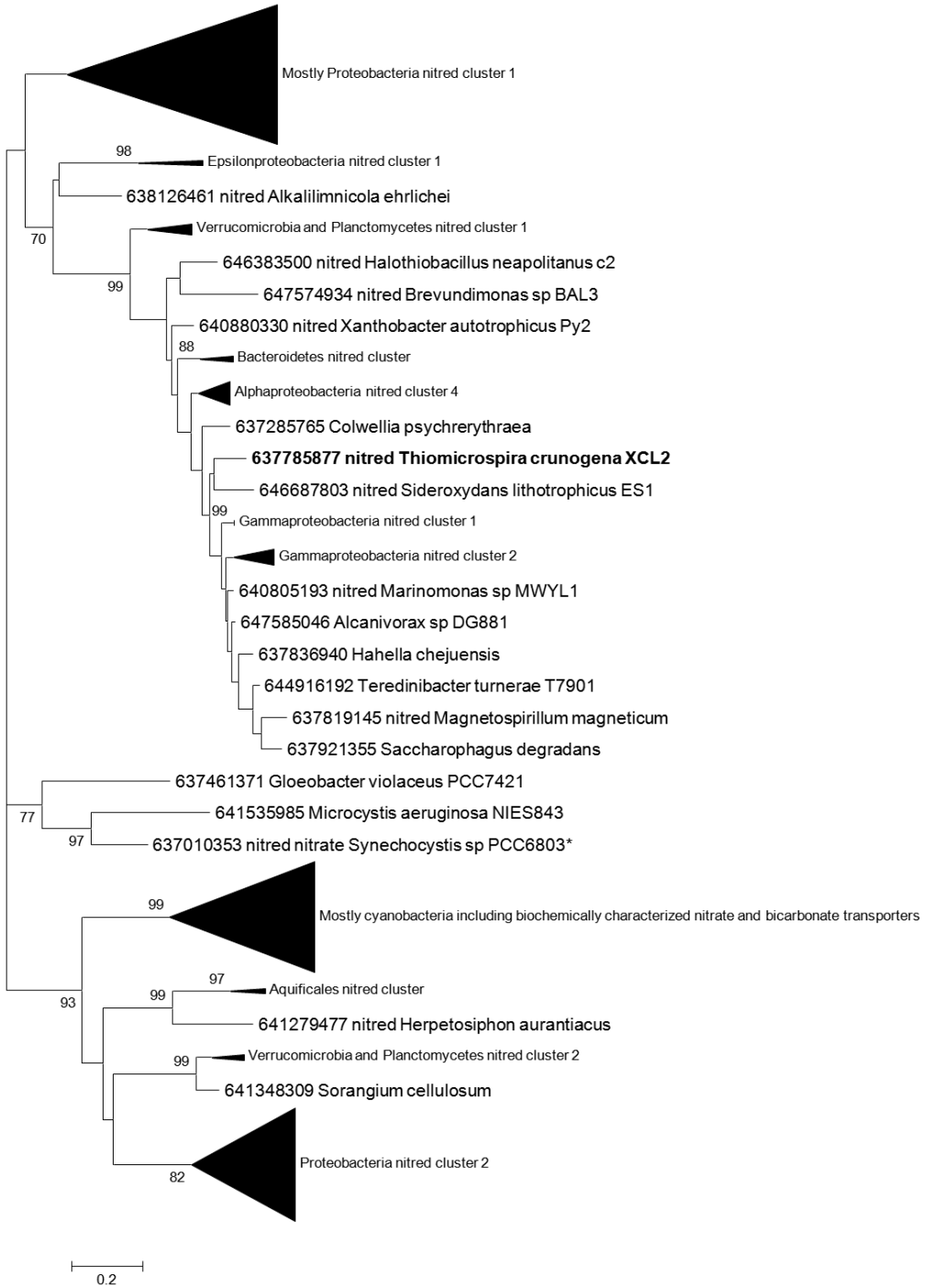

Figure 6. Maximum likelihood analysis of *Tcr_1153*, which encodes a solute-binding protein from an ABC transporter. Genes whose products have been biochemically characterized with respect to substrate specificity are marked with asterisks. 'Nitred' in the taxon title indicates that the gene is present in a likely operon with genes encoding nitrate reductase. Numbers by clades indicate bootstrap values from 1000 resamplings of the alignment.

Chapter 4- Discussion

A comparison of enzyme and whole cell K_{CO2} values is consistent with active DIC transport by *T. crunogena*. The carboxysomal RubisCO K_{CO2} (250 μM) was much higher than the K_{CO2} of whole cells (1.03 μM; 16). The carboxysomal RubisCO K_{CO2} value is rather high for a form I RubisCO, which typically ranges from 22-180 μM in bacteria (6, 32, 34, 35, 46, 84) but is on par with other form I RubisCOs found in organisms with CCMs (173-293 μM; 2, 3, 19, 34 50). A form I RubisCO K_{CO2} has been measured as high as 750 μM in an organism that likely has a CCM (69). In the absence of active uptake, the K_{CO2} for whole cells should be similar to or greater than that of the carboxysomal RubisCO since the intracellular CO_2 will be similar to, or lower than extracellular. Active transport is also supported by the energy-requiring ability of *T. crunogena* to generate intracellular DIC concentrations 100 times greater than extracellular, despite intracellular pH (~7) being lower than extracellular (~8; 16).

Similar to some cyanobacteria, DIC accumulation is coupled to ATP hydrolysis (59). In *T. crunogena*, when intracellular ATP concentrations were diminished in cells grown under low-DIC conditions, either by adding CCCP or DCCD, intracellular DIC concentrations also fell (Fig. 4A), suggesting reliance on an ATP-sensitive transporter for DIC uptake when extracellular DIC concentrations are low. Both intracellular ATP and DIC concentrations, as well as fixed carbon, were less sensitive to DCCD than to CCCP (Fig. 4A). The

Calvin cycle maybe less susceptible to the impact of DCCD than CCCP, since it requires both ATP and NAD(P)H. DCCD only affects intracellular ATP, and not NAD(P)H, while CCCP inhibits both ATP synthase as well as reverse electron transport by collapsing $\Delta\Psi$, resulting in less NAD(P)H and ATP. It is also possible that DCCD may not have completely inhibited ATP synthase; both Na^+ and alkaline pH have been shown to protect ATP synthase from inhibition by DCCD (42, 43, 52, 85, 86). Inactivation of the complex in *Propionigenium modestum* was achieved in 10 seconds between pH 5-7 but, at pH 9, the same level of inactivation was not achieved until 250 seconds (42). In *P. modestum*, 0.5 mM Na^+ protected ATPase inactivation by 50% (43). Indeed, in preliminary experiments with *T. crunogena* in which the NaCl concentration was ~20% of the concentration used for the experiments reported here, the ATP concentration in the DCCD treatment was diminished to less than that seen in the unenergized control group (unpublished data). Unfortunately, particularly for NH_3-limited cells, it was necessary to increase the NaCl concentration in the growth and incubation media so that the cells would be dense enough to travel through the silicone oil used for silicone oil centrifugation.

It is also possible that *T. crunogena* cells have a novel way of synthesizing ATP (aside from ATP synthase) that is not apparent from the genome sequence (e.g. there are no genes encoding the APS system, in which sulfite oxidation is coupled to ADP phosphorylation in a process similar to substrate-level phosphorylation; 68). Additionally, a secondary transporter

(relying on $\Delta\Psi$) may also be involved in DIC uptake and work in tandem with the ATP-sensitive transporter.

Although *T. crunogena* cells cultivated in high-DIC (NH_3-limiting) conditions can also generate somewhat elevated intracellular DIC concentrations, these cells are unable to accumulate intracellular DIC concentrations as high as those present in cells grown under low-DIC conditions (Table 1). Cells grown with an abundance of DIC have a lower affinity for DIC than cells grown in low-DIC conditions (Table 1; 16), which likely reflects diminished CCM activity. DIC transport in NH_3-limited cells does not appear to be ATP-sensitive (Fig. 4B), in contrast to its behavior in DIC-limited cells. In cyanobacteria with CCMs, the ATP-dependent HCO_3^- transporter BCT1 has only been shown to be induced under low-DIC conditions (58). It is possible that, in *T. crunogena* cells, an ATP-sensitive HCO_3^- transporter is also only induced under DIC-limited conditions and may account for the increase in cellular affinity for DIC.

It is possible that a secondary transporter is partially responsible for DIC uptake in NH_3-limited cells. Intracellular DIC concentration was significantly decreased when the $\Delta\Psi$ was collapsed. However, when the $\Delta\Psi$ was collapsed, intracellular pH also decreased (Fig. 4B), which is likely to have turned more intracellular DIC into CO_2, which could diffuse out of the cell. The fixed carbon concentration for the energized control group was very low (Table 1) since these cells lack a CCM, and these incubations are conducted under DIC-limited conditions; it is not surprising that the fixed carbon concentrations are very similar despite the presence of an inhibitor.

It is possible that ATP-dependent DIC uptake in *T. crunogena* is mediated by an ABC transporter, similar in function to the one found in β-cyanobacteria (BCT1, encoded by the *cmpABCD* genes). Ten ABC transporter candidate operons are present in the *T. crunogena* genome, each of which includes a gene for a solute-binding protein, suggesting that they are import ABC transporters, and not exporters (14). Microarray analysis of transcript abundance of these ABC transporter genes in *T. crunogena* did not show an increase in transcript abundance under DIC limitation (18). It is possible that this ATP-sensitive HCO_3^- transporter is post-transcriptionally regulated and, therefore, transcript abundance would not be different between cells grown in high versus low-DIC conditions.

The ABC transporter candidate operon from *T. crunogena* whose components had highest sequence similarity to the genes encoding the cyanobacterial BCT1 transporter was scrutinized further for evidence that it might encode a HCO_3^- transporter. Phylogenetic analysis suggests this gene is a nitrate transporter. It is not surprising that *T. crunogena* appears to be lacking a BCT1 transporter as this ATP-dependent transporter has only been found in β−cyanobacteria and a single α-cyanobacterium (63).

It is interesting to note that reliance on an ATP-sensitive transporter is likely to be a more energetically expensive proposition in this cellular context than utilizing a secondary transporter; it is unfortunate that there are no studies available to confirm this. Genome data are consistent with *T. crunogena* relying on the Sox system to generate transmembrane Δp by oxidizing reduced sulfur compounds (68). The most efficient strategy would be to use a secondary

transporter to directly couple this Δp to bicarbonate uptake. Instead, the energy stored in the transmembrane proton gradient is communicated to the bicarbonate uptake system by using the Δp to power ATP synthase. Introducing an added step of energy conversion (ATP synthase) will result in some energy loss as heat (First Law of Thermodynamics). Having such a system suggests that energy limitation and carbon limitation may not co-occur in *T. crunogena in situ.* In organisms that utilize poor electron donors (e.g. Fe^{+2}, NH_3, reduced sulfur compounds), it would be difficult to sustain an ATP-dependent DIC-transporter as a portion of Δp and ATP must be used to run reverse electron transport for reduction of NAD(P) for biosynthesis (89). Obligate chemolithoautotrophs may need to rely on efficient DIC uptake to overcome the energetic requirement of this type of system. For chemolithoautotrophs living in habitats where their electron donors are in scant quantities, increased rates of electron donor oxidation may also provide the additional energy requirement.

A comparison of the carboxysomal RubisCO K_{CO2} to the whole cell K_{CO2} makes it clear that HCO_3^- transport is essential to the function of the CCM in *T. crunogena* but the identity of the transporter or transporters responsible for HCO_3^- uptake is still unclear, though work is currently underway to identify them. Once found, the DNA sequences of these genes will make it possible to find homologs in other organisms. The phylogenetic distribution of these genes is certain to provide insight into the ecophysiology and evolution of carbon uptake in autotrophic microorganisms across the many phyla and habitats where they are present.

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