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Novel CO_2 transporters from autotrophic bacteria from extreme environments

by

Sarah E. Schmid

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Biology
with a concentration in Environmental and Ecological Microbiology
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ABSTRACT

Autotrophic organisms are responsible for introducing carbon into food webs. These organisms take up dissolved inorganic carbon (DIC) and use it as their major carbon source, converting it to biomass using a variety of carbon fixing pathways (e.g., the Calvin-Benson-Bassham cycle). In nature, DIC concentrations can be low or temporally heterogeneous, so autotrophic organisms adapt with CO_2 concentrating mechanisms (CCMs) to facilitate the acquisition and fixation of this dissolved gas. CCMs consist of membrane-spanning DIC transporters, which elevate intracellular DIC concentrations, which in turn are harvested by carboxysomes, proteinaceous microcompartments that contain the carboxylase Ribulose-1,5-bisphosphate carboxylase-oxygenase (RubisCO) and carbonic anhydrase. CCMs have been well-studied in *Cyanobacteria*, but much remains to be learned about their presence and activity in other bacterial phyla. Indeed, a novel multisubunit DIC transporter (MDT) was recently discovered, and its activity has been preliminarily described in three organisms. It is broadly distributed throughout a diversity of phyla and consists of either one, two or three subunits. Given the breadth of MDT distribution among organisms with diverse physiologies, and its extensive differences in structure, it was important to determine whether DIC uptake was universal among these transporters. To study these transporters, a carbonic anhydrase-deficient strain of *E. coli* Lemo21(DE3)*yadF⁻cynT⁻* that is unable to grow under low-CO_2 conditions was created, and can be rescued via heterologous expression of DIC transporters. We chose seven transporters from the MDT family from four phyla (*Actinobacteria*, *Aquificae*, *Proteobacteria* and *Campylobacterota*), representative of organisms from a variety of habitats (acidic to basic, cold to hot), with all three subunit configurations found among MDT. Expression of all seven transporters rescued the ability of *E. coli* Lemo21(DE3)*yadF⁻cynT⁻* to grow under low

CO_2 conditions. Six of the seven constructs showed increased intracellular DIC. In MDT with multiple subunits, all subunits were necessary to transport DIC. CO_2 appeared to be the source of DIC used by the MDT. In addition, collapsing the proton gradient using carbonyl cyanide m-chlorophenyl hydrazone (CCCP) prevented the accumulation of intracellular DIC, suggesting this family of transporters is using cellular proton potential to drive CO_2 transport. The MDT is the first CO_2 transporter described for bacteria and is confirmed to function as a DIC transporter throughout its broad taxonomic distribution.

CHAPTER 1: INTRODUCTION

Autotrophs are responsible for fixing inorganic carbon, such as bicarbonate (HCO_3^-) or carbon dioxide (CO_2), into organic carbon in ecosystems. The organic carbon synthesized by these organisms forms the base of the food web in many habitats. Conditions in these habitats can be extreme, with pH values ranging from 2 to 12 and temperatures up to 88 °C (1-3). Given these conditions, CO_2 supply from the environment can be an issue and it is likely these organisms have adaptations to overcome a scarcity of inorganic carbon. One adaptation is likely to be active transport of dissolved inorganic carbon (DIC; CO_2 + HCO_3^- + CO_3^{2-}) across the cell membrane, though such transport has not been well-studied in autotrophs outside the *Cyanobacteria*. Recently, Multisubunit DIC Transporters (MDT) were discovered as new transporter family (4). The objective of the work conducted here is to clarify the role of MDT in diverse bacteria.

CHEMOLITHOAUTOTROPHY AND CHEMOLITHOAUTOTROPHIC ECOLOGY

Chemolithoautotrophs use inorganic compounds as a source of electrons, such as sulfur, thiosulfate, ferrous iron and hydrogen, and CO_2 as a carbon source (5). Electrons are fed into the electron transport chain to create a proton gradient, which can be used to fuel essential cellular processes (e.g., ATP formation via ATP synthase). Chemolithoautotrophs thrive in a diverse array of habitats. To provide examples of their diverse ecologies and physiologies, the seven organisms whose MDTs were chosen for study are described in detail here (Table 1). *Acidimicrobium ferrooxidans* is a thermophile from the phylum *Actinobacteria*, isolated from a hot spring where it is subjected to acidic pH ranges and uses ferrous iron as its electron donor (6). The following four bacteria are all from the phylum *Proteobacteria*. *Acidithiobacillus*

ferrooxidans is found in acid mine drainage and is also exposed to low pH ranges (<3), using hydrogen, sulfur or ferrous iron as its electron donor (7). This bacterium is of particular interest because of its importance in bioleaching copper (8). *Acidithiobacillus thiooxidans* is an acidophile found in a wide range of acid habitats including copper mines, coal dumps, and a clay formation, using sulfur as the reducing agent (9). *Hydrogenovibrio crunogenus* is a hydrothermal vent-associated bacterium that oxidizes sulfur and was the original bacterium in which the MDT was discovered (10). *Halothiobacillus neapolitanus* oxidizes sulfur and the type strain was isolated from dissolved concrete (11). It was also the first organism in which carboxysomes were described and has become a model organism for their study (12). *Sulfurovum* sp. AR from the phylum *Campylobacterota* is a sulfur oxidizer that was isolated from marine sediment within the Arctic Circle (13). Lastly, *Thermocrinis ruber* from the phylum *Aquificae* is a hyperthermophile isolated from a hot spring and grows on hydrogen, thiosulfate and elemental sulfur (3).

CO_2 FIXATION

There are six pathways of carbon fixation known thus far, and more are likely to be discovered (14). One of the best characterized is the Calvin Benson Basham (CBB) cycle (5). The CBB cycle is energetically expensive but has the advantage over some of the other autotrophic pathways in that its enzymes are active in the presence of oxygen (15). As a result it is found in *Cyanobacteria* and chloroplasts, in addition to many α, β, and γ *Proteobacteria* and some *Actinobacteria* (16). A key enzyme in the CBB cycle is RubisCO, which carboxylates ribulose 1,5-bisphosphate (RuBP) with CO_2. However, it can also use oxygen as a substrate, resulting in the production of phosphoglycolate, from which the regeneration of RuBP is energetically expensive (17). Another limitation for RubisCO is that it has a low affinity for CO_2 with K_{CO2} values ranging from 30-140 μM in form 1A RubisCO; therefore, high concentrations of CO_2 are needed for rapid rates of carbon fixation to occur (18). Microbes inhabiting an

environment with a variable concentration of CO_2 benefit from a mechanism that aids in raising the concentration of CO_2 inside the cell when the environmental concentrations are low (4).

CARBON CONCENTRATING MECHANISM

Carbon concentrating mechanisms (CCMs) are likely to be prevalent in autotrophs that inhabit environments with low or variable CO_2 concentrations (19). For example, due to the alkaline pH values in marine environments, HCO_3^- is the dominant form of DIC, and CO_2 concentrations are low. To adapt to these conditions, *Cyanobacteria* and other autotrophs utilize a CCM (Figure 1) to increase the efficiency of carbon fixation via active transport of DIC (19). Membrane transporters deposit DIC in the cytoplasm, raising the intracellular concentration of DIC (10, 20). Under DIC limited conditions, some cells are able to generate intracellular concentrations of DIC up to 100 times higher than the external concentrations (10).

Carboxysomes, also critical for CCM function, act to facilitate the fixation of the large pool of intracellular DIC generated by the transporters. These bacterial microcompartments are made up of a proteinaceous shell, containing RubisCO and carbonic anhydrase (CA) (21). The two enzymes act together to facilitate carbon fixation. CA converts some HCO_3^- to CO_2, generating an elevated concentration of CO_2 in the carboxysome. The carboxysome shell keeps CO_2 from diffusing out of the compartment (22). The elevated concentration of CO_2 in the carboxysome results in more rapid rates of carbon fixation by RubisCO (23). The product of the RubisCO reaction, 3-phosphoglycerate, is used by the cell to synthesize biomass (17).

DIC TRANSPORTERS IN *CYANOBACTERIA*

CCMs have been particularly well-characterized in *Cyanobacteria*. HCO_3^- transporters found in the *Cyanobacteria* fall into three evolutionarily distinct, nonhomologous transporter families: BCT1, SulP and SbtA (19). BCT1 is a multi-subunit ATP-binding cassette transporter, while both SulP and SbtA are sodium-bicarbonate symporters (24). These three transporters

differ in their affinity for HCO_3^-, and because of this, organisms often encode more than one transporter and differentially express them when grown in the presence of various concentrations of DIC (24). *Cyanobacteria* also have several modified NADH dehydrogenase (NDH) complexes (CupA/ChpY or CupB/ChpX) that rapidly convert intracellular CO_2 to HCO_3- to prevent CO_2 from leaking out of the cell (16, 24).

DIC TRANSPORTERS IN *GAMMAPROTEOBACTERIA*

Most research on CCMs has been focused on *Cyanobacteria,* but DIC transporters have also been found in the *Gammaproteobacteria* (16). Genomic studies within the *Thiotrichales* have shown a diversity of transporters (25). These transporters include members of the SulP and Sbt transporter families that are only ~25-30% identical in amino acid sequence to those present in members of *Cyanobacteria.* They also include members of the Chr family of transporters, as well as a novel multisubunit DIC transporter (MDT; see below) (25). Many of these transporter genes within the *Thiotrichales* are upregulated when cells are grown under low DIC conditions, suggesting a role in DIC uptake (25). When these transporters are expressed in *E. coli*, they produce elevated intracellular DIC concentrations, confirming their ability to transport DIC (25).

MDT FAMILY OF TRANSPORTERS

The first MDT was described in *H. crunogenus,* a mesophilic hydrothermal vent Gammaproteobacterium. When this organism was subjected to random mutagenesis, strains that had interruptions in either of two genes (*Tcr_0853* or *Tcr_0854*) were unable to grow under low CO_2 conditions. These mutant strains were unable to accumulate elevated concentrations of DIC in their cytoplasm. When wild-type versions of these genes were expressed in CA-deficient *E. coli,* they conferred an ability to generate elevated concentrations of intracellular DIC, indicating their potential role as DIC transporters (4). The *Tcr_0853* subunit belongs to a family

of proteins that includes proton transporters and is predicted to have 13 transmembrane helices, and *Tcr_0854* belongs to a family of proteins of unknown function and is predicted to reside in the cytoplasm (4, 26).

Homologs of *Tcr_0853* and *Tcr_0854* were found in 14 other phyla, including representatives from the *Proteobacteria, Actinobacteria*, and *Firmicutes* among many others (4). MDTs are predicted to have three different subunit compositions, based on genome data. Many are predicted to consist of two subunits, as the homologs to *Tcr_0853* (M subunit; membrane-spanning) and *Tcr_0854* (C subunit; cytoplasmic) are always adjacent to each other on the chromosome. Others include a third short gene (T subunit; tiny) between the M subunit and C subunit homologs and are predicted to consist of three subunits. For some, the M subunit and the C subunit are homologous to the 5' and 3' region of a single gene, suggesting that the transporter in this case consists of a single subunit (4). It has been proposed, though not demonstrated, that the M subunit transports CO_2 into the cell (27). The C subunit is distantly related to β-carbonic anhydrase, and has been proposed to convert CO_2 into HCO_3^- (27).

OBJECTIVES OF THIS STUDY

MDT transporters are widespread and diverse, and it is unclear whether all of them transport DIC. Verifying the role of MDT transporters in DIC transport is the central objective of this project. More specifically, our objectives for this project are to:

1) Verify the function of a range of members of the MDT family as DIC transporters.
2) Biochemically characterize these transporters to determine what form of DIC they transport and what mechanisms of transport are they using.

Transporters from seven species of bacteria were chosen because of their host organisms' wide taxonomic diversity. Species from the phyla *Actinobacteria*, *Aquificae*, *Proteobacteria* and *Campylobacterota* were chosen from a variety of environments with a wide range of pH values, from alkaline-neutral to acidic, and temperatures (Table 1). *H. crunogenus* was chosen as a

positive control for these experiments as heterologous expression of its M subunit and C subunit genes has already proved successful (25). Most of the species utilize the CBB cycle for carbon fixation, except for *Sulfurovum.* sp. AR and *T. ruber* which use the reverse tricarboxylic acid (rTCA) cycle. In addition, among the seven transporters chosen to study, there is at least one of each of the three M, C and T subunit compositions (1 subunit, 2 subunits, and 3 subunits).

Choosing a diversity of transporters allows us to evaluate whether members of the MDT family all share the ability to transport DIC. The possibility of members of the MDT family transporting a variety of substrates is suggested by the observation that most families of transporters include members that transport different substrates (28). So far, the MDT family has only been studied in *H. crunogenus, H. neapolitanus* and *Staphylococcus aureus* (4, 27, 29), where it has been confirmed to transport DIC. However, it is still unknown which form of DIC the MDT is transporting, HCO_3^- or CO_2, though currently all known DIC transporters in Bacteria are HCO_3^- transporters. It is unknown if this transporter will transport DIC in other organisms as well and have a similar mechanism for transport despite the diversity apparent in this transporter family.

Table 1: Taxonomy and habitats of organisms hosting the seven MDT homologs that are the subjects of this study.

Organism	Phylum-Class-Order-Family	Environment	pH	Temperature range
Acidimicrobium ferrooxidans	*Actinobacteria-Acidimicrobia-Acidimicrobiales-Acidimicrobiaceae*	Hot spring	Acidic	Thermophile
Acidithiobacillus ferrooxidans	*Proteobacteria-Acidithiobacillia-Acidithiobacillales-Acidithiobacillaceae*	Acid mine drainage	Acidic	Mesophile
Acidithiobacillus thiooxidans	*Proteobacteria-Acidithiobacillia-Acidithiobacillales-Acidithiobacillaceae*	Clay formation	Acidic	Mesophile
Hydrogenovibrio crunogenus	*Proteobacteria-Gammaproteobacteria-Thiotrichales-Piscirickettsiaceae*	Hydrothermal vent	Neutral, alkaline	Mesophile
Halothiobacillus neapolitanus	*Proteobacteria-Gammaproteobacteria-Chromatiales-Halothiobacillaceae*	Dissolved concrete	Neutral	Mesophile
Sulfurovum sp. AR	*Campylobacterota-Unclassified-Unclassified-Unclassified*	Marine sediments	Neutral, alkaline	Mesophile
Thermocrinis ruber	*Aquificae-Aquificae-Aquificales-Aquificaceae*	Hot spring	Neutral, alkaline	Hyper-thermophile

Table 2: C1 metabolism strategy of host organisms, and subunit compositions of the seven MDT homologs that are the subjects of this study.

Species	C1 metabolism[a]	Subunit composition[b]
Acidimicrobium ferrooxidans	CBB	MC[c]
Acidithiobacillus ferrooxidans	CBB	M, C
Acidithiobacillus thiooxidans	CBB	M, T, C
Hydrogenovibrio crunogenus	CBB	M, C
Halothiobacillus neapolitanus	CBB	M, T, C
Sulfurovum sp. AR	rTCA	M, T, C
Thermocrinis ruber	rTCA	M, TC

[a]CBB, Calvin-Benson-Bassham cycle; rTCA, reverse tricarboxylic acid cycle

[b]M, membrane-spanning subunit; T, tiny subunit; C, cytoplasmic subunit

[c]Transporter exists as a single fused protein

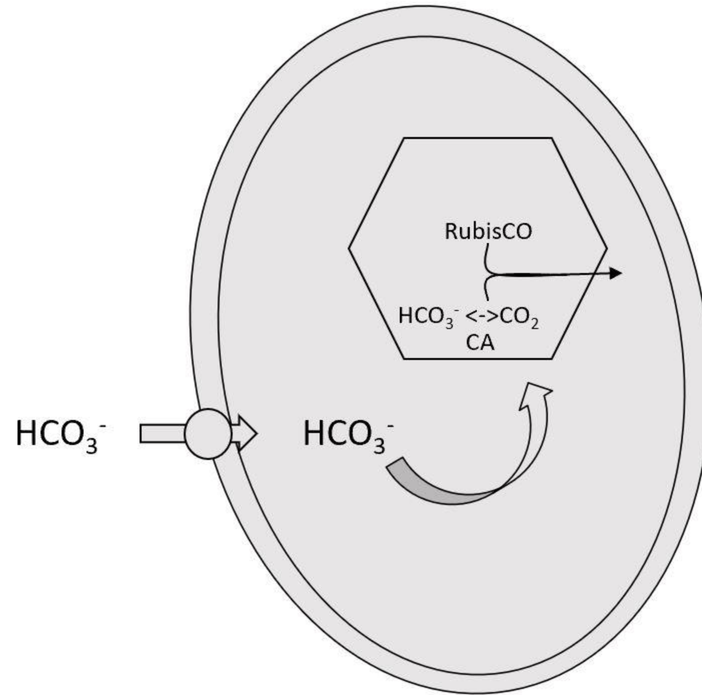

Figure 1: Components of a carbon concentrating mechanism. DIC is transported into the cytoplasm, and from there it enters the carboxysome, where it is converted to CO_2 and is then used as a substrate for RubisCO. Only bicarbonate uptake is shown here for simplicity, but CO_2 can also be taken up directly.

CHAPTER 2: CHARACTERIZATION OF MDT

INTRODUCTION

Chemolithoautotrophs link redox and carbon cycles by fixing dissolved inorganic carbon (DIC; = CO_2 + HCO_3^- + CO_3^{2-}) using reduced inorganic compounds as electron donors. These microorganisms play an essential role in carbon cycling in environments where compounds such as H_2S or H_2 are abundant, including hydrothermal vents (30) or hot springs (31). The type of DIC available depends on the pH of the system, with more CO_2 available at lower pH values, HCO_3^- most prevalent at neutral pH and CO_3^{2-} dominating at alkaline pH. The availability of DIC may also vary spatially and temporally, as it does at hydrothermal vents (32, 33).

One adaptation employed by autotrophs to ensure the cells can fix DIC despite low or variable DIC concentrations is a carbon concentrating mechanism (CCM). CCMs have been studied primarily in *Cyanobacteria* but are also found in *Proteobacteria*, and are likely to be present in a number of other phyla including *Actinobacteria* (34). CCMs are typically composed of two parts: transmembrane transporters that bring DIC into the cell, and carboxysomes, proteinaceous microcompartments that contain RubisCO, the carboxylase of the Calvin-Benson-Bassham cycle, and carbonic anhydrase to interconvert CO_2 and HCO_3^- (19). To date there are four known DIC transporter families found in *Bacteria*: the ABC transporter BCT1, sodium-bicarbonate symporters BicA and SbtA, and the newly discovered Multisubunit DIC Transporters (MDT). Additionally, some *Cyanobacteria* with CCMs use modified NADPH dehydrogenase (NDH) complexes to convert intracellular CO_2 into HCO_3^- to prevent diffusion out of the cell (35).

MDT were originally discovered in the hydrothermal vent Gammaproteobacterium *Hydrogenovibrio crunogenus* and have since been found in 14 phyla (4). Genes encoding MDT are often found near other genes involved in carbon fixation (4). MDT consist of one to three

subunits. One subunit is a transmembrane protein (M subunit; membrane-spanning, Pfam00361), the other is a cytoplasmic protein belonging to a family of unknown function (C subunit; cytoplasmic, Pfam10070) (25). In some organisms, e.g., *Acidimicrobium ferrooxidans* these two subunits are fused together into one protein. In other organisms, a third small subunit (T subunit; tiny) is encoded by a gene that is found between those encoding the M and C subunits. In addition to *H. crunogenus*, a MDT has been described in *Staphyloccocus aureus* where it was predicted to be a sodium bicarbonate cotransporter (29). In *Halothiobacillus neapolitanus,* it has been predicted to couple the uptake of CO_2 and protons (27).

Despite their ubiquity among microorganisms, few MDT have been studied; prior to this work it was unclear how widespread DIC transport was among members of this transporter family, and the actual form of DIC transported (CO_2 or HCO_3^-) had yet to be determined. This study further investigates a diversity of MDT transporters from seven taxonomically diverse bacteria and their mechanism of transport. Included are four *Proteobacteria*: acidophiles *Acidithiobacillus ferrooxidans* (7) and *Acidithiobacillus thiooxidans* (9), and neutrophiles *Hydrogenovibrio crunogenus* (36) and a model organism for carboxysome study *Halothiobacillus neapolitanus* (37, 38). Additionally, transporters from *Sulfurovum* sp AR (neutrophile (39)) from the phylum *Campylobacterota,* hyperthermophile *Thermocrinis ruber* (neutral-alkaline pH (3)) from the phylum *Aquificae,* and a thermophile *Acidimicrobium ferrooxidans* (acidophile (6)) from the phylum *Actinobacteria* were used. Transporters with all three subunit compositions (1 subunit, 2 subunits, and 3 subunits) were represented.

The objective of this study was to confirm whether MDT transporters from a variety of organisms are indeed DIC transporters. Transporters were heterologously expressed in *Escherichia coli* to facilitate their characterization. I investigated whether DIC transport is sensitive to the proton motive force and the form of DIC on which the transporter is acting (CO_2, HCO_3^-).

MATERIALS AND METHODS

Construction of plasmids for expressing DIC transporters

Homologs of the Multisubunit DIC Transporter (MDT) genes from seven taxonomically diverse organisms were inserted into three vectors, each with a different promoter: native (pENTR), T7 (pET28) and araBAD (pBAD202). These constructs were synthesized by the Department of Energy Joint Genome Institute and transformed into *E. coli* TOP10.

Additional JGI constructs were made using these vectors with only the M subunit present to investigate if both parts of the transporter were necessary for DIC transport. An additional construct was made with a deletion of the T subunit in *A. thiooxidans.*

Construction of *E. coli* host strain for expressing DIC transporters

An *E. coli* strain was created to test the ability of the MDT to transport DIC. Like other heterotrophs, *E. coli* requires bicarbonate for essential biological processes, and thus requires a carbonic anhydrase (CA) to convert cytoplasmic CO_2 to HCO_3^-. *E. coli* uses two different forms of CA: YadF, which is expressed when cells are growing slowly and CynT, a paralog of *yadF* that is expressed when growing on cyanate (40). Others had previously constructed *E. coli* EDCM636, in which *yadF* had been deleted, to measure DIC uptake by putative DIC transporters (40). This strain has been used to demonstrate DIC uptake by transporters from the MDT, Chr, Sbt and SulP families (25). Without YadF, the strain relies on the chemical conversion of CO_2 to HCO_3^-, the rate of which depends on the concentration of CO_2. This strain cannot grow under atmospheric CO_2 conditions, and requires a high CO_2 concentration for growth. Since the *cynT* gene is still intact in this strain, pseudorevertants are common, in which *cynT* becomes constitutively expressed, allowing the cells to recover the ability to grow under low CO_2 conditions.

For our experiment, we created a strain in which both carbonic anhydrase genes were deleted in *E. coli* Lemo21(DE3). It carries a plasmid, pLysS, which confers chloramphenicol

resistance, but also encodes a T7 lysozyme to facilitate attenuation of gene expression if toxic (41).

Overlap extension PCR was used to create the *yadF* and *cynT* knockouts in *E. coli* Lemo21(DE3) (42). Two PCR reactions were run to amplify the regions upstream and downstream of the gene to be removed (Tables 3 and 4). The two PCR products were gel-purified. The forward primer of the downstream region has a tail that is complementary to the tail of the reverse primer of the upstream region (Figure 2). A third PCR reaction was used to join the upstream and downstream genes together, and the two PCR products act as primers for this PCR. The product of this reaction, which joined the upstream and downstream regions to each other, no longer contains the target gene. To amplify this PCR product further, another round of PCR was undertaken, in which the 5' primer from upstream, and the 3' primer from the downstream region, were used. Both primers included SpeI sites at their 5' end to facilitate cloning into a vector (see below). PCR products were purified using QIAquick PCR purification kit.

Amplicons in which the target genes (*yadF* or *cynT)* were deleted were cloned into the plasmid pLD55, a suicide vector with replication origin oriR$_{R6K\gamma}$ and ampicillin and tetracycline resistance genes (43) (Figure 3). The pLD55 plasmid and PCR products were digested with the restriction enzyme SpeI (New England Biolabs). PCR products were purified using Phenol:chloroform and chloroform, and then ethanol precipitation. PLD55 was treated with Antarctic phosphatase (New England Biolabs), and purified with QIAquick PCR purification kit (Qiagen). The insert was incorporated into the vector with T4 DNA ligase (New England Biolabs). Two plasmids were constructed: pLD55 in which upstream and downstream regions for *cynT* had been connected (pLD55/ *cynT*$^{-}$) and pLD55 in which regions upstream and downstream from *yadF* had been connected (pLD55/*yadF*$^{-}$).

These plasmids were then transformed into the *pir+ recA-* strain *E. coli* BW20767 to maintain high copy numbers and prevent RecA- mediated recombination into the chromosome

(43). Plasmid pLD55/*cynT*- was mated from *E. coli* BW20767 into *E. coli* Lemo21(DE3) (Figure 4). To prepare for mating, cells were grown to late exponential phase and washed three times with sterile PBS via centrifugation and brought to OD_{600}~20. Washed *E. coli* BW20767 carrying pLD55/*cynT*- and *E. coli* Lemo21(DE3) were mixed 1:1 V:V, 50 µl was dotted onto lysogeny agar (LA) without antibiotics and grown overnight at 37 °C. Patches were streaked to isolation on LA with chloramphenicol (30 mg/L) and ampicillin (100 mg/L). Colonies capable of growing in the presence of ampicillin and chloramphenicol were derived from *E. coli* Lemo21(DE3) that had successfully conjugated with *E. coli* BW20767, and in which the plasmid had integrated into the chromosome via a RecA- mediated single crossover event. To select for double crossovers, five colonies were selected and grown overnight in lysogeny broth (LB) with chloramphenicol and autoclaved chlortetracycline to induce the expression of tetracycline resistance. The overnight culture was spread onto tetracycline sensitive selective (TSS) plates for fusaric acid counterselection and grown overnight at 42 °C (43). Cells expressing the tetracycline resistance gene have less stable cell membranes and are more sensitive to higher temperature and calcium chelation by fusaric acid (44). Cells which have undergone a second crossover event will lose the integrated plasmid, and therefore also the tetracycline and ampicillin resistance genes. These cells will be more resistant to fusaric acid and high temperature. Single colonies were selected and streaked to isolation on another TSS plate for another round of selection. Isolated colonies were grown on LA plates with chloramphenicol only and with both chloramphenicol and ampicillin to check they were no longer resistant to ampicillin, indicating that a second crossover event had removed the integrated plasmid from their chromosome. This strain was screened by PCR to verify that the *cynT* gene had been deleted. The *cynT*- strain was then subjected to a second round of gene deletion, in which it was mated with *E. coli* BW20765 carrying (pLD55/*yadF*-) and subjected to conditions identical to those used to remove the *cynT* gene. The successful deletion of both CA-genes resulted in a requirement of high CO_2 conditions for growth (5% headspace CO_2).

Chemically competent *E. coli* Lemo21(DE3)*yadF⁻cynT⁻*

E. coli Lemo21(DE3)*yadF⁻cynT⁻* was made chemically competent using the $CaCl_2$ method, which has proven to be more effective than $MgCl_2$-$CaCl_2$ or DMSO methods for *E. coli* BL21(DE3)-PLysS (45). Preparation of competent cells was as per (46) with the following modifications. Bacteria were pelleted from 200 ml LB supplemented with chloramphenicol (30 mg/L) under a 5% CO_2 headspace. The culture was resuspended in 12 ml 0.1 mM $CaCl_2$ then incubated on ice for 30 minutes. Cells were harvested via centrifugation (3000 rpm, 10 minutes). The pellets were resuspended in 3.2 ml 1 M $CaCl_2$ with 15% glycerol (v/v) and stored at -80 °C.

Expressing transporter genes in *E. coli* Lemo21(DE3)*yadF⁻cynT⁻*

Plasmids carrying transporter genes (see Construction of plasmids for expressing DIC transporters, above) were purified from *E. coli* Top10 using the QIAprep spin mini kit (Qiagen) and introduced into the chemically competent *E. coli* Lemo21(DE3)*yadF⁻cynT⁻* cells described above. Transformations were conducted as described in (46) with the following modifications. Mixed DNA and competent cells were stored on ice for 15 minutes. A recovery period of one hour at 37 °C was used. Each reaction was plated on solid LA supplemented with the appropriate antibiotic (depending on the vector; 25 mg/L kanamycin for pENTR vectors, 25 mg/L kanamycin for pET28 vectors, and 30 mg/L apramycin for pBAD202 vectors) and incubated for 24 hours at 30 °C in a high CO_2 incubation chamber. PCR assays (Tables 3 and 4) were used to confirm that the pLD55 plasmids containing the C and M subunits were successfully carried by the *E. coli* Lemo21(DE3)yadF⁻cynT⁻ cells after transformation.

Screening of cells expressing transporters for the ability to grow under low-CO_2 conditions

As an initial screen to determine whether expression of putative DIC transporters stimulated growth under low DIC conditions, strains were propagated on solid lysogeny

medium: low CO_2 (ambient CO_2, ~400 ppm) and high CO_2 (5% CO_2). For transporters whose expression was driven by the T7 promoter, IPTG (1 mM) was added to the solid medium to induce expression, while media for transporters whose expression was driven by araBAD was supplemented with arabinose (6 mM) to induce expression. Once growth was confirmed on solid medium, cells were cultivated in liquid medium and growth monitored via OD_{600} to measure the effects of DIC, inducer (1 mM IPTG or 6 mM arabinose; see above), or repressor (2 mM rhamnose for pET28, 0.2% glucose for pBAD202). Constructs showing control over regulation of MDT using either the pBAD202 or pET28 vectors were chosen for each of the seven organisms.

Quantification and biochemical characterization

To quantify and characterize DIC uptake by these transporters, we used a silicone oil centrifugation technique as previously described (10). Cells were grown in LB under a 5% CO_2 atmosphere with the appropriate antibiotic and inducers. Eppendorf tubes were prepared with 20 µl dense killing solution (2:1 [vol/vol] 1 M glycine, pH 10, Triton) overlain with 65 µl of medium-density silicone oil, and a top layer of 200 µl of HEPES-buffered LB (50 mM, pH 8) supplemented with $DI^{14}C$ (0.2 mM). Cells were added to the top layer, incubated for 30 seconds, pelleted for 30 seconds (14000 x g), flash-frozen with liquid nitrogen, and clipped into a scintillation vial (10, 25). Scintillation cocktail was added to the vials and radioactivity read with a scintillation counter.

Complete transporters from three bacteria, *Am. ferrooxidans, H. crunogenus* and *Sulfurovum* sp. AR, were chosen for further investigation. These provide a subsample of each subunit composition type (1-subunit, 2-subunit, and 3-subunit) and a diversity of phyla (*Actinobacteria*, *Proteobacteria*, and *Campylobacterota*) and habitat types (neutral and alkaline). These transporters were tested to determine if the MDT transports CO_2 or HCO_3^- by performing isotopic disequilibrium experiments as described in (10). This relies on the slowness of

interconversion of CO_2 and HCO_3^- in the absence of CA (47). To prepare for isotopic disequilibrium experiments, a solution of $^{14}CO_2$ was prepared by adding DI^{14}C stock solution to 1 mM H_3PO_4 in a sealed serum vial and allowing it to equilibrate for 5 minutes. To measure CO_2 uptake, cells were suspended in 200 μl of 50 mM HEPES-buffered LB (pH 8) and layered on top of silicone oil and killing solution as described above. Ten μl of $^{14}CO_2$ solution was added, and after a 20 second incubation, cells were centrifuged into the killing solution and processed as described above. To measure $H^{14}CO_3^-$ uptake, the incubation solution also included bovine carbonic anhydrase (0.1 mg/ml) which instantaneously converts ~99% of the CO_2 to HCO_3^- and CO_3^{2-}, assuming a pKa of 6.1, and an incubation pH of 8. Intracellular DIC was measured via silicone oil centrifugation as described above.

To investigate the mechanism of DIC uptake, the proton potential was collapsed using the protonopore carbonyl cyanide-m-chlorophenylhydrazone (CCCP) as in (4). CCCP solutions were prepared in DMSO (100 mM). Cells were incubated for 2 minutes in the presence of 0.1% DMSO (solvent control) or 0.1 mM CCCP dissolved in DMSO before intracellular DIC concentrations were measured as described above. To verify that the CCCP affected proton potential in these cells, the effect of this inhibitor was also measured on intracellular ATP with the ATP Bioluminescent assay kit (Sigma). Effects on the proton potential were also verified by measuring intracellular pH using ^{14}C-methylamine, an ammonia derivative that accumulates in the cytoplasm to a level proportional to pH (48).

Verification of gene expression in constructs lacking the ability to accumulate intracellular DIC

Proteomic analysis was used to verify that proteins were successfully expressed in constructs without an apparent ability to accumulate DIC. Cells were harvested from 20 ml cultures by centrifugation for 5 minutes at 10,000 rpm. Cells were then washed with 20 mM

$MgCl_2$, and pellets were stored at -80 °C. Peptides were processed according to (4) with the following modifications: a 120-minute gradient was used to separate peptides and data files were processed using MaxQuant, version 1.6.17.0 (www.maxquant.org) including predicted M subunit homolog or T subunit sequences.

RESULTS

Growth of *E. coli* constructs expressing putative DIC transporters

E. coli carrying plasmids encoding complete transporters were able to grow under low CO_2 conditions. When driven by native promoters, expression of these transporters facilitated growth on solid media under low CO_2 conditions (Figure 5). Since we anticipated that expression from native promoters would be low, we also monitored growth when gene expression was controlled by stronger promoters (T7 and araBAD). When cells with gene expression under control of promoters were cultivated in liquid media, response to repressors and inducers varied among the transporters, potentially due to toxic effects of the higher levels of expression. Four of the transporters expressed well when in pBAD202 vectors: the presence of arabinose (inducer) stimulated growth (Figure 6), while glucose (repressor) inhibited it. For the remaining three transporters, expression in pET vectors was successful: growth was stimulated by the presence of IPTG (inducer) and inhibited by rhamnose (repressor). Constructs with either the C subunit or the T subunit deleted were unable to grow under low CO_2 conditions, regardless of the inclusion of an inducer (Figure 7). These constructs were only able to grow when provided with a high CO_2 environment. Mass spectrometry of cell proteins confirmed the expression of these genes (Table 5).

Intracellular DIC concentrations in *E. coli* expressing putative DIC transporters

Six of the seven constructs were able to generate measurably elevated intracellular DIC concentrations when cultivated in the presence of inducer to stimulate expression of DIC transporter genes (Figure 8). Constructs grown in the presence of repressor, as well as

constructs expressing incomplete transporters, were unable to generate elevated intracellular DIC concentrations.

A two-way ANOVA was run to determine factors resulting in statistically distinguishable concentrations of intracellular DIC. For each transporter, intracellular DIC concentrations measured for each of the following four samples were compared: complete transporter with inducer, complete transporter with repressor, incomplete transporter with inducer, and incomplete transporter with repressor. Only two samples (complete transporter with inducer, complete transporter with repressor) were compared for constructs from *Am. ferrooxidans* because this transporter consists of a single subunit (Table 6). Bonferroni post-hoc tests were used to determine which factors resulted in statistically distinguishable differences in intracellular DIC concentrations. When constructs with the complete transporter were grown with inducer, intracellular DIC was higher than those grown with repressor in all but the *T. ruber* construct ($p = 1.0$). Constructs that contained only the M subunit or were missing the T subunit did not have appreciably elevated intracellular DIC concentrations.

Specificity of transporters: HCO_3^- or CO_2

Three transporters representing one, two, and three subunit forms of MDT, were chosen for further experiments. When cells were provided with CO_2, intracellular DIC concentrations were higher than when provided with HCO_3^- (Figure 9). A two-way ANOVA was used to compare DIC species (CO_2 and HCO_3^-) and media (inducer or repressor). As above, Bonferroni post-hoc tests were used to determine which variables resulted in statistically distinguishable differences in intracellular DIC concentrations. For all three transporters, intracellular DIC concentrations were significantly higher when their expression was induced, and the cells were provided with CO_2 instead of HCO_3^- (Table 7). There was no significant difference between the intracellular DIC concentration of cells provided with HCO_3^- in the presence of inducer and the presence of repressor in *H. crunogenus* and *Sulfurovum* sp. AR. Cells grown in the presence of

inducer do have measurable accumulation of DIC when HCO_3^- is provided, though this amount is far less than when CO_2 is provided (Figure 9). This may be due to low amounts of CO_2 present in incubations in which HCO_3^- was provided as the dominant form of DIC.

Use of proton potential for DIC transport

For cells expressing the three transporters selected for further study, the addition of protonopore CCCP diminished intracellular DIC concentrations (Figure 10). The difference between intracellular DIC concentrations in cells provided with CCCP (dissolved in DMSO) and those supplied with DMSO only (solvent control) was significant (t-test $\alpha = 0.05$) in *Am. ferrooxidans* ($p = 7.15e\text{-}6$) and *H.crunogenus* ($p = 4.90e\text{-}5$), but not in *Sulfurovum* sp. *AR* ($p = 0.07$).

The effect of CCCP on the intracellular DIC concentration was likely due to its effect on the proton motive force. Cells incubated with CCCP had lower intracellular pH values (Table 8; $p = 2.6e\text{-}8$, ANOVA and Bonferroni tests as described above). Furthermore, cells treated with CCCP also had lower intracellular ATP concentrations compared to the control medium (Table 8; $p = 2.0e\text{-}11$), as expected if ATP synthase was inhibited by a diminishment in proton motive force. Interestingly, DMSO itself also affected intracellular ATP concentrations when compared with control medium (Table 8; $p = 1.2e\text{-}11$), though less so than when CCCP was also present. This may explain why intracellular DIC concentrations are lower for our solvent control experiments (Figure 10) than when DMSO was absent (Figure 8).

DISCUSSION

MDT have been identified in 14 phyla (4), and the results here suggest that they all act as DIC transporters. Here, we demonstrate that MDT with all three subunit configurations, gathered from members of phyla *Actinobacteria*, *Proteobacteria*, *Camplylobacterota*, and *Aquificae* inhabiting diverse habitats (e.g. hot springs, acid mine drainage, marine sediments)

with a broad range of pH values (acidic to basic), all transport DIC. The organisms hosting the seven transporters chosen here grow autotrophically via the Calvin-Benson-Bassham (CBB) cycle or reverse tricarboxylic acid (rTCA) cycle, suggesting that these transporters are compatible with a variety of autotrophic metabolisms. Furthermore, MDTs are found in heterotrophic organisms as well, suggesting that they contribute to cellular growth in general (29).

Despite their origins in phylogenetically diverse organisms from very different habitats, six of the seven transporters chosen for study here could be successfully expressed in *E. coli.* Unsurprisingly, their 'preferred' promoters varied; different promoters resulted in different growth yields (four were higher with the araBAD promoter, three were higher with T7). This difference may be due to the effect of overproduction of the transporter protein, as has been documented in membrane proteins using pET vectors with IPTG as an inducer (49). In order for any of these transporters to rescue *E. coli* Lemo21(DE3)*yadF*$^{-}$*cynT*$^{-}$, it is necessary for it to be able to generate a high enough intracellular DIC concentration to support metabolism. However, accumulating too much intracellular DIC might affect intracellular pH or have other physiological effects. Too much transporter expression could also overtax the Sec system (responsible for placing integral membrane proteins into the cell membrane) and inhibit the translocation of other membrane proteins necessary for cellular function. Protein expression is a balancing act and the actual level of expression that facilitates growth without inhibiting growth is going to vary from transporter to transporter.

With respect to the *T. ruber* MDT, it is not clear whether expression was successful. Expression behind a native promoter rescued CO_2-sensitive *E. coli,* and growth was robust when controlled by a T7 promoter. However, intracellular DIC concentrations were not measurably higher when this transporter was expressed, and presence of the MDT could not be confirmed via mass spectrometry. It is possible that this transporter was expressed too weakly to provide a signal for the proteomic analysis and DIC measurement, while still providing the

small amounts of DIC uptake necessary to sustain growth by *E. coli.* Another factor could be related to the host organism's habitat; *T. ruber* was the only hyperthermophile in this study. It is likely that the temperature used for expression (37 °C) and measuring DIC uptake (21 °C) were not optimal for its activity.

For MDTs comprised of two or three subunits, all subunits are needed for DIC transporting activity. The M subunit, though predicted to have multiple membrane-spanning alpha helices, cannot act as a permease by itself. Nor, for three-subunit MDTs, can M and C subunits transport DIC without the T subunit; expression of the M subunit and the C subunit without the T subunit from *A. thiooxidans* also failed to accumulate intracellular DIC. The T subunit is also found in *H. neapolitanus* and *Sulfurovum* sp. AR and all have predicted transmembrane helices, suggesting it may be involved in transport across the membrane. Alternatively, it may play a regulatory role like that of the Sbt transporter regulatory subunit, SbtB. The SbtB subunit acts as like PII regulatory protein, modulating HCO_3^- transport by adenylylation (34, 50).

It is quite surprising that MDTs use CO_2 as a substrate, and not HCO_3^- . All the transporters associated thus far with bacterial CCMs (SbtA, SulP, and BCT1) transport HCO_3^-. The other CO_2-active membrane proteins present in bacterial CCMs, Cup systems in *Cyanobacteria*, do not transport CO_2, but act to convert intracellular CO_2 to HCO_3^- to prevent it from leaking from the cell (24, 35). This makes MDT unique among DIC transporters currently characterized in *Bacteria*. Though it seems counterintuitive that a CO_2 transporter would facilitate CO_2 entry into a cell, since CO_2 can diffuse through cell membranes, CO_2 entry and capture when these transporters are expressed is much higher than in their absence (Figure 8). CO_2 transport seems a problematic mechanism for DIC accumulation in the cytoplasm since CO_2 can also diffuse back out of the cell. To overcome this, the cytoplasmic subunit might act as a carbonic anhydrase and convert CO_2 to HCO_3^- (27). MDTs are likely to transport CO_2 and trap it in the cytoplasm as HCO_3^-, resulting in elevated intracellular DIC concentrations.

DIC uptake by MDT appears to rely on proton motive force. The M subunit belongs in a protein family that includes subunits from other complexes that act to transport protons. One of these is the NADH dehydrogenase complex (including NuoL, ND5 and NdhF) which oxidizes NADH while contributing to cellular proton potential via proton expulsion. The other homologous subunit belongs to Multiple resistance and pH (Mrp)-type Na+/H+ antiporters (4). This antiporter contains either six or seven transmembrane subunits that function to maintain intracellular pH and sodium homeostasis (51).

This sub-sample of seven MDTs indicates that other members of this transporter family throughout the phylogenetic tree likely serve the same purpose for the autotrophic organisms in which they are found. MDTs are also encoded in the genomes of many heterotrophic organisms and have been demonstrated to transport DIC in *S. aureus* (29). DIC accumulation by heterotrophs likely stimulates the activity of metabolic processes that use DIC as a substrate, including purine and pyrimidine synthesis, as well as oxaloacetate replenishment (40). Thus, MDTs, while first uncovered in a deep-sea sulfur chemolithoautotroph, are widely distributed transporters for a metabolite (DIC) of near-universal importance.

Table 3: Primer sequences.

Purpose	F	R
Amplifying region upstream of *yadF*	GAG AAC TAG TTT GCG TTT TCC CCA TAG ATC GAG TTG TTT AAG ATA T	GCA TCC GGC ATG GCA TTT GGA GGT TAA CGA CCT GTA ACC
Amplifying region downstream of *yadF*	AAA TGC CAT GCC GGA TGC AAC A	GAG AAC TAG TTG AGC GTT ACA AAG ACA GTG GC
Amplifying region upstream of *cynT*	GAG AAC TAG TTT CTC CTG CGA CAT TTC CTG TAG CTG	TGG AAC TCC TGA TGG TTT AAA AAT AAG GCG TTA ACC TCT GTC TGT CTC TG
Amplifying region downstream of *cynT*	CCT TAT TTT TAA ACC ATC AGG AGT TCC A	GAG AAC TA TCA GAA CGG TTT GTT CGG CAG ATA TTT ACC
Purification PCR *yadF*	GAG AAC TAG TTT GCG TTT TCC CCA TAG ATC GAG TTG TTT AAG ATA T	GAG AAC TAG TTG AGC GTT ACA AAG ACA GTG GC
Purification PCR *cynT*	GAG AAC TAG TTT CTC CTG CGA CAT TTC CTG TAG CTG	GAG AAC TA TCA GAA CGG TTT GTT CGG CAG ATA TTT ACC
Verify presence of OE PCR product in pLD55	ATG ACC ATG ATT ACG CCA AGC	CCC CGA TTT AGA GCT TGA CG
Verify presence of plasmids carrying DIC transporters (complete constructs)	Am. fer: TTC GCA TAG CGG AAG TGT ATT	Am. fer: GAA GGC ACC CGT GTA GAT G
	At. fer: CGA CGA TCC AGA AGC TCA ATA C	At. fer: AAA GTA CGC CGC GCA ATA
	A. thio: CGG GCG TTG GCA TTA TTT AC	A. thio: GGG TTT CGT CGT CTT GAT CT
	H. cru: GAT GGT TGT GGG CTT GAT TTG	H. cru: TCG CCA ACC ACT CAT GAT AAA
	H. nea: GGC GCA TAG CTC CAT CTT AT	H. nea: GAG TCA GTT CGC GGG TAA TC
	S. AR: TTG ATC ACC CTT GCA CTC TAT C	S. AR: AAT CCT GCC CAA CCA TGA A
	T. rub: TGC CTA TCT ACT CCC TTC TGT	T. rub: GGG TTA TGT ATC CCT CCC AAA G
Verify presence of plasmids encoding incomplete DIC transporters	At. fer: ATGATTACATCCTCTCTTCTTATGTTGGT	At. fer: GTA CGG CGA TAC TGT ACA AAC C
	A. thio (M subunit): ATG GGT AAT TGG GCA ATT GCA	A. thio (M subnit): AAC CGT GGG CGA TCA G
	A. thio (T subunit deletion): ATG GGT AAT TGG GCA ATT GCA	A. thio (T subunit deletion): AAC CGT GGG CGA TCA G
	H. cru: ATG AAT ATG CAA TGG GTA GGG G	H. cru: TTA AGA AGG CAT AAG CTT TGT AAC AAG AG

Table 3: Primer sequences (Continued).

	H. nea: ATG ATG AAC CTG CAA TGG TTA ATT CC	H. nea: GAC CAT GCG CAA TGA GAT GG
	S. AR: ATG GAA AAG ATT ATA TTG CTC ATT CCA GC	S. AR: CCA AGA AGA GTG TTG CTT TAA AGA CAC
	T. rub: ATG GTT CTT GAA GCA ATC ATC GT	T. rub: GGT CTT TTC TTG CCT CGT GT

All primer sequences are written 5’ to 3’

Table 4: PCR conditions.

Purpose	Denaturing temperature (time)	Annealing temperature (time)	Extension temperature (time)	Number of cycles
Amplifying upstream regions	95 °C (1 min)	60 °C (6 min)	72 °C (2 min)	10
	95 °C (1 min)	60 °C (3 min)	72 °C (2 min)	20
Amplifying downstream regions	95 °C (1 min)	60 °C (1 min)	72 °C (2 min)	25
Overlap PCR	95 °C (1 min)	55 °C (1 min)	72 °C (1.5 min)	15
Purification PCR	95 °C (1 min)	60 °C (1 min)	72 °C (1.5 min)	30
Verify presence of OE PCR product in pLD55	95 °C (1 min)	50 °C (1 min)	72 °C (1.5 min)	25
Verify presence of plasmids carrying bicarbonate transporters	95 °C (1 min)	50 °C (1 min)	72 °C (1 min)	25

Table 5: Confirmation of transporter gene expression in *E. coli* via mass spectrometry.

Species	Protein of interest	Protein detected
Am. ferrooxidans	M subunit	Yes
At. ferrooxidans	M subunit	Yes
A. thiooxidans	M subunit C subunit	Yes Yes
H. crunogenus	M subunit	Yes
H. neapolitanus	M subunit	Yes
Sulfurovum sp. AR	M subunit	Yes
T. ruber	M subunit C subunit	Yes Yes

Table 6: *P*-values from analysis of DIC accumulation by cells expressing DIC transporters

	ANOVA		Bonferroni	
	Construct (complete vs. incompete)	Condition (induced vs. represed)	Complete transporter induced vs. repressed	Complete transporter + inducer vs incomplete transporter + inducer
Am. ferrooxidans	7.68e-06 (t-test)			
At. ferrooxidans	<2e-16	<3.27e-15	<2e-16	<2e-16
A. thiooxidans	0.000127	0.23	0.0015	0.03 (C subunit deletion) 1.2e-06 (T subunit deletion)
H. crunogenus	5.00e-12	1.91e-12	3.1e-15	5.3e-15
H. neapolitanus	4.03e-15	8.03e-09	6.5e-13	5.4e-15 (C subunit deletion) <2e-16 (T subunit deletion)
Sulfurovum sp. AR	<2e-16	<2e-16	<2e-16	<2e-16 (C subunit deletion) <2e-16 (T subunit deletion)
T. ruber	0.007	0.84	1	0.09

Table 7: *P*-values from analysis of DIC accumulation by cells provided with either CO_2 or HCO_3^-.

	ANOVA		Bonferroni		
p-value	Condition (inducer vs repressor)	DIC species (HCO_3^- vs. CO_2)	HCO_3^- + inducer vs. CO_2 + inducer	HCO_3^- + inducer vs. HCO_3^- + repressor	CO_2 + inducer vs. CO_2 + repressor
Am. ferrooxidans	1.19e-08	5.91e-07	2.3e-07	0.0087	2.1e-08
H. crunogenus	2.84e-08	4.97e-07	9.4e-08	0.17	1.8e-08
Sulfurovum sp. AR	0.0007	0.121	0.04	1	0.001

Table 8: Effect of CCCP and its solvent (DMSO) on the intracellular pH and ATP content of *E. coli.*

	pH	Percent of ATP remaining	*P*-values, Bonferroni comparison of intracellular pH vs. control	*P*-values, Bonferroni comparison of intracellular ATP vs. no addition control
No additions	7.58 ± 0.13	100% ± 0%	-	-
0.1% DMSO	7.66 ± 0.18	39.4% ± 2.93%	0.97	1.2e-11
0.1 mM CCCP, 0.1% DMSO	6.92 ± 0.12	4.35% ± 1.10%	2.6e-08	2.0e-13

pH and ATP values are provided with standard deviations of three measurements.

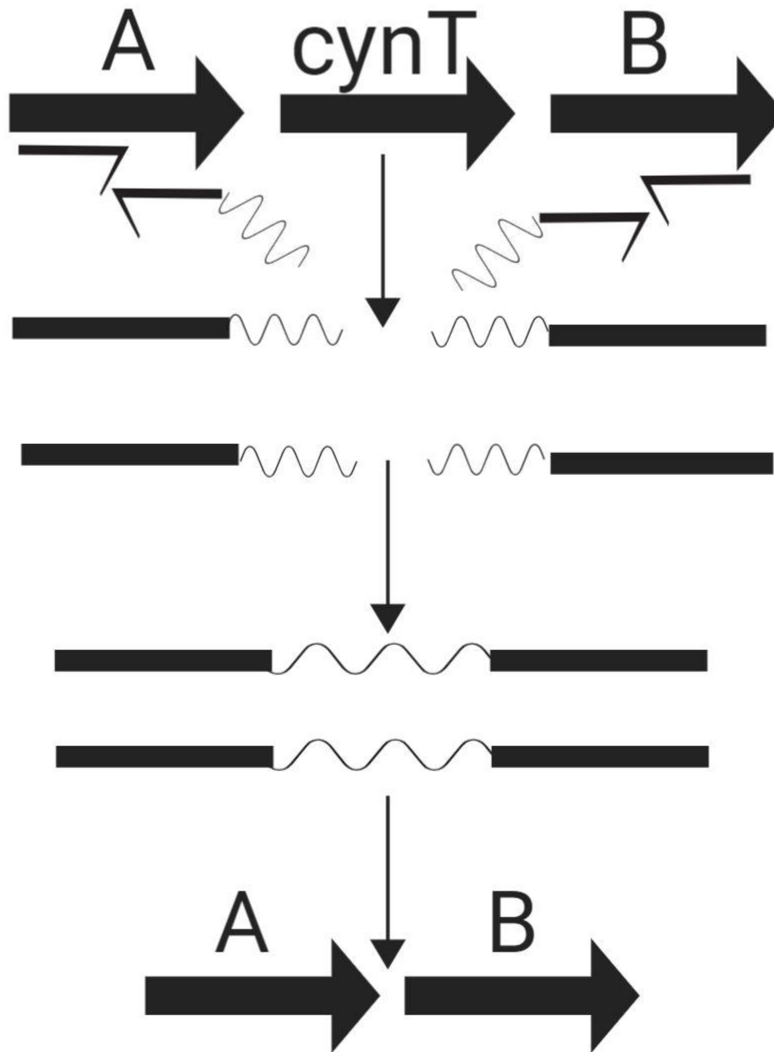

Figure 2: Steps in overlap extension PCR. The black arrows (A and B) represent the genes upstream and downstream of *cynT*. Below these genes the single-barbed arrows are the forward and reverse primers for the genes A and B. The waved lines on the forward primer for A and reverse primer for B are the complementary tails. Step 2 shows the amplified upstream and downstream regions with the modified tail. Step 3 shows the third PCR reaction in which the upstream and downstream products are joined. Step 4 shows the final product with the *cynT* deletion. The same process was repeated for *yadF*.

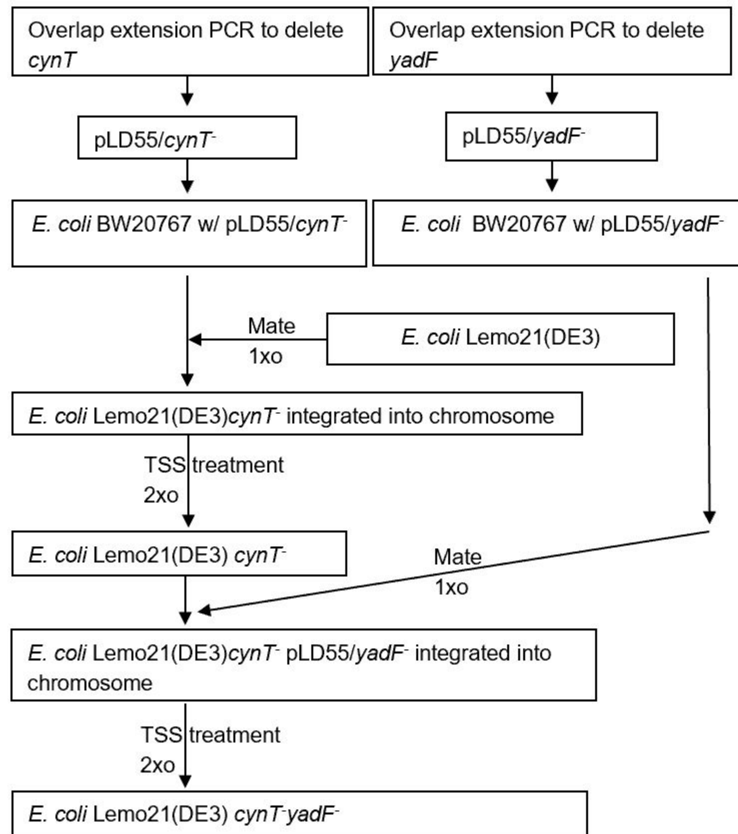

Figure 3: Process for deleting *cynT* and *yadF* from *E. coli* Lemo21(DE3). Crossover events are denoted as “xo”.

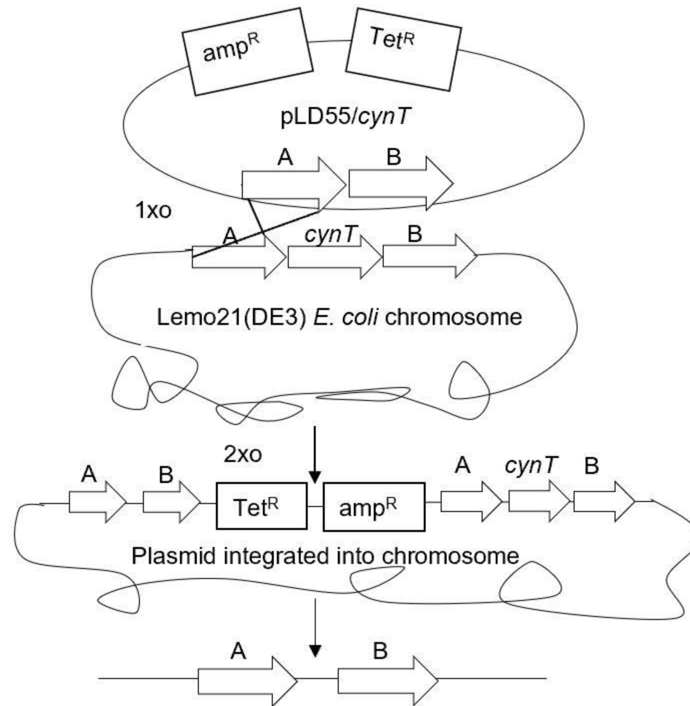

Figure 4: Double crossover event mediated by RecA. The first crossover (1xo) between pLD55/*cynT* and *E. coli*. This results in a merodiploid bacterium. A second crossover (2xo) results in the loss of duplicate flanking genes, *cynT* and the ampicillin and tetracycline resistance genes.

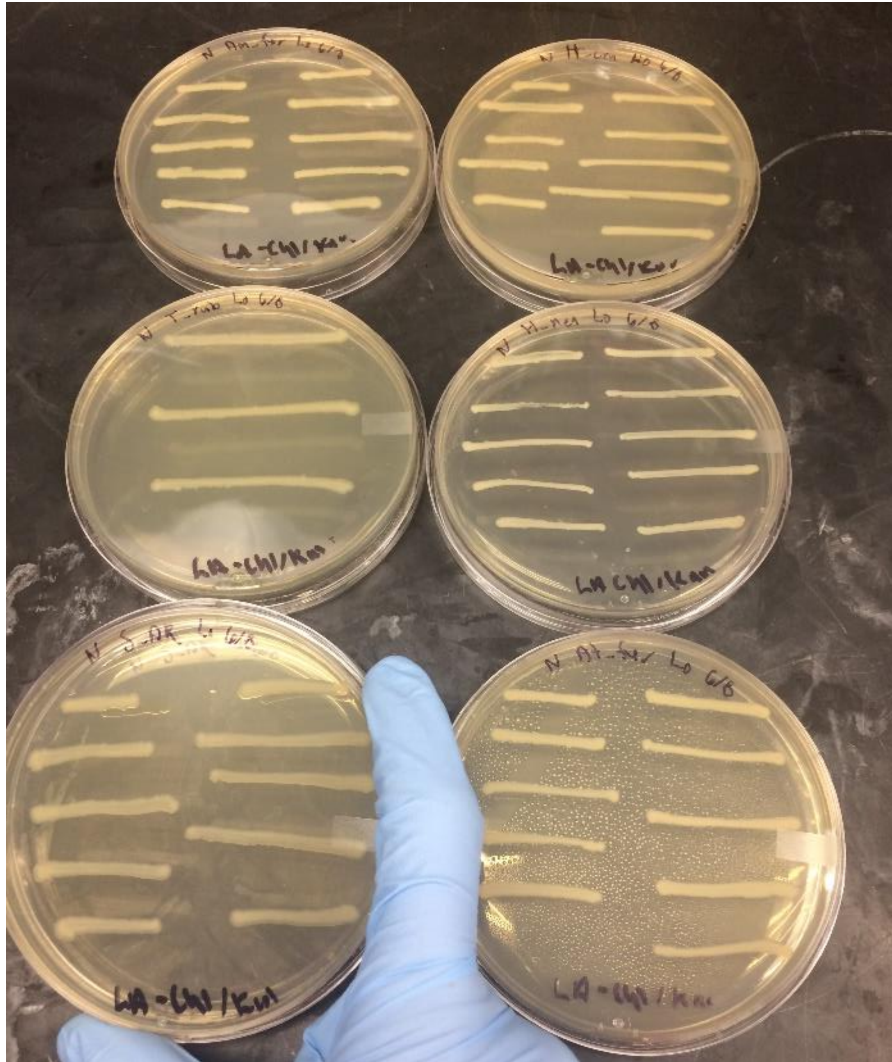

Figure 5: Rescue of *E. coli* Lemo21(DE3)yadF$^-$cynT$^-$ under low CO_2 conditions, when MDT transporter expression is driven by native promoters. Each plate has line colonies of *E. coli* expressing transporters from a different organism. The plates in order from left to right, top to bottom are *Am. ferrooxidans*, *H. crunogenus*, *T. ruber*, *H. neapolitanus*, *Sulfurovum* sp. AR, and *At. ferrooxidans*. Each line is a different colony isolated from the original transformation plate.

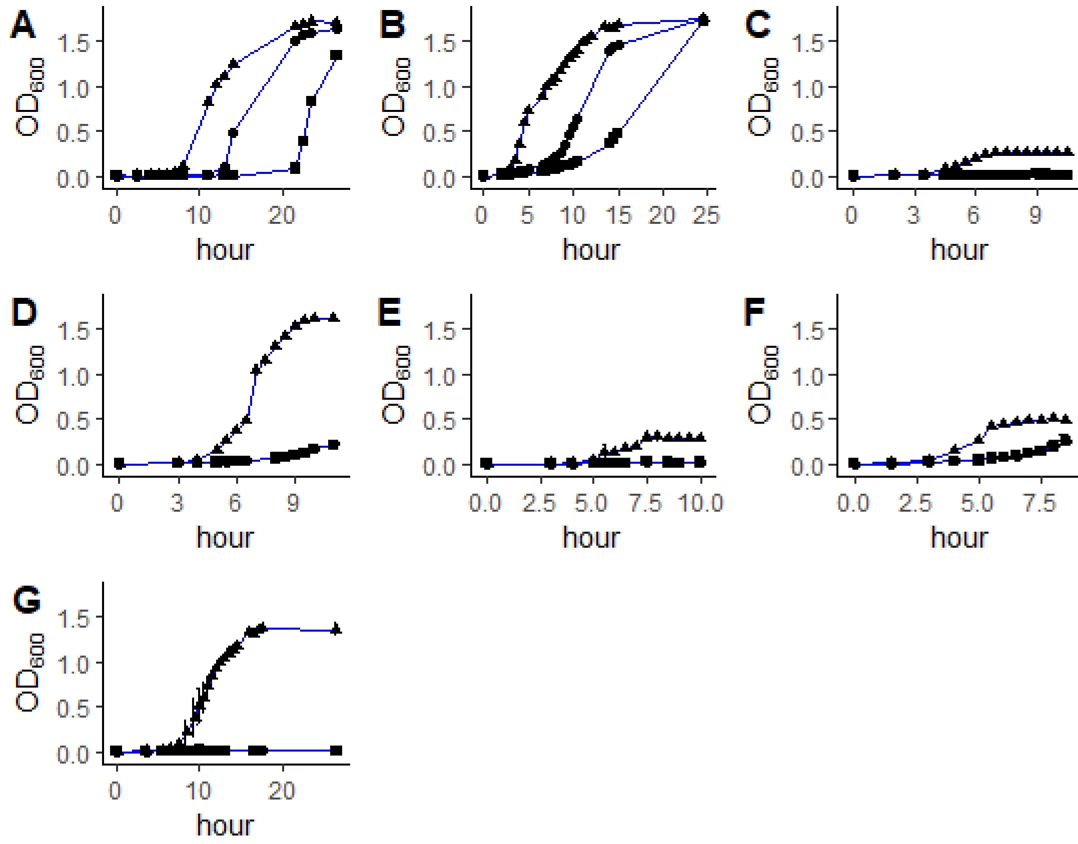

Figure 6: Growth curves of *E. coli* expressing complete MDTs under low CO_2 conditions. Each plot represents growth of a construct expressing a transporter from a different species: A) *Am. ferrooxidans* in pET B) *At. ferrooxidans* in pET C) *A. thiooxidans* in pBAD202 D) *H. crunogenus* in pbad202 E) *H. neapolitanus* in pBAD202 F) *Sulfurovum* sp. AR in pBAD202 G) *T. ruber* in pET28. Circles = no inducer or repressor, triangles = inducer (IPTG for pET28 constructs; arabinose for pBAD202 constructs), and squares = repressor (rhamnose for pET28 constructs; glucose for pBAD202).

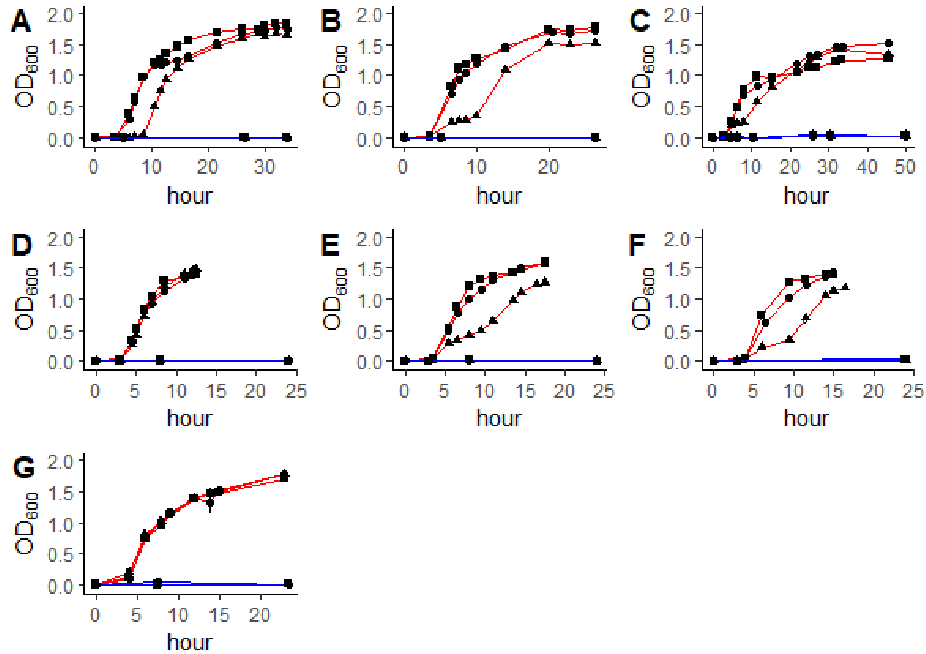

Figure 7: Growth curves of *E. coli* expressing transporters with missing subunits under high CO_2 (red) and low CO_2 (blue) conditions. Each plot represents growth of a construct expressing a transporter from a different species: A) *At. ferrooxidans* in pET28 B) *A. thiooxidans* in pBAD202 C) *A. thiooxidans* in pBAD202 (without middle subunit) D) *H. crunogenus* in pBAD202 E) *H. neapolitanus* in pBAD202 F) *Sulfurovum* sp. AR in pBAD202 G) *T. ruber* in pET28. Circles = no inducer or repressor, triangles = inducer (IPTG for pET28 constructs; arabinose for pBAD202 constructs), and squares = repressor (rhamnose for pET28 constructs; glucose for pBAD202).

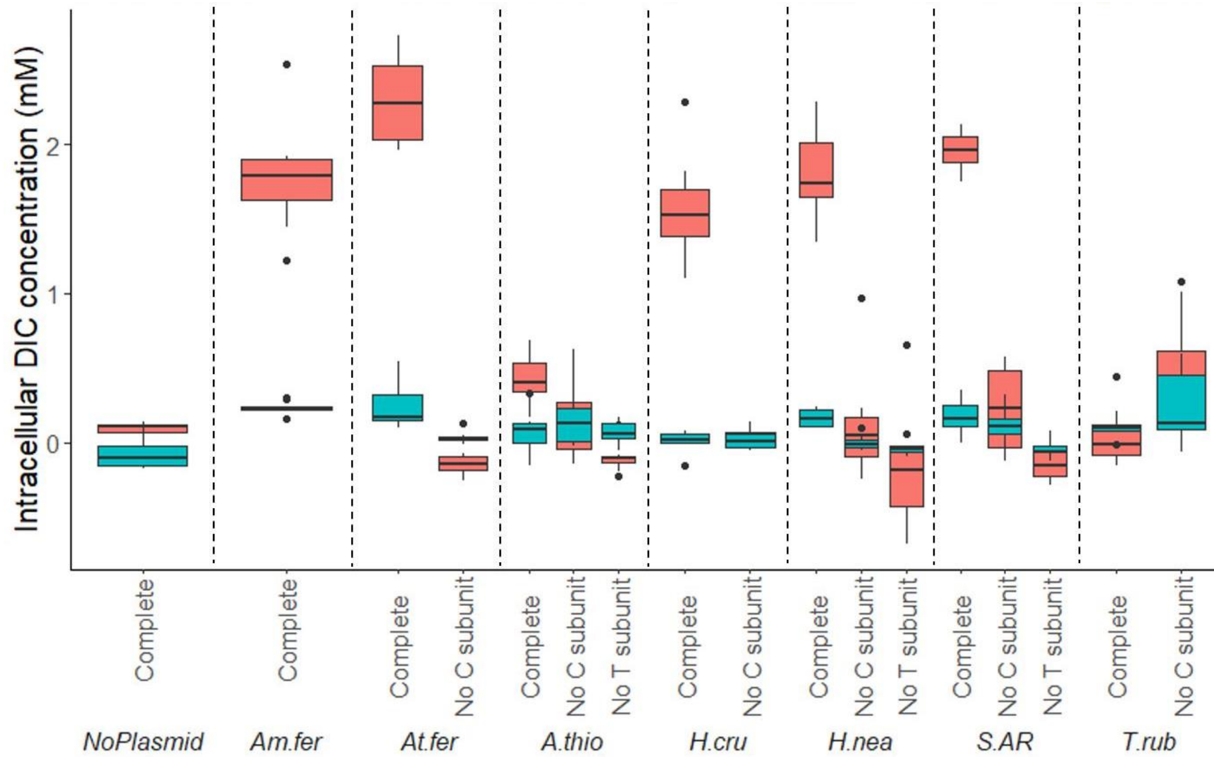

Figure 8: Intracellular DIC concentrations measured in each of the constructs with a complete transporter or missing either the C subunit or the T subunit. Results are shown for cells grown in the presence of inducer (orange) or repressor (blue).

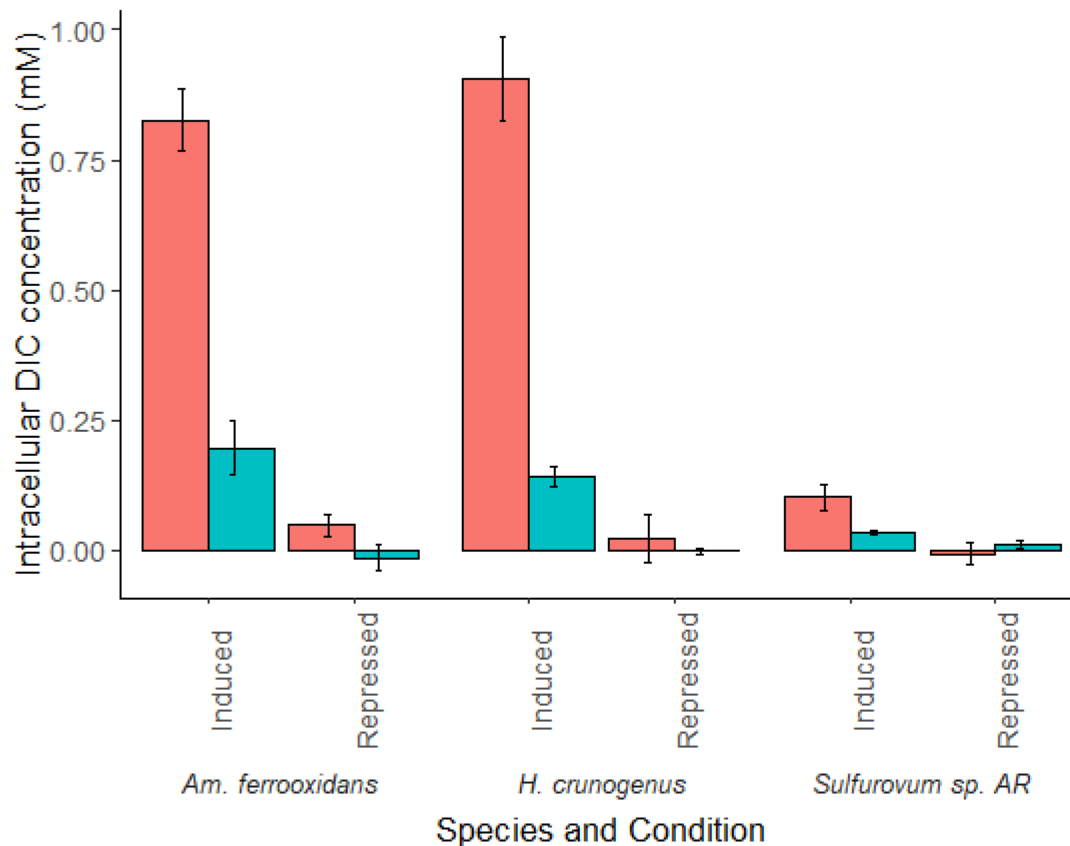

Figure 9: Intracellular DIC concentrations when cells expressing transporters were provided with either CO_2 or HCO_3^-. Results are shown for cells grown in the presence of CO_2 (orange) or HCO_3^- (blue). Error bars show standard error.

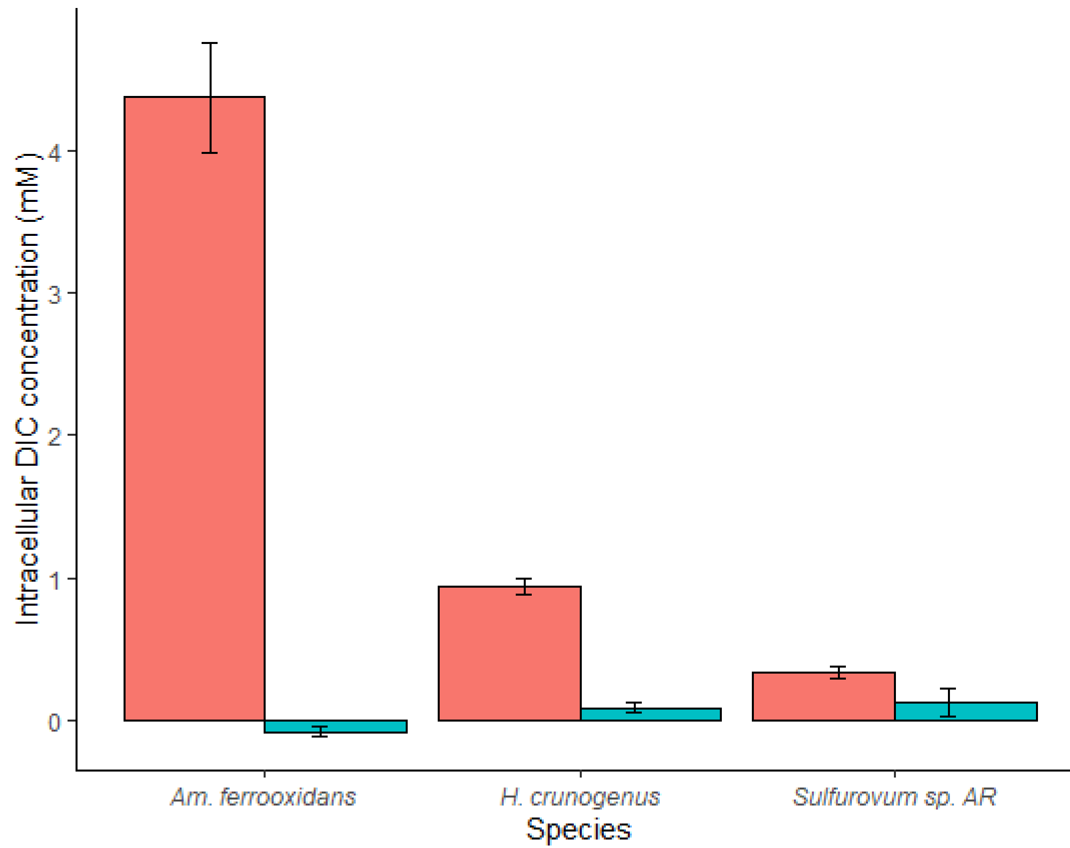

Figure 10: Intracellular DIC concentrations measured in cells expressing DIC transporters incubated in the presence of either DMSO (solvent control) (orange) or CCCP (proton motive force inhibitor)+DMSO (blue). Error bars show standard error.

CHAPTER 3: CONCLUSION

Many autotrophs depend on a CCM to cope with low or variable concentrations of DIC. The DIC transporters of the CCM allow autotrophs to accumulate intracellular DIC and drive carbon fixation. We have expanded on previous research showing that the MDT transports DIC in *H. crunogenus* (4), *S. aureus* (29), and *H. neapolitanus* (27) to include a greater diversity of bacteria. The aim of this study was to address the following objectives:

Objective 1: Verify the function of MDT as DIC transporters in a diversity of bacteria

Six of out of the seven transporters were able to generate elevated intracellular DIC concentrations in *E. coli.* The transporter from the hyperthermophile *T. ruber* did not measurably increase intracellular DIC concentrations, however it was still able to rescue *E. coli* Lemo21(DE3)*yadF*$^-$*cynT*$^-$ under low CO_2 conditions.

Objective 2: Biochemically characterize the MDT transporters

For MDT predicted to consist of multiple subunits, all subunits were required to transport DIC. Constructs that either had only the M subunit or were missing the T subunit failed to accumulate intracellular DIC. The C and M subunits have been shown to form a heterodimeric complex (27) and our results are consistent with interactions between the subunits of these complexes being necessary for their activities.

MDT appears to transport CO_2 as opposed to HCO_3^-, using cellular proton potential. All other bacterial DIC transporters characterized to date (SulP, SbtA, and BCT1) transport HCO_3^- (19). MDT is also unique among these transporters in that it couples CO_2 uptake to cellular

proton potential. The other transporters couple HCO_3^- uptake to Na+ uptake (SulP, SbtA) or ATP hydrolysis (BCT1) (24). Given that electron transport in these organisms generates a proton gradient (26), using this proton gradient directly instead of secondarily via sodium gradient or ATP hydrolysis avoids energy losses associated with conversion.

Future directions for studying MDT transport

The function of the T subunit found in some MDT is yet to be determined. Future experiments could examine the function of this subunit, possibly looking to see if the T subunits act as regulatory subunits, in a manner analogous to the regulation of Sbt transporters by smaller SbtB proteins (50). This three-subunit variety of MDT appears to be spread throughout several phylogenetic classes including the *Alphaproteobacteria, Betaproteobacteria* and *Acidithiobacilla* (34). Determining the role of the T subunit will clarify why some MDT have this subunit, while others do not.

In autotrophic *Proteobacteria,* MDTs are frequently present in genomes that encode other DIC transporters. In *Cyanobacteria*, several DIC transporter genes likewise coexist within many genomes, and are induced by different growth conditions, as the transporters have different affinities for HCO_3^- (19). Perhaps *Proteobacteria* tailor the expression of different transporters to different growth conditions. Since MDTs transport CO_2, they may be particularly useful when the pH of the environment is 7 or lower. Further research could investigate if the MDT is a high or low-affinity transporter.

Based on the current understanding of CCMs (transporters plus carboxysomes), it is not surprising that autotrophs that use the CBB cycle have transporters like these to facilitate growth under low DIC conditions. However, MDTs are also encoded in genomes from organisms using other pathways like the reverse tricarboxylic acid (rTCA) cycle and hydroxypropionate cycle for CO_2 fixation (4). These other autotrophic pathways are not associated with carboxysomes or other subcellular structures with which DIC transport can be

coupled to facilitate carbon fixation, so it would be interesting to determine how MDTs benefit these organisms. MDTs are also encoded in genomes from pathogens *Legionella pneumophila* and *Vibrio cholerae* (29), where they likely function to replenish intracellular DIC for use in synthesis of metabolic intermediates such as oxaloacetate, or in purine and pyrimidine synthesis. It would be interesting to determine whether MDT play a role in pathogenicity.

Successful heterologous expression of these transporters in *E. coli* suggests that integrating these transporters into engineered organisms for bioengineering applications will be successful. The presence of these transporters in bacteria from acidic and high temperature environments is of interest because microbes used in industrial processes might be subjected to these conditions. In addition, there is current research into the expression of DIC transporters in plants in hopes of increasing photosynthetic efficiency, particularly in hot, dry environments (19). The addition of MDT family transporters to the arsenal of other DIC transporters (SbtA, SulP, and BCT1) increases the toolkit of DIC transporters that can be integrated into engineered organisms. Its unique capability of CO_2 transport may provide advantages, particularly under low pH conditions.

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