FEMS Microbiology Letters, 367, 2020, fnaa106

doi: 10.1093/femsle/fnaa106
Advance Access Publication Date: 26 June 2020
Research Letter

RESEARCH LETTER – Physiology & Biochemistry

Ubiquity and functional uniformity in CO_2 concentrating mechanisms in multiple phyla of *Bacteria* is suggested by a diversity and prevalence of genes encoding candidate dissolved inorganic carbon transporters

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One sentence summary: Carbon dioxide concentrating mechanisms are common in carboxysome-using autotrophs.

Editor: Rich Boden

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ABSTRACT

Autotrophic microorganisms catalyze the entry of dissolved inorganic carbon (DIC; $= CO_2 + HCO_3^- + CO_3^{2-}$) into the biological component of the global carbon cycle, despite dramatic differences in DIC abundance and composition in their sometimes extreme environments. *"Cyanobacteria"* are known to have CO_2 concentrating mechanisms (CCMs) to facilitate growth under low CO_2 conditions. These CCMs consist of carboxysomes, containing enzymes ribulose 1,5-bisphosphate oxygenase and carbonic anhydrase, partnered to DIC transporters. CCMs and their DIC transporters have been studied in a handful of other prokaryotes, but it was not known how common CCMs were beyond *"Cyanobacteria"*. Since it had previously been noted that genes encoding potential transporters were found neighboring carboxysome loci, α-carboxysome loci were gathered from bacterial genomes, and potential transporter genes neighboring these loci are described here. Members of transporter families whose members all transport DIC (CHC, MDT and Sbt) were common in these neighborhoods, as were

Received: 13 March 2020; **Accepted:** 23 June 2020

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members of the SulP transporter family, many of which transport DIC. 109 of 115 taxa with carboxysome loci have some form of DIC transporter encoded in their genomes, suggesting that CCMs consisting of carboxysomes and DIC transporters are widespread not only among *"Cyanobacteria"*, but also among members of "*Proteobacteria*" and "*Actinobacteria*".

Keywords: CO2 concentrating mechanism; autotroph; carbon fixation; carboxysome

INTRODUCTION

Autotrophic microorganisms fix dissolved inorganic carbon (DIC; CO_2 + HCO_3^- + CO_3^{2-}) in an incredible array of habitats, including hydrothermal vents (Jannasch and Mottl 1985), terrestrial hot springs (Ward *et al.* 1998), the oligotrophic open ocean (Chisholm *et al.* 1988; Wuchter *et al.* 2006), acid rock drainage areas (Edwards *et al.* 2000) and soda lakes (Sorokin *et al.* 2003). Since the pH range of these habitats is 1–12 (Edwards *et al.* 2000; Sorokin *et al.* 2003), the concentration and composition of DIC also vary markedly; at acidic pH, CO_2 dominates, at near-neutral pH, HCO_3^- is abundant and at more alkaline pH values, CO_3^{2-} is the major form present.

Organisms that use the Calvin–Benson–Bassham (CBB) cycle for CO_2 fixation in these varied habitats must grapple with the catalytic constraints of ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO; EC 4.1.1.39). RubisCO has poor substrate specificity; it catalyzes both the carboxylase reaction of the CBB cycle, as well as a wasteful oxygenase reaction, which results in added energetic expense to regenerate the ribulose 1,5 bisphosphate (RuBP) necessary for the CBB cycle (Tabita 1999). In addition, RubisCO enzymes have relatively low affinities for CO_2 (5–250 μM; (Tabita 1999). RubisCO affinities for CO_2 are particularly low for autotrophic bacteria (25–250 μM; tabulated in (Horken and Tabita 1999). Furthermore, RubisCO is only able to use CO_2 as a substrate, and not HCO_3^- (Cooper and Filmer 1969), despite the scarcity of CO_2 and abundance of HCO_3^- at the circumneutral pH typical for cytoplasm.

It is likely that many autotrophic bacteria using the CBB cycle have CO_2-concentrating mechanisms (CCMs) that compensate for RubisCO. CCMs are nearly universal among *"Cyanobacteria"*, in which it has been demonstrated that CCMs consist of two components: (1) membrane transporters for DIC, which generate high concentrations of cytoplasmic HCO_3^-, and (2) carboxysomes, which are present in the cytoplasm and facilitate high rates of CO_2 fixation by RubisCO (Fig. 1; reviewed in Price *et al.* (2009); Rae *et al.* (2013); Long *et al.* (2016)). Carboxysomes are a type of bacterial microcompartment, and consist of a protein shell filled with RubisCO and a trace of carbonic anhydrase activity (EC 4.2.1.1; Kerfeld *et al.* 2018). Cytoplasmic HCO_3^- enters carboxysomes, where carbonic anhydrase converts some of it to CO_2, which is then fixed by RubisCO. Carboxysomes are also present in many autotrophic members of "*Proteobacteria*" (Cannon *et al.* 2002); it is reasonable to suggest that they play a key role in CCMs in members of this phylum as well. Indeed, *Hydrogenovibrio crunogenus*, a member of *Gammaproteobacteria* has a CCM consisting of carboxysomes and DIC transporters (Dobrinski, Longo and Scott 2005; Dobrinski *et al.* 2012), and members of genera *Hydrogenovibrio, Thiomicrorhabdus* and *Thiomicrospira* upregulate genes encoding carboxysome components and DIC transporters when cultivated under low DIC conditions (e.g., (Dobrinski *et al.* 2012; Esparza *et al.* 2019; Scott *et al.* 2019).

Two types of carboxysomes (α and β) are currently recognized (reviewed in (Cannon, Heinhorst and Kerfeld 2010; Kerfeld and Melnicki 2016). These types can be distinguished by the form of RubisCO they carry (α-carboxysomes carry form IA RubisCO; β-carboxysomes carry form IB RubisCO), as well as differences in carbonic anhydrases and carboxysome shell components (Kerfeld and Melnicki 2016). Members of "*Proteobacteria*" and certain marine members of "*Cyanobacteria*" have α-carboxysomes, while the remaining members of "*Cyanobacteria*" have β-carboxysomes (Price *et al.* 2009; Scott *et al.* 2019).

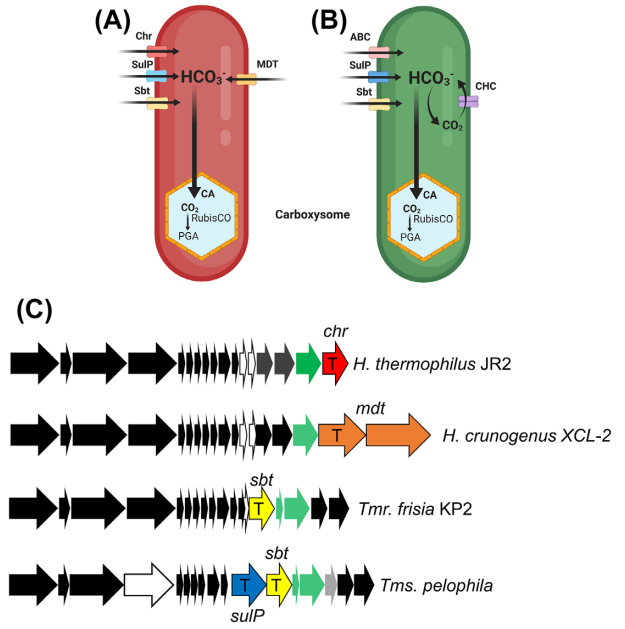

Figure 1. CO_2 concentrating mechanisms (CCMs) in members of *"Proteobacteria"* **(A)** and *"Cyanobacteria"* **(B)**, and their chromosomal loci **(C)**. For both CCMs, DIC transporters generate elevated concentrations of bicarbonate, which facilitate CO_2 fixation by carboxysomes. *CA* – Carbonic anhydrase; *CHC* – CO_2 hydration complex; *chr* - chromate family transporter; *mdt* - multisubunit dissolved inorganic carbon transporter; *pga* – phosphoglyceric acid; *sbt* – sodium bicarbonate transporter; *sulP* – SulP-family transporter. Genes encoding carboxysome structural and enzyme components (black) with transmembrane proteins (T) encoded nearby, from the genomes of *Hydrogenovibrio thermophilus, Hydrogenovibrio crunogenus, Thiomicrorhabdus frisia* KP2 and *Thiomicrospira pelophila*. Genes encoding regulatory (green), conserved hypothetical (grey) and hypothetical (white) proteins are also depicted.

DIC transporters also vary among these organisms. Among members of phylum *"Cyanobacteria"*, three evolutionarily independent lineages of bicarbonate transporters have been identified. An ATP-binding cassette transporter couples bicarbonate uptake to ATP hydrolysis (Omata *et al.* 1999). Transporters belonging to the SulP family (named for the sulfate transport activity of the founding member), couple bicarbonate uptake to sodium uptake (Price *et al.* 2004; Du *et al.* 2014), as do SbtA transporters, the third type of cyanobacterial bicarbonate transporter (Shibata *et al.* 2002b; Du *et al.* 2014). In addition, some members of *"Cyanobacteria"* also have 'CO_2 traps', which are located at the thylakoid or cell membrane, and consist of a carbonic anhydrase activity associated with NAD(P)H dehydrogenase. These CO_2-hydrating complexes (CHC) couple electron transfer to plastoquinone with the conversion of CO_2 to HCO_3^-, perhaps via localized alkalinization around their associated carbonic

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anhydrase enzymes, which minimizes CO_2 losses from these cells (Price et al. 2002; Shibata et al. 2002a; Battchikova, Eisenhut and Aro 2011; Han et al. 2017; Schuller et al. 2020).

A growing body of evidence suggests that mechanisms for DIC transport by autotrophic organisms from other phyla are also diverse, and currently include members of four evolutionarily distinct transporter families: multi-subunit DIC transporters (MDT), Chr-, SulP- and Sbt-family transporters (Fig. 1; Scott et al. 2019). MDT activity was first described in *Gammaproteobacterium Hydrogenovibrio crunogenus* (Mangiapia et al. 2017), confirmed to transport DIC (Scott et al. 2019), and subsequently found to be active in *Halothiobacillus neapolitanus* (Price, Long and Förster 2019) and also in phylum "*Firmicutes*" (Fan et al. 2019). Indeed, homologs of MDT are widespread in many phyla (Mangiapia et al. 2017). A Chr-family transporter from *Hydrogenovibrio thermophilus* was found to be able to transport DIC (Scott et al. 2019). SulP- and Sbt-family transporters deeply divergent from DIC transporters from "*Cyanobacteria*" (~25–30% identical amino acid sequences) also demonstrate measureable DIC transport. SulP-family DIC transporters in *Escherichia coli* and *Salmonella typhimurium* may play a role in lipid synthesis (Babu et al. 2010; Srinivasan et al. 2016), while one from *H. thermophilus* likely facilitates CO_2 fixation, as it is upregulated under low DIC conditions and is capable of DIC uptake (Scott et al. 2019). Since SulP-family transporters are sometimes fused to carbonic anhydrase, it has been suggested that DIC uptake by these transporters may be common (Felce and Saier 2004). A Sbt-family transporter in *Thiomicrorhabdus frisia* also transports DIC and is upregulated under low DIC conditions (Scott et al. 2019).

Several things remain to be clarified about DIC transporters in autotrophic *Bacteria*. The prevalence of different types of DIC transporters among autotrophs, the partnering of carboxysomes with DIC transporters in CCMs and taxonomic distribution of DIC transporters have yet to be described. The prevalence of DIC transport capability among members of the Chr- and SulP-family is unknown. Unlike Sbt and MDT transporters, whose characterized members only transport DIC, members of Chr- and SulP-families are known to transport other compounds (Pimentel, Moreno-Sanchez and Cervantes 2002; Zolotarev et al. 2008). It also seems likely that yet more types of DIC transporters remain to be discovered.

One attractive option is to use genome data to address these unknowns. Indeed, a genomic approach has been used to successfully identify candidate DIC transporters in members of "*Cyanobacteria*" with α-carboxysomes (Gaudana et al. 2015). Genomes from these organisms were queried for homologs of DIC transporters characterized in "*Cyanobacteria*" with β-carboxysomes, and several likely DIC transporters were uncovered (Gaudana et al. 2015). Gene neighborhood information gathered from genome sequences can also be helpful in inferring gene function. In members of domain "*Bacteria*", genes whose products function together are often collocated in operons (Price et al. 2005). Furthermore, correlations in transcript abundance have been noted for operons that are adjacent to each other on a bacterial chromosome (Junier and Rivoire 2016). This collocation of functionally related genes at the operon and supra-operon level has been used to predict functions of bacterial microcompartments by examining the genome neighborhood adjacent to genes encoding the structural components of these subcellular structures (Axen, Erbilgin and Kerfeld 2014). In the course of that study, it was noted that many microcompartment-related loci were collocated with putative transporters related to microcompartment functions, including NADH dehydrogenase (EC 1.6.5.11) subunit homologs now known to comprise MDT (Axen, Erbilgin and Kerfeld 2014). Indeed, genes encoding MDT, Chr-, SulP- and Sbt-family DIC transporters are present downstream of carboxysome loci in members of *Thiomicrospira, Hydrogenovibrio* and *Thiomicrorhabdus* (Scott et al. 2018, 2019).

For the study presented here, a genome context approach was taken to clarify the prevalence and distribution of potential DIC transporters in autotrophic "*Bacteria*" beyond phylum "*Cyanobacteria*", and to identify new families of transporters that might be capable of DIC uptake. α-carboxysome loci encoding RubisCO, carbonic anhydrase and shell proteins were collected, and their neighborhoods were examined for potential DIC transporters.

METHODS

Selecting carboxysome loci for analysis

Carboxysome loci were selected from sequenced microbial genomes since the genome neighborhoods surrounding these loci were going to be examined for potential DIC transporters. These loci were found by gathering all members of Pfam12288 (Carboxysome shell peptide mid-region, *CsoS2_M*) from the Integrated Microbial Genomes & Microbiomes system (IMG; https://img.jgi.doe.gov/; Chen et al. 2019). Pfam12288 was selected because all known α-carboxysomes contain the CsoS2 protein, which is necessary for carboxysome assembly (Cai et al. 2015). *csoS2* genes closer than 25 000 nucleotides from the end of a contig were removed, as their neighborhoods were truncated. The remaining *csoS2* genes were present in genomes from members of phyla "*Cyanobacteria*", "*Proteobacteria*" and "*Actinobacteria*", and certain genera were overrepresented (124 members of *Prochlorococcus*, 36 members of *Synechococcus* and 52 members of *Thioalkalivibrio*), reflecting bias in genome representation in the database. To select members from these three genera, and to diminish overrepresentation, members from each genus were placed into clusters via the 'Genome Clustering' tool at IMG, using COG presence to cluster genomes by principal components analysis. Members of each cluster were compared to eachother via pairwise average nucleotide identities (ANI) by using the IMG 'Compare Genomes' tool. To select members of different species within each genus, members of each cluster that had at most 95% ANI relative to eachother were selected for further study (Konstantinidis and Tiedje 2005; Rodriguez-R and Konstantinidis 2014). After vetting as described above, 115 carboxysome loci remained for analysis (Table 1; Table S1, Supporting Information).

Gathering carboxysome loci and their neighborhoods, and screening for potential DIC transporters

Previously it had been shown that genes encoding DIC transporters are found up to 15 genes downstream from *csoS2* in genera *Thiomicrospira, Thiomicrorhabdus* and *Hydrogenovibrio* (Scott et al. 2019). Therefore, for this analysis, gene neighborhoods that included all genes up to 20 genes upstream, and 20 genes downstream, from *csoS2* were gathered from the 115 genomes selected above to search for potential DIC transporters (Fig. 2, blue circle; Table S2, Supporting Information; referred to subsequently as 'carboxysome gene neighborhoods'). From these, genes with Pfam domains were collected (Fig. 2, orange circle; Table S3, Supporting Information; assignment based on Pfam v30; https://img.jgi.doe.gov/docs/pipelineV5/; Huntemann et al. 2015).

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Table 1. Taxonomic distribution of organisms whose genome sequences were used for this study.

Phylum	Class	Order	Family
"Actinobacteria" (1)	*"Acidimicrobia"* (1)	*Acidimicrobiales* (1)	*Acidimicrobiaceae* (1)
"Cyanobacteria" (22)	unclassified (22)	*Chroococcales* (1)	*Aphanothecaceae* (1)
		"Synechococcales" (21)	*Prochloraceae* (5)
			Synechococcaceae (15)
"Proteobacteria" (93)	*Acidithiobacillia* (4)	*Acidithiobacillales* (4)	*Acidithiobacillaceae* (4)
	Alphaproteobacteria (6)	*Rhizobiales* (4)	*Bradyrhizobiaceae* (4)
		Rhodobacterales (2)	*Rhodobacteraceae* (2)
	Betaproteobacteria (22)	*Burkholderiales* (8)	*Comamonadaceae* (2)
			unclassified (6)
		"Ferrovales" (2)	*"Ferrovaceae"* (2)
		Nitrosomonadales (11)	*Gallionellaceae* (3)
			Nitrosomonadaceae (5)
			Thiobacillaceae (3)
		unclassified (1)	unclassified (1)
	Gammaproteobacteria (61)	*Acidiferrobacterales* (1)	*Acidiferrobacteraceae* (1)
		Alteromonadales (1)	*Alteromonadaceae* (1)
		Chromatiales (40)	*Chromatiaceae* (13)
			Ectothiorhodospiraceae (21)
			Granulosicoccaceae (1)
			Halothiobacillaceae (3)
			Thioalkalispiraceae (1)
			unclassified (1)
		Thiotrichales (18)	*Piscirickettsiaceae* (16)
			Thiotrichaceae (2)
		Unclassified (1)	Unclassified (1)

Numbers in parentheses indicate the numbers of members in each taxonomic unit. A full list of organisms is provided in Table S1 (Supporting Information).

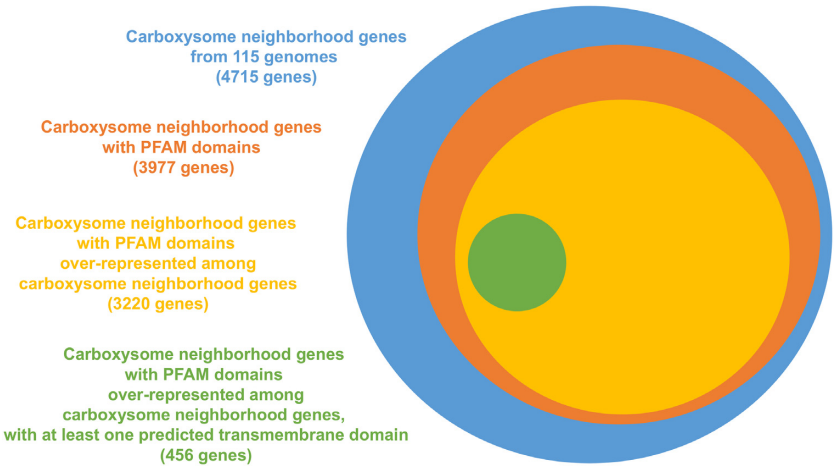

Figure 2. Process for collecting potential transporter genes collocated with carboxysome genes. Numbers of genes are indicated for each of the four steps of the process.

A bootstrap sampling procedure was used to determine whether certain Pfams were more common in these carboxysome gene neighborhoods than elsewhere in the genomes. To determine how common Pfams were throughout the genome, random genes were selected with replacement, and the Pfams present in the 41 gene region centered on each gene (referred to subsequently as 'random gene neighborhoods') were tabulated (Pfam assignment based on Pfam v30; https://img.jgi.doe.gov/docs/pipelineV5/; Huntemann *et al.* 2015). This procedure was repeated 10 000 times for each organism's genome to build the distribution of number of times each Pfam was observed in random gene neighborhoods, from which the 0.975 quantile was calculated to obtain the upper portion of the 95% confidence interval (CI) for each Pfam. This was done in R (R version 3.6.1, 2019) and code is available in the supplementary information (Supplementary Methods 1).

The Pfams occurring in each carboxysome gene neighborhood were counted in the same way, and the number of occurrences of each Pfam in each carboxysome gene neighborhood was compared to the upper bound of the 95% CI from the random gene neighborhoods from elsewhere in the same genome. If the Pfam occurred more often in the carboxysome gene neighborhood, it was scored as over-represented in the carboxysome gene neighborhood (Fig. 2, yellow circle). To determine if particular Pfams were over-represented in multiple organisms, over-represented Pfams were summed across organisms, counting once for each organism in which a Pfam was over-represented (Table S3, Supporting Information). To select potential DIC

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transporters, genes falling into these Pfams that were predicted to encode proteins with transmembrane domains were collected (Fig. 2, green circle; transmembrane domains were predicted based on TMHMM; Krogh et al. 2001).

Making a 16S rRNA gene tree for determining taxonomic distribution of potential DIC transporters

Genes encoding 16S rRNA were collected from IMG, and aligned via SINA 1.2.11 (https://www.arb-silva.de/aligner/job/748971; Pruesse, Peplies and Glöckner 2012). The alignment was trimmed via GBLOCKS using stringent criteria (Talavera and Castresana 2007). PhyML 3.0 was used to construct phylogenetic trees (Guindon et al. 2010) via Maximum Likelihood (ML) analysis. Smart Model Selection (SMS) was used to evaluate best-fit models of evolution (GTR + G + I, G = 0.633, I = 0.541, where GTR = generalized timer-reversible model, G = gamma distribution parameter; I = proportion of invariant sites; Tavare 1986; Lefort, Longueville and Gascuel 2017). Clade robustness was assessed with 1000 bootstrap replicates, and the consensus tree was visualized using FigTree (Version 1.4.3; A. Rambaut).

RESULTS AND DISCUSSION

Predicted protein families for DIC transporters that are over-represented near carboxysome loci

A total of 456 genes were found, that met all selection criteria described above (Fig. 2). These genes, collected from carboxysome gene neighborhoods, encode proteins with transmembrane domains, and belong to 157 Pfams that were over-represented in the carboxysome gene neighborhood in at least one genome (Fig. 3; complete list in Table S4, Supporting Information). Some of proteins encoded by these genes contain more than one Pfam domain (Fig. 4). The Pfams discussed below are those that were present in at least five of these 456 proteins, and were found to be over-represented in the carboxysome gene neighborhoods in at least two genomes.

87 proteins were identified as members of Pfam00361 (Proton-conducting membrane transporter). Of these, 53 also had an N-terminal Pfam00662 domain, as is common for members of Pfam00361 (https://pfam.xfam.org/family/PF00662#tabview=tab1). Some were judged to be unlikely to be DIC transporters, as they were in the midst of genes encoding the other subunits of multisubunit sodium/proton antiporters (12 genes), formate-hydrogen lyase (EC 1.17.99.7; 2 genes), or NADH dehydrogenase (3 genes). The remaining 70 of the genes could be predicted to encode subunits of CHC or MDT based on their juxtaposition to genes encoding other subunits of CHC (Shibata et al. 2002b; Han et al. 2017) or MDT (Mangiapia et al. 2017; Scott et al. 2019): Pfam10216 CO_2 hydration protein ChpXY (CHC; 28 genes) or Pfam10070 Uncharacterized protein conserved in bacteria (DUF2039) (MDT; 42 genes). MDT appear to have at least three forms, encoded by one, two or three subunits (Fig. 4). MDT were found to be widespread in *Proteobacteria*, while CHC were found exclusively in "*Cyanobacteria*" (Fig. 5).

A total of 29 genes were identified as coding for Pfam05982 Na^+-dependent bicarbonate transporter superfamily. This Pfam includes the Sbt-type transporters found in "*Cyanobacteria*" (Shibata et al. 2002b; Du et al. 2014), and members of this Pfam had previously been noted to be encoded downstream from carboxysome loci in some "*Cyanobacteria*" (Gaudana et al. 2015). Among members of "*Cyanobacteria*", Sbt-type transporters are encoded by *sbtA* genes; *sbtB* genes are adjacent to *sbtA* genes, and encode PII-type regulatory proteins, which regulate Sbt-mediated HCO_3^- transport by binding adenylnucleotides (Kaczmarski et al. 2019). Interestingly, the genes found here in members of "*Proteobacteria*" also have genes encoding PII-type regulatory proteins following them, so it seems likely that transporter regulation by the cellular adenylnucleotide pool is universal for these transporters. They were less common in carboxysome gene neighborhoods than MDT or CHC for both "*Proteobacteria*" and "*Cyanobacteria*" (Fig. 5). Their presence in these neighborhoods was common in genera *Thiomicrospira* and *Thioalkalivibrio*.

Genes for proteins that contain Pfam00916 (Sulfate permease, SulP) were almost as common, with 27 of them falling into this protein family (Fig. 3), and 22 of these also contain Pfam01740 (STAS domain). The STAS domain, like SbtB, may regulate SulP activity by interacting with the cellular adenylnucleotide pool (Aravind and Koonin 2000). The members of Pfam00916 found here had three major types of domain structure: SulP only, SulP with a C-terminal STAS domain, or two SulP domains followed by a C-terminal STAS domain (Fig. 4). None were fused to carbonic anhydrase domains, or adjacent to genes encoding carbonic anhydrase, unlike other SulP transporters implicated in DIC uptake (Felce and Saier 2004; Price et al. 2004). However, SulP transporters from "*Cyanobacteria*" (Price et al. 2004) and "*Proteobacteria*" (Scott et al. 2019) that have measureable DIC uptake activity lack this carbonic anhydrase domain, so its presence is not necessary for DIC transport. This absence of carbonic anhydrase domain in SulP capable of DIC transport makes it quite difficult to predict substrates transported by different SulP proteins based on their sequences, and phylogenetic analysis also does not cluster genes by substrate (Scott et al. 2019). Accordingly, it is not possible to predict with confidence whether these genes encode DIC transporters in the members of *Thiomicrospira*, *Thiocapsa*, or *Marichromatium* where they are present near carboxysome loci (Fig. 5).

Pfams of other transporters and membrane channels are less broadly distributed in carboxysome gene neighborhoods. Genes encoding the membrane component of ATP-binding cassette transporters (ABC membrane, Pfam00664) were found near the carboxysome locus in 15 organisms (Fig. 3), 12 of which were members of "*Cyanobacteria*" (Table S5, Supporting Information). The ABC transporters specific for HCO_3^- that have been studied in members of this phylum consist of four subunits: a solute-binding protein, a membrane-spanning permease and two ATPase subunits, one of which may play a regulatory role (Omata et al. 1999; Price 2011). The 15 ABC transporter genes found in this study to be present near carboxysome loci encode a transporter with a different subunit structure; no genes encoding solute-binding proteins are found nearby, and these genes encode both permease (Pfam00664) and ATP-binding domains (Pfam00005) as a fusion protein. If these transporters are specific to DIC, that would be interesting, as they are structurally distinct from the HCO_3^- transporting ABC transporters that have already been characterized.

Members of Pfam01925 (sulfite transporter TauE) were found in the carboxysome gene neighborhoods of 11 of the organisms studied here, ten of which were "*Cyanobacteria*" (Fig. 3, Table S5, Supporting Information). In other organisms, these transporters export sulfite produced from metabolizing sulfonates (Weinitschke et al. 2007). The genes neighboring these potential sulfite transporters do not encode enzymes for metabolizing these compounds, though in *Ectothiorhodosinus mongoli-*

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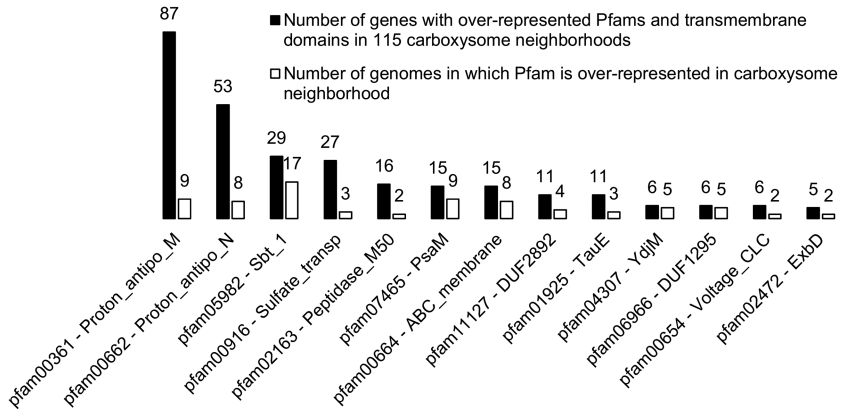

Figure 3. Tally of genes whose products are predicted to have transmembrane domains, whose Pfams that are enriched in carboxysome neighborhoods, and the number of genomes in which these Pfams are enriched in carboxysome neighborhoods. Genes that are present in at least five copies, and in at least two genomes, are represented here. A full list of all genes is provided in Table S4.

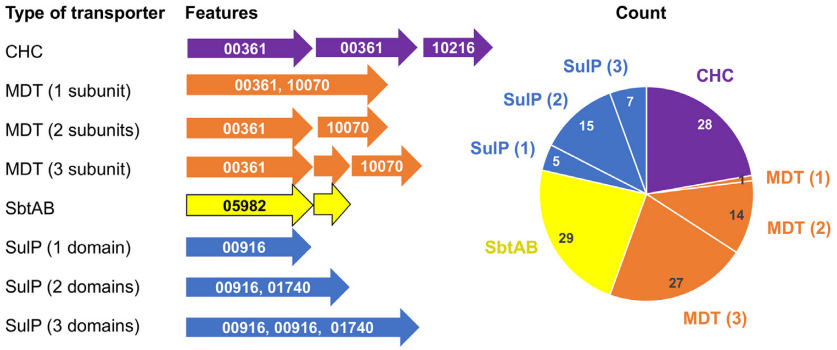

Figure 4. Features and counts of transporter types present in carboxysome neighborhoods. Transporter types shown here are those for which DIC transport activity has been measured for at least one member. Under features, each arrow indicates a gene, and each gene is labeled with the Pfam domains predicted from the amino acid sequence. The pie chart indicates the number of each type of transporter found in carboxysome gene neighborhoods from the 115 genomes studied here. Abbreviations for transporter types: CHC, CO_2-hydration complex; MDT, multisubunit DIC transporter; SbtAB, sodium bicarbonate transporter; SulP, SulP-family transporter. Pfam names: 00361, Proton-conducting membrane transporter; 10216, CO_2 hydration protein (ChpXY); 10070, Uncharacterized protein conserved in bacteria (DUF2309); 05982, Na^+-dependent bicarbonate transporter superfamily; 00916, sulfate permease family; 01740, STAS domain.

cus a neighboring gene encodes sulfide:quinone reductase (E.C. 1.8.5.4), suggesting that this transporter may be active on sulfite or other product of sulfide oxidation.

Genes encoding six proteins belonging to Pfam00654 (voltage-gated chloride channel) are present in four members of "*Cyanobacteria*", and two copies are present in *Acidothiobacillus ferrivorans* (Fig. 3; Table S5, Supporting Information). Some members of this protein family are capable of facilitating the diffusion of other anions besides chloride across the membrane, including HCO_3^- (Suzuki, Morita and Iwamoto 2006). Since channels such as these allow substances to travel down their gradients, it seems unlikely that they play a role in CCMs by generating elevated intracellular DIC concentrations.

A total of five genes encoding members of Pfam02472 (biopolymer transport protein ExpD/TolR) are present in one cyanobacterium and two species of *Marichromatium* (two copies each; Table S5, Supporting Information). Since genes encoding ExpB and TonB neighbor them, it is likely that they function as part of a Ton complex, to bring large molecules past the outer membrane and deliver them to inner membrane transporters (Maki-Yonekura *et al.* 2018). They therefore seem less likely to function as part of a CCM.

Some of these genes belong to Pfams whose members are not known to act as transporters. 16 genes coding for proteins with a Pfam02163 domain (Peptidase family M50) were found downstream from carboxysome loci in some members of "*Cyanobacteria*". When these proteins were aligned, the HEXXH motif necessary for metal binding by the active site was apparent (Rawlings and Barrett 1995), so it does not seem likely that these genes are involved in DIC uptake. 15 genes, all from members of "*Cyanobacteria*", belong to Pfam07465 (PsaM, photosystem I reaction center subunit XII). These single transmembrane domain proteins are 60–70% identical to the biochemically characterized PsaM protein from *Synechococcus elongates* (Uniprot P0A404), so it is likely that they are a component of photosystem I.

All six members of Pfam04307 (YdjM; LexA-binding, inner membrane-associated putative hydrolase) and Pfam06966 (Protein of unknown function DUF1295) are present near the carboxysome loci of members of *Thioalkalivibrio* (Fig. 5). The chances that either of these include DIC transporters is diminished by a few factors. Homologs of these genes are not collocated with genes encoding enzymes relevant to DIC metabolism in other organisms; also, their collocation with carboxysome loci in a single genus makes it seem more likely that this collocation is by chance, not by shared function.

The 11 members of Pfam11127 (protein of unknown function DUF2892) are found among "*Proteobacteria*", and have one or two transmembrane domains (Fig. 3 Table S5, Supporting Infor-

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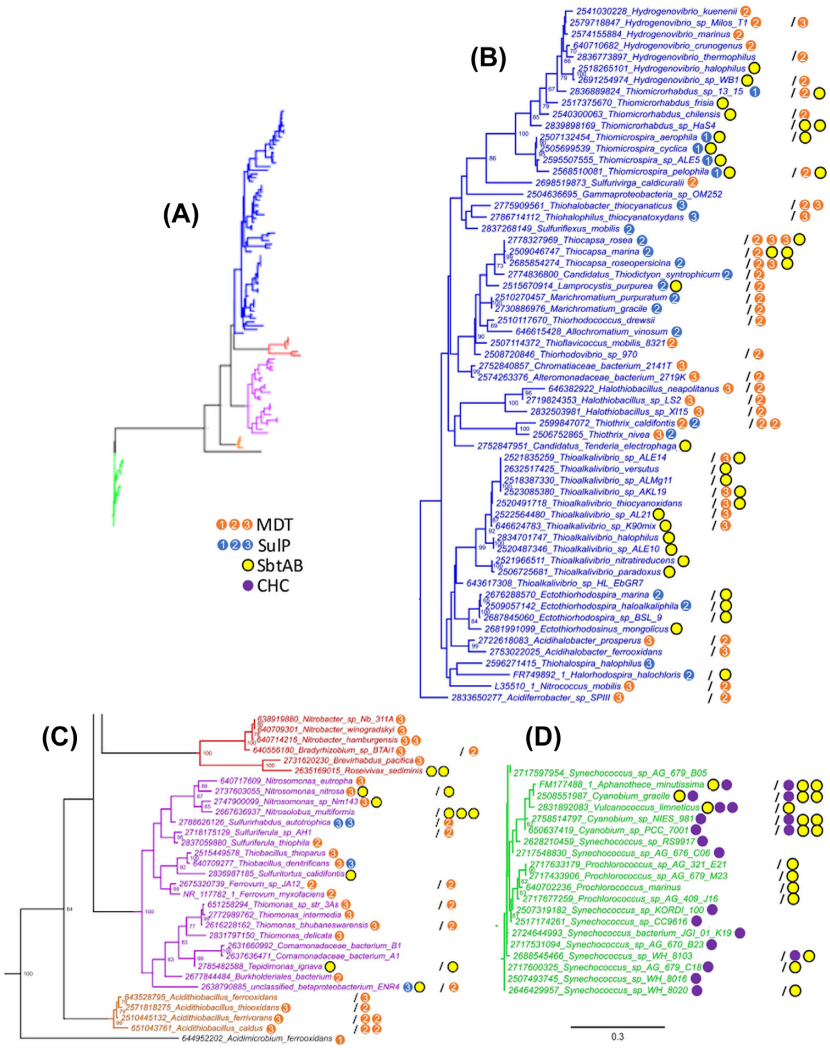

Figure 5. Transporter types distributed on an unrooted maximum likelihood tree based on 16S rRNA gene sequences of host organisms. Transporter types shown here are those for which at least one member has demonstrated DIC transport activity. Taxon names are preceded by gene object ID numbers from Integrated Microbial Genomes and Microbiomes. Taxon names are followed by transporter types whose genes are present in carboxysome gene neighborhoods. Transporter types following '/' are CHC, MDT or SbAB encoded by genes located elsewhere on the genome. Abbreviations for transporter types: CHC, CO_2-hydration complex; MDT, multisubunit DIC transporter (1-, 2-, or 3-subunit type); SbtAB, sodium bicarbonate transporter; SulP, SulP-family transporter (1-, 2- or 3-domain type). **A.** Intact tree to show original topology of the tree. **B.** Members of *Gammaproteobacteria*. **C.** Members of *Alpha-* (red) and *Betaproteobacteria* (purple), *Acidithiobacillia* (orange) and *Actinobacteria* (black). **D.** Members of *Cyanobacteria*. The tree is based on 1006 sites, bootstrap values greater than 60 are displayed at the nodes, and the scale bar indicates the number of substitutions per site.

mation). Given the absence of functional information about members of this Pfam, it is not possible to infer whether they might be involved in CCMs.

It is apparent that transporter gene type near the carboxysome locus follows from host taxonomy. Members of the same genus often have the same type of transporter encoded near their carboxysome locus (Fig. 5). This pattern results from gene synteny conserved among members of the same genus (Figure S1, Supporting Information). Between genera, synteny is not conserved outside the carboxysome locus (Figure S1, Supporting Information). Despite this lack of synteny between genera, the 51 genera selected for this study share a pattern of having transporters in the neighborhood of the carboxysome locus, suggesting selective pressure for them to be encoded there. This collocation across genera of genes encoding MDT, Sbt and SulP transporters and carboxysome loci strengthens the case that these transporters act together with carboxysomes in CCMs.

Proteins from Pfams whose members solely transport DIC, that are present elsewhere on the chromosome besides the carboxysome loci neighborhoods

Members of Pfams 00361 (verified to have either members of Pfam10216 (CHC), or 10070 (MDT) nearby; see above), and 05982 (Sbt) are also encoded on the genome away from carboxysome loci. Since all biochemically characterized Sbt, CHC and MDT transporters are capable of DIC transport, it seems reasonable to suggest that the proteins encoded by these non-carboxysome loci associated genes are also capable of DIC transport.

Non-carboxysome loci associated MDT are particularly prevalent in phylum "*Proteobacteria*". Genes encoding these transporters are present in 65 of 92 members of *Acidithiobacillia, Alpha-, Beta-* and *Gamma-proteobacteria* queried here, and also in a member of phylum "*Actinobacteria*". CHC encoded elsewhere on the chromosome cluster with genes encoding CHC that are

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inducible in other "*Cyanobacteria*", which are expressed under extreme DIC limitation (Shibata *et al.* 2002b; Battchikova, Eisenhut and Aro 2011). For organisms with multiple types of DIC transporters, and multiple copies of a single type of DIC transporter, having the possibility of independent regulation, as is possible if genes are distantly located on the chromosome, may lend more versatility to an organism's CCM, as different transporters may have different affinities for DIC, V_{max} values, or energetic expenses.

Diversity of DIC transporters argues against physical interaction of DIC transporters with carboxysomes

Intuitively, it would seem that docking transporters to carboxysomes would facilitate the transfer of DIC to these microcompartments. If such physical docking would occur, one would anticipate that genes encoding transporters and microcompartments would be tightly co-regulated, and therefore colocated on the chromosome, as genes encoding carboxysomes and DIC transporters frequently are (Fig. 5).

The advantage that docking could confer would be greatest if there were a possibility that the time it could take for DIC to diffuse from the transporter to the carboxysome would be lengthy in the absence of docking. HCO_3^- has a diffusion coefficient of approximately 1000 $\mu m^2/s$ in water (roughly double that of CO_2). In bacterial cytoplasm, small molecules tend to have a diffusion coefficient approximately 25% of that in water (Verkman 2002); a realistic estimate for the diffusion coefficient of HCO_3^- is 250 $\mu m^2/s$ within a cell. The approximate timescale for a molecule to traverse a cell is

$$\tau = R^2/6D,$$

where τ is the time scale of diffusion, R is the traverse distance and D is the diffusion coefficient (Milo and Phillips, 2015). This gives an estimate of only 0.16 ms for an idealized molecule of HCO_3^- to diffuse across a cell of 0.5 μm width. This short timescale of diffusion over this small distance suggests that docking of the carboxysomes to transporters is not necessary to enhance flux of HCO_3^- to these microcompartments. The diversity of DIC transporters also makes carboxysome-transporter docking seem less likely, as it would have to include mechanisms to accommodate at least three different evolutionarily distinct DIC transporters (CHC, MDT, Sbt, as well as some members of SulP).

CONCLUSIONS

Based on the results presented here, MDT are very common among autotrophic members of "*Proteobacteria*" and CHC among "*Cyanobacteria*", while Sbt-type transporters are common among members of both phyla. SulP-type transporters are also well-represented in carboxysome loci, though the broad substrate specificity of members of this transporter family makes it unwise to suggest these all transport DIC. Of the 115 taxa queried here, 77 of them have confirmed DIC-transporters (MDT, CHC, Sbt) encoded near carboxysome loci, and 32 more have these transporters encoded elsewhere on their chromosomes (Fig. 5). Therefore, in total, 109 of 115 taxa with carboxysome loci have some form of DIC transporter encoded in their genomes, which is a conservative estimate since it seems likely that more types of DIC transporter remain to be found. While it is accepted that CCMs in "*Cyanobacteria*" consist of DIC transporters acting with carboxysomes, whether this was also the case in other phyla has not been determined. The genome data presented here strongly suggest a uniformity in CCM components including and beyond "*Cyanobacteria*": carboxysomes partnering with DIC transporters.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSLE* online.

ACKNOWLEDGMENTS

We are grateful for insightful discussions with Gordon Cannon, and the helpful comments of the anonymous reviewers. KSB, SB, JWB, CPC, JFG, JKJ, AL, JMM, PWR, JMS, SS, CT, KFW and RW contributed to this study as students in the USF Genomics class in Fall 2018.

FUNDING

This work was supported by the National Science Foundation [grant numbers NSF-MCB-0643713 and MCB-1952676] to KMS and the Office of Science of the U.S. Department of Energy DE-FG02-91ER20021 to CAK.

Conflicts of Interest. None declared.

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