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Comparative genomic analyses of transport proteins encoded within the red algae *Chondrus crispus, Galdieria sulphuraria* and *Cyanidioschyzon merolae*[1]

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Abstract

Galdieria sulphuraria and *Cyanidioschyzon merolae* are thermo-acidophilic unicellular red algal cousins capable of living in volcanic environments, although the former can additionally thrive in the presence of toxic heavy metals. Bioinformatic analyses of transport systems were carried out on their genomes, as well as that of the mesophilic multicellular red alga *Chondrus crispus* (Irish moss). We identified transport proteins related to the metabolic capabilities, physiological properties, and environmental adaptations of these organisms. Of note is the vast array of transporters encoded in *G. sulphuraria* capable of importing a variety of carbon sources, particularly sugars and amino acids, while *C. merolae* and *C. crispus* have relatively few such proteins. *C. crispus* may prefer short chain acids to sugars and amino acids. Additionally, the number of encoded proteins pertaining to heavy metal ion transport is highest in *G. sulphuraria* and lowest in *C. crispus*. All three organisms preferentially utilize secondary carriers over primary active transporters, suggesting that their primary source of energy derives from electron flow rather than substrate-level phosphorylation. Surprisingly, the percentage of inorganic ion transporters encoded in *C. merolae* more closely resembles that of *C. crispus* than *G. sulphuraria* but only *C. crispus* appears to signal via voltage-gated cation channels and possess a Na^+, K^+-ATPase and a Na^+-exporting pyrophosphatase. The results presented in this report further our understanding of the metabolic potential and toxic compound resistances of these three organisms.

Keywords

genomes; metabolic potential; proteomes; red algae; transmembrane transport systems

Introduction

Genomes provide valuable insight into the ways in which organisms carve niches for themselves in the world. Various adaptations required for survival are reflected by the contents of genomes. Physiological differences stem from the production of unique sets of proteins, and thus the best way to understand the adaptations enabling an organism to

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survive in its chosen environment is to carefully consider its encoded proteins (Berner & Salzburger 2015, Wang et al. 2015, Tigano & Friesen 2016).

The ability of some algae, such as *Galdieria sulphuraria* (Galdieri) Merola (hereafter referred to as Gsu; Merola et al. 1982), to not only live, but thrive in volcanic localities is quite unexpected when we consider typical eukaryotic characteristics. Gsu is a unicellular thermo-acidophilic red alga that lives in the springs, rivers, and soils of these environments where it can represent nearly all of the biomass (Schonknecht et al. 2013). Extremophiles such as Gsu can live under conditions considered hostile to most other species. The specifications that define these environments can include acute high and low temperatures, extreme pH ranges, and high salt and metal ion concentrations (Canganella & Wiegel 2011). In order to survive in these drastic habitats, the ancestral predecessors of Gsu must have adapted their phenotypes, and therefore their genotypes, accordingly to their surroundings. These changes might not merely allow these organisms to weather extreme conditions, they may also have enabled Gsu to out-compete neighboring species (Wang et al. 2015).

Like other rhodophytes (red algae), Gsu uses phycobilisomes, protein complexes that capture light energy for photosynthesis (Su et al. 2010). Gsu usually lives in minimal light environments that are characteristic of their murky volcanic habitats. One feature that sets Gsu apart from other autotrophic red algae is that the subunits of its light harvesting complex display tighter couplings, and so are more efficient in energy retention than homologous protein complexes from other species (Thangaraj et al. 2011). Yet, while this alga is an asexual photosynthetic extremophile, it is also a heterotroph that can obtain carbon and energy from exogenous sources (Schonknecht et al. 2013). The fact that Gsu has heterotrophic abilities, and that it retains the metabolic pathways lost in other algae, seems to give credence to the strong evolutionary pressures that its volcanic habitats provide (Schonknecht et al. 2014).

Gsu is a member of the Cyanidiophyceae class, which diverged about 1.3 billion years ago from other rhodophytic classes (Ciniglia et al. 2004). However, Gsu has also been the recipient of multiple horizontal gene transfers (HGT), having acquired genes encoding non-native enzymes that promote its high metal and halo-tolerance (Rothschild & Mancinelli 2001, Schonknecht et al. 2014). Recent genome analyses have revealed the presence of sequences encoding archaeal ATPases, bacterial antiporters, and bacterial pumps in Gsu (Schonknecht et al. 2014). These non-native proteins seem to give Gsu not only an edge in survival, but also the ability to become the majority species in its unusual environments. Additionally, the fact that the estimated divergence of *Galdieria* from its closest characterized cousin, *Cyanidioschyzon merolae* P. DeLuca, R. Taddei & L. Varano (hereafter referred to as Cme), occurred about 1 billion years ago, shows that Gsu is a unique rhodophyte even among its closest recognized relatives (Schonknecht et al. 2014).

Cme is another red alga that can be found inhabiting extremely acidic hot springs. For example, it has been found at the Phlegraean Fields, a volcanic area west of Naples, Italy (Yoon *et al.* 2006). The pH of the springs in which it lives is extremely low, usually around 1.5, and the temperatures are around 45°C (Matsuzaki et al. 2004). Cme has been considered to be one of the most primitive photosynthetic eukaryotes, especially when considering its

genomic simplicity (Cunningham et al. 2007). It is a small, club-shaped, unicellular alga with a 2 μm diameter (Yagisawa et al. 2009). Due to its size, it lacks a rigid cell wall and only carries one nucleus, one mitochondrion, and one plastid in addition to one Golgi apparatus, one endoplasmic reticulum, and some polyphosphate-rich compartments (Yagisawa et al. 2009). This is a far smaller organelle composition than is generally present within animal or plant cells, and this minimal composition means that determining the behavior of organelles during mitosis and the rest of the cell cycle is simplified (Misumi et al. 2005). Its genome is also the smallest amongst photosynthetic eukaryotes but is highly specialized for its environment. The complete nucleotide sequence of its nuclear genome, as well as its plastid genome, is available for analysis (Ohta *et al.* 2003).

Few genes within Cme contain introns, and they are in small numbers, unlike most other eukaryotes (Nozaki et al. 2007). Previous BLAST searches and annotation results determined that Cme has genes in common with plants and animals, implying that it is related to a common prokaryotic ancestor (Misumi et al. 2005). Due to its small genome and its classification as a primitive alga, it may be a good model organism for the study of the origin of eukaryotes and various types of endosymbiosis (Misumi et al. 2005). Its nucleus, mitochondrion, and plastid are spherical or disk-like in shape. Their divisions can be synchronized using light treatment (Moriyama et al. 2010). Cellular mechanisms studied using Cme are present in both higher animals and plants, making Cme a unique candidate for studying mitochondrial and plastid divisions (Kuroiwa *et al.* 1998).

Because transport proteins are integral to nutrient acquisition, waste removal, and signaling, they can provide a connection between an organism's genome and its environment (Getsin et al. 2013). Focusing on their characterization in Gsu and Cme might therefore elucidate aspects of their physiologies. They might also reveal how horizontally transferred genes were selectively retained in Gsu for its survival under extreme conditions (Lam et al. 2011).

Over time, transporters evolve complexity with altered substrate specificities, and studying these features might provide molecular insight as to how extremophilic red algae became different from their rhodophytic relatives (Lam et al. 2011). As such, proteome analyses were performed for transporters in Gsu and Cme, as well as the mesophilic red alga, *Chondrus crispus* Stackhouse (Gigartinales) or Irish moss (hereafter referred to as Ccr). In contrast to Gsu and Cme, Ccr is a member of the Florideophyceae class, and is a macroalga. Yet, this organism has fairly small and compact gene families (Collen et al. 2013). A comparison between the transporters found in these three rhodophytes and their characterizations could assist in furthering our understanding of the adaptations of Gsu and Cme to extreme environments, as well as the processes that allowed them to branch away to become their own class.

Methods

Gsu and Ccr proteomes were retrieved on September, 2014 from the protein database of the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov) website and were screened by our genome BLAST (GBLAST) program against the transporters tabulated in the Transporter Classification Database (TCDB; www.tcdb.org; (Reddy & Saier

2012). The same procedure was performed for Cme in January, 2015. Each putative open-reading frame was used as a query in the BLASTP software to search for proteins homologous to those in TCDB under a stringent comparative e-value score cutoff of 0.001. The low complexity filter was not used as it is normally of value for larger datasets that include proteins with multiple repeat elements. The information provided includes suspected query transporters, their top TC hits, TC hit accession numbers and short descriptions, protein sequence match lengths, e-values, numbers of transmembrane segments (TMSs) in both query and hit proteins, and numbers of overlapping TMSs. The second-to-last feature was accomplished through the HMMTOP 2.0 program, which scans each open reading frame to predict the number of putative TMSs (Tusnady & Simon 1998). The resulting Gsu, Cme and Ccr lists of putative transport proteins were screened for false positives, and those that displayed 0 or 1 TMSs through the HMMTOP program were removed unless their identified protein families were shown to have membrane insertion capabilities. This would eliminate non-integral and non-multispanning membrane proteins, but still retain 0 or 1 TMS proteins such as the β–barrel porins. In cases where TMS numbers needed to be resolved between the query and hit, the Web-based Hydropathy, Amphipathicity and Topology (WHAT) program (Zhai & Saier 2001) was used with a window size of 19 residues and an angle of 100 degrees to display the protein's hydropathy and amphipathicity plots. Though the WHAT program uses the HMMTOP program to highlight regions where predicted TMSs could be, this program sometimes predicts incorrectly, and it is ultimately up to the user to judge the actual TMS numbers (Reddy et al. 2014). An arbitrary criterion for a TMS would be a hydrophobic peak with a value of greater than 1 (Zhai & Saier 2001). In certain cases where the hydrophobicity plots were unclear, the TOPCONS web server program was used to provide a consensus TMS prediction. The current TOPCONS uses an algorithm that combines the prediction of five programs, which include SCAMPI, OCTOPUS, ΔG-scale, ZPRED, and PRO/PROVID-TMHMM (Bernsel et al. 2009, Reddy et al. 2014).

When comparative e-value scores retrieved from the GBLAST program were particularly low, yet the TMS numbers and sequence locations were similar, the Global Sequence Alignment Tool (GSAT) was used to check for statistical significance. The GSAT program utilizes the EMBOSS Needleman-Wunsch algorithm to provide a global alignment and gives a standardized comparison score based on a user-defined number of shuffles (Reddy & Saier 2012). An additional measure of verification is the use of the NCBI website's Conserved Domain Database (CDD) search, which allows visualization of the conserved domains within a query transporter, and this can be compared to the motifs/domains in a hit protein family.

To find novel transport proteins, distantly related to any in TCDB, GBLAST searches of the Gsu, Cme, and Ccr proteomes were performed again with a poorer e-value cutoff of 0.1. This produced a larger list of putative transporters. Only the proteins with comparative scores between 0.001 and 0.1 were scrutinized via the aforementioned steps for detecting false positives and negatives, and verified candidate transporters were entered into TCDB. For each of the proteins in the verified list of transporters, the scientific literature was checked for substrate specificities and mechanism, and annotated with a UniProt accession number in Table S1 in the Supporting Information. It should be noted that protein sequences

with the same UniProt accession number were only counted as a single entry, and this is also reflected in the total count of proteins in the three red algal proteomes.

Results

Statistical analyses of transport proteins found in Ccr, Gsu and Cme

The genomes of the three red algae were scrutinized for transport proteins by using the proteomes of these organisms as separate queries in comprehensive BLAST searches against the Transporter Classification Database using the GBLAST program (TCDB; www.tcdb.org; (Saier et al. 2016). Table 1 summarizes our analyses according to TC class and subclass, while Table 2 summarizes these results according to substrate specificity. Finally, Table S1 presents the detailed information upon which Tables 1 and 2 were based, that is, the predicted TC classified transport-related proteins found in each of the three red algal genomes.

The percentages of the TC classes and subclasses are depicted for the three red algae in Figure 1. It appears that of Ccr's 9836 predicted proteins, only 312 proteins or about 3.17% are recognized multispanning integral membrane transport proteins. Gsu has 477 such proteins, about 6.47% of its 7375 predicted proteins. In comparison, Cme has 294 integral membrane transport proteins, but also has a much smaller complete proteome, containing 5044 proteins. Therefore, approximately 5.83% of Cme's proteome corresponds to transporters (Table 1). While Ccr has the largest genome of the three red algae, the percentage of its proteins that conforms to the prediction of known integral membrane transport proteins is less than half of that of Gsu, and despite Cme having the smallest genome of the three, its transport protein percentage is still less than that of Gsu's.

Class 1 proteins in TCDB include those that either form permanent transmembrane channels, or are capable of forming transient membrane pores. The transport mechanism used by these proteins is, in general, free diffusion, being energy independent (Spencer & Rees 2002, Zeth & Thein 2010). When the numbers of Class 1 proteins are compared, the relationship between Ccr and Gsu seems closer than that of either alga to Cme (44, 50, and 31 respectively; Table 1). However, the corresponding percentages of class 1 proteins with respect to the total number of transporters are 14.1% for Ccr, 10.4% for Gsu, and 10.5% for Cme, revealing closeness between the latter two Cyanidiophyceae (Figure 1). While passive diffusion-mediating transporters are important for red algae in general, they do not seem to confer particular benefits to the survival of extremophilic red algae. However, they may be important for maintenance of the multicellular state.

Types of channel proteins are further distinguished by their subclasses, of which 1.A, 1.B, and 1.F are relevant due to their presence in the three red algal genomes. Subclass 1.A transporters are characterized by transmembrane α-helical secondary structure-forming sequences. The numbers of these proteins in Ccr, Gsu, and Cme are 32, 36, and 22, respectively. Thus, Gsu has more than Ccr, which has many more than Cme (Table 1). Subclass 1.B includes outer membrane porins that are typically made up of transmembrane β-barrels. They usually exhibit a single N-terminal α-helical transmembrane segment (TMS) as the targeting sequence to an organelle. Both Ccr and Gsu have five of these proteins,

while Cme has four. Subclass 1.F includes the vesicle fusion pore proteins, which form complexes that promote interaction of vesicles with the cell membrane for transient creation of pores, usually for solute exocytosis (Fuhrmans et al. 2015). Between Ccr and Gsu, there is a difference of two in the number of proteins of this subclass (7 and 9, respectively). Cme has five such proteins. Of the percentages of each aforementioned subclass, relative to the total number of transport proteins in the three red algae, TC subclass 1.A α-type channels have the greatest difference of 2.8% between Ccr and the two Cyanidiophyceae algae, while TC subclasses 1.B and 1.F have no more than 0.6% and 0.5% differences, respectively (Figure 1).

Class 2 proteins are those that function by secondary active transport, usually energized by ion (H^+ or Na^+) electrochemical gradients (Shi 2013). Of the class 2 transporters, only the porters of subclass 2.A, which include uniporters, antiporters, and symporters, have been found in the three genomes (Table 1). Ccr, Gsu, and Cme respectively have 121, 252, and 144 such porters, largely accounting for the differences in transport protein numbers between Gsu and the other two algae. These surprising differences contrast with the relevant proportions of channel proteins, where Ccr had far more channels than Cme. The corresponding subclass 2.A percentages of 38.8% for Ccr, 52.8% for Gsu, and 49.1% for Cme reveal that porters are a major fraction of the transporters in the three genomes. The percent difference of 3.7% between Gsu and Cme pales in comparison to the 10.3% difference between Cme and Ccr, again suggesting the closeness between the two Cyanidiophyceae (Figure 1). The 14% difference between Ccr and Gsu is the largest found within any of the various subclasses. This suggests that catalysis by secondary active transport is most important for red algal growth and survival, and that photosynthesis and electron transfer processes are preferentially used for energy generation compared with substrate level phosphorylation.

Class 3 proteins are those that function by primary active transport mechanisms in order to move solutes through the membrane against large concentration gradients (Enkavi et al. 2013). Many class 3 proteins are subunits of transport complexes, where only the channel-forming integral membrane constituents are tabulated. The numbers of these transport proteins are 90, 98 and 71 for Ccr, Gsu, and Cme, respectively (Table 1). Class 3 proteins are the second largest transporter group in all three algae, with percentages being 28.8%, 20.5%, and 24.1% for Ccr, Gsu, and Cme respectively (Figure 1). Thus, although Gsu has the largest number of these proteins, it has the smallest percentage. With the largest percent difference being no more than 8.3%, it seems that transporters utilizing primary energy sources play a less important role to the survival of extremophilic algae, than secondary carriers.

Of the primary active transport subclasses, 3.A, 3.D, and 3.E appear in the genomes of Ccr, Gsu, and Cme. Subclass 3.A includes proteins that energize substrate transport by hydrolysis of pyrophosphate bonds. Ccr has 67 transport proteins in this subclass, Gsu has 73, and Cme has 48 (Table 1). These numbers account for 21.5%, 15.3%, and 16.4% of the total number of transport proteins within the respective genomes (Fig. 1). Subclass 3.D includes transport proteins that are energized by redox reactions, and Ccr, Gsu, and Cme have 19, 20, and 17 of these proteins (6.1%, 4.2%, 5.8% of the total transporter count), respectively. Subclass 3.E includes transport systems that use solar energy for transport, and Ccr has four proteins in

this category, while Gsu and Cme both have five. This results in percentages of 1.3%, 1.0%, and 1.7%, respectively. Therefore, of the class 3 subclasses, 3.A phosphate bond hydrolysis-driven transporters display the greatest difference of 6.2% between Ccr and Gsu; other percentage differences do not exceed more than 2.1% (Figure 1). The sum total of these differences is probably not significant, suggesting that these organisms have comparable abilities to generate electro-chemical gradients using electron flow.

Class 4 proteins are those that catalyze substrate transport accompanied by substrate modification via some coupled process. Subclass 4.C proteins, which utilize Coenzyme A to form carboxylic acid thioesters, were found in Ccr and Gsu, but not in Cme. The included Fatty Acid Transporter (FAT) Family proteins (TC #4.C.1) have been thought to allow coupled fatty acid uptake through the use of acyl-CoA synthetases (Schneider et al. 2014). There appears to be one such FAT protein in Ccr and two in Gsu (Tables 1 and S1). With these proteins contributing an overall low percentage of the total protein transporters in their respective algae, it seems unlikely that they play a role in providing a special benefit for Gsu's survival in extreme environments (Fig. 1). An interesting feature of Gsu's FAT proteins is that they were reportedly obtained laterally from the thermophilic bacterium *Roseiflexus castenholzii* (Schonknecht et al. 2013).

Proteins in subclass 4.D can be involved in group translocation of polysaccharides to the extracytoplasmic environment (Davis 2012, Hubbard et al. 2012, Weigel 2015). Members of the Putative Vectorial Glycosyl Polymerization (VGP) Family (TC #4.D.1) appear in all three algae (2 for Ccr, 2 for Gsu, and 1 for Cme; Table S1). Ccr seems to be unique in having 2 proteins associated with the Glycan Glucosyl Transferase (OpgH) Family (TC # 4.D.3). As a whole, Class 4 proteins represent 1.6%, 0.8%, and 0.3% of the total transporters in Ccr, Gsu, and Cme, respectively.

Class 5 proteins transport electrons from one side of the membrane to the other, thereby influencing the membrane potential. Ccr has 12 of these proteins, whereas Gsu and Cme have nine and seven, respectively (Table 1). Proportionally, however, this results in 3.8%, 1.8%, and 2.4% of their respective transporters. It seems unlikely that these proteins promote the ability of Gsu and Cme to live in extreme climes (Fig. 1). Gsu and Cme lack proteins of the 5.A subclass, which transport electron pairs, although Ccr has one such protein, and all other class 5 proteins are of subclass 5.B, which transport single electrons. Most likely, transmembrane electron-flow processes do not play a significant role in fitness in extreme environments, but they may serve specific functions, influencing cellular energetics and regulation.

Class 8 includes auxiliary proteins that facilitate substrate transport, usually by enhancing the activity of a transporter. Ccr, Gsu, and Cme appear to have one, five, and one, respectively, of the relevant class 8 proteins (Table 1), of which all belong in the 8.A subclass (see below). The corresponding class 8 percentages are therefore small (0.3% Ccr, 1.0% Gsu, and 0.3% Cme; Fig. 1).

The class 9.A proteins of TCDB include those that are known transporters that function by an unknown mechanism, while those within subclass 9.B are putative transporters with

insufficient evidence to establish a transport function. Ccr has 39 class 9 proteins, Gsu has 59, and Cme has 39 (Table 1). By looking at the class 9 percentages of each alga, one can see that the difference is no greater than 0.9% between any two algae (12.5% for Ccr, 12.4% for Gsu, 13.3%; Fig. 1).

Table 2 categorizes the substrates of transport systems identified in the genomes of Ccr, Gsu, and Cme according to TC class. It can be seen that Ccr's 312 transport proteins form a total of 283 transport systems, while Gsu has 440 systems formed by its 477 transport proteins and Cme has 269 systems formed by its 292 transport proteins. The Class 1 channels and pores of the three algae are dedicated mostly to the transport of inorganic substrates, there being a two-to-one ratio for those transporting cations versus anions (Table 2). A notable feature of Gsu is its number of channel systems transporting carbohydrate polyols (Gsu's 5 versus Ccr's 1 and Cme's 0). These belong to the Major Intrinsic Protein (MIP) Family (TC# 1.A.8) of aquaporins, which often transport both water and glycerol (Bienert et al. 2008). Some of *G. sulphuraria's* MIP proteins were reportedly obtained laterally from the mesophilic bacterium *Xanthomonas axonopodis* (Schonknecht et al. 2013).

A comparison between the numbers of class 2 secondary carriers in the three red algae shows that Gsu has more than the other two in nearly all substrate types except for anions, peptides and their conjugates, lipids, vitamins, and the unknowns (Table 2). Compared to the other two, Gsu displays moderate increases of transporters for inorganic cations, carboxylates, amines, amides, polyamines, organocations, nucleic acid constituents and their conjugates, proteins, drugs, and cofactors. Gsu has a far greater number of systems involved in the transport of organoanions (Gsu's 27 versus Ccr's 0 and Cme's 6), as well as of amino acids and conjugates (Gsu's 24 versus Ccr's 6 and Cme's 8). The greatest numerical increase can be seen in the approximately three-to-one ratio of the sugar and polyol-specific systems (Gsu's 62 versus Ccr's and Cme's 21 and 22, respectively). The secondary active transporter family most responsible for the increase in Gsu's sugar and polyol transporting systems is the Major Facilitator Superfamily (MFS; TC# 2.A.1). This particular superfamily is extremely large and diverse with respect to substrate types (Yan 2013). Whereas Ccr and Cme show four and three members of this family contributing to sugar transport, Gsu has a total of 39 systems involved in this function.

Three Class 2 protein families have been found in the two Cyanidiophyceae algae that are involved in organoanion transport. These include certain members of the MFS, the Organic Solute Transporter (OST) Family (TC# 2.A.82), and the Aromatic Acid Exporter (ArAE) Family (TC# 2.A.85). Such systems could not be found in the genome of Ccr. System numbers are substantially greater for Gsu than for Cme: 15 vs. 2 for MFS and 12 vs. 3 for ArAE.

Two families mostly account for the increase in Gsu's transport systems acting on amino acids and their conjugates (Table S1). They are the Amino Acid-Polyamine-Organocation (APC) Family (TC# 2.A.3), and the Amino Acid/Auxin Permease (AAAP) Family (TC# 2.A.18), both members of the large APC superfamily which display a common 5+5 or 7+7 TMS repeat topology (Vastermark et al. 2014). Gsu has four APC family amino acid transporters while Ccr and Cme both have one (Table S1). Members of the AAAP family

transport a variety of amino acids and their derivatives including the plant hormone, auxin (Fischer et al. 2002). Gsu has 14 such symporters, while Ccr has none and Cme has one (Table S1).

There are similar numbers of protein subunits of subclass 3.A primary active transporters in Ccr and Gsu, while Cme has noticeably fewer (Table 1). The total numbers of transport systems reflects this (Table 2). Similarities in numbers can be seen for transport systems specific for cations and proteins in all three algae. Differences include Ccr lacking transport systems for anions (whereas Gsu and Cme have six and two, respectively), and Ccr having one primary active transport system involved in organoanion transport (whereas the other two have none). Additionally, transport systems in Ccr involved in lipid movement are twice as numberous compared to the other two algae. Gsu has the greatest percentage of primary active transport systems devoted to drug/metabolite efflux.

There are three primary active transporter families that contribute to Gsu's anion transporting capability, one of which is shared with Cme. The first, the Sulfate/Tungstate Uptake Transporter (SulT) Family (TC# 3.A.1.6), is a member of the ATP-binding Cassette (ABC) Superfamily and is expressed in bacteria and plastids (Aguilar-Barajas et al. 2011). The second, the Nitrate/Nitrite/Cyanate Uptake Transporter (NitT) Family (TC# 3.A.1.16), allows cyanobacteria and plastids to assimilate bicarbonate, nitrate, nitrite, and cyanate (Maeda & Omata 2009). Gsu has three proteins and one protein from the two aforementioned ABC families, respectively, whereas Cme has two SulT family members (Table S1). The third family catalyzing anion transport is the Arsenite-Antimonate (ArsAB) Efflux Family (TC# 3.A.4; Yang et al. 2012). Gsu has two such proteins (Table S1). An interesting feature of *G. sulphuraria*'s ArsAB proteins is that they were reportedly obtained laterally from the thermo-acidophilic bacterium *Leptospirillum ferriphilum* (Schonknecht et al. 2013).

All class 5 electron carriers that catalyze transmembrane electron transport. Protein TC# 5.B.2.2.4, found in Gsu, can be conjectured to additionally transport Cu^{+} ions based on the fusion of a 5 TMS N-terminal ferric chelate reductase to a 3 TMS copper transporter. A highly similar protein has been found in the genome of Cme (Table S1). Differences between the three red algae are mostly associated with three protein families, the Disulfide Bond Oxidoreductase D (DsbD) Family (TC# 5.A.1), the gp91phox Phagocyte NADPH Oxidase-associated Cytochrome b558 (Phox) Family (TC# 5.B.1), and the Eukaryotic Cytochrome b651 (Cytb561) Family (TC# 5.B.2).

Some members of the DsbD family catalyze electron transfer from cytoplasmic thioredoxins to periplasmic thioredoxins in Gram-negative bacteria, but other functions are known (see TCDB; (Appia-Ayme & Berks 2002). Gsu and Cme lack DsbD members, but Ccr has one (Table S1). Phox family members are respiratory burst oxidases that transfer single electrons from NADPH to O_2, thus generating superoxides for development, protection, and signaling (Cheng et al. 2001, Cheng et al. 2013). Whereas Ccr has four Phox homologues, Cme has two and Gsu has only one (Table S1). Lastly, Cytb561 proteins are ferric chelate reductases that transfer single electrons from cytoplasmic L-ascorbate to extracellular Fe^{3+} for a variety of purposes (Asard et al. 2013). Plant homologues usually have an N-terminal dopamine-β-

hydroxylase regulatory region (DOMON) for heme or sugar-binding (Luthje et al. 2013), and two of the three Ccr Cytb561 proteins have this feature. The genomes of Gsu and Cme both contain one of these proteins, albeit fused with a putative copper transporter as noted above (Table S1).

Class 8 auxiliary proteins found in Gsu, but not Ccr and Cme, include the Stomatin/Podocin/Band 7/Nephrosis.2/SPFH (Stomatin) Family (TC# 8.A.21) and the Tetraspanin Family (TC# 8.A.40). Stomatin homologues are capable of modulating the Mechanical Nociceptor (Piezo) Family (TC# 1.A.75) ion channels through unknown mechanisms (Poole et al. 2014). Gsu has at least three stomatin proteins. Tetraspanin family members, represented only in Gsu, create physical cell interactions and interact with each other to bind ligands and move intracellular contents (Wang et al. 2012).

Within TC subclass 9.A, only transport systems mediating cation movement have been found in all three algae, while systems that transport lipids have been found only in the two Cyanidiophyceae algae. Ccr, Gsu, and Cme have 11, 10, and seven proteins, respectively, for cation transport, and Gsu and Cme have four proteins and one protein, respectively, for lipid transport. Within subclass 9.B, exclusive to Ccr are members of the Putative Mg^{2+} Transporter-C (MgtC) Family (TC# 9.B.20), and members of the Acyltransferase-3/Putative Acetyl-CoA Transporter (ATAT) Family (TC# 9.B.97), whereas proteins from the Integral Membrane CAAX Protease-2 (CAAX Protease2) Family (TC# 9.B.2) and the Endoplasmic Reticulum Retrieval Protein1 (Rer1) Family (TC# 9.B.82) are missing, though present in the other two red algae. CAAX Protease2 proteins cleave certain C-terminal protein sequences in a pathway involved with substrate trafficking (Pryor et al. 2013). The ability of these proteases to transport the peptide products of hydrolysis has been proposed but not established (Pryor et al. 2013).

Regarding Cme, absent are members of the Integral Membrane CAAX Protease (CAAX Protease) Family (TC# 9.B.1), the Regulator of ER stress and autophagy TMEM208 (TMEM208) Family (TC#9.B.26) and the Rhomboid Protease (Rhomboid) Family (TC# 9.B.102). These families are putatively involved in protein, lipid, and protein transport, respectively. Exclusive to Gsu are transport proteins of the VAMP-associated protein (VAP) Family (TC# 9.B.17) and the Hly III (Hly III) Family (TC# 9.B.30). The former is involved in lipid regulation, while the latter is involved in pore formation and nonspecific osmolyte transport.

Four families may flip lipids in Gsu, but these families are absent from Ccr (Table 2). They include G-protein-coupled receptors (GPCR; TC# 9.A.14), several of which have been shown to flip lipids (Sanyal & Menon 2009, Menon et al. 2011), the Outer Membrane Mitochondrial Cholesterol/Porphyrin Uptake Translocator Protein (TSPO) Family (TC# 9.A.24; (Batarseh & Papadopoulos 2010), of which Cme also has one member, the Ca^{2+}-dependent Phospholipid Scramblase Family (TC# 9.A.36; (Posada et al. 2014), and the aforementioned VAP proteins in the endoplasmic reticulum that function in vesicle biogenesis, exocytosis, and protein stabilization (Lev et al. 2008).

Figure 2 displays the percentages of transport substrate categories in the three red algae studied. A look at the major substrate groups shows that nearly half of the transporters in Ccr and Cme are involved with inorganic molecular transport, while only a third are dedicated to the same in Gsu (44.9% Ccr, 43.1% Cme, 32.5% Gsu; Figure 2). Gsu devotes larger percentages to the transport of carbon sources as well as amino acids and their derivatives than Ccr or Cme. Similar percentages can be seen in all three algae towards transport systems that handle macromolecules, drugs, vitamins, and cofactors. Overall, it seems that Gsu preferentially utilizes organic substrates, while Ccr and Cme prefers inorganic molecules. Thus, the percentage devoted to the transport of sugars and amino acids is nearly double for Gsu compared to the other two algae. For most other categories, the percentages do not differ appreciably.

Figure 3 summarizes the distribution of transporter families encoded within the genomes of Ccr, Gsu, and Cme and organizes them according to occurrence in the three organisms. In all, there are 183 different transporter families represented. 18 are specific to Ccr, 15 are Gsu-specific, and seven are Cme-specific. Gsu shares 19 families exclusively with Ccr and 28 families exclusively with Cme. Ccr shares only one family exclusively with Cme. All three organisms share 95 transport protein families. Some of these families have been described above. Gsu has about 85.8% of the represented families, while Ccr and Cme have 72.7% and 71.6%, respectively.

Channel proteins

Table S1 presents the detailed information about multispanning transport proteins found in the three red algal species analyzed. Several observations are worthy of comment because they are likely to have physiological significance. Ccr has seven paralogues of the Voltage-gated Ion Channel (VIC) Family (TC# 1.A.1), while Gsu and Cme have only one and two, respectively. The fact that the different paralogues in Ccr show greater sequence similarity to different TC entries suggests that these proteins did not merely arise by gene duplication events following the divergence of Ccr from other red algae. They probably serve dissimilar functions. We suggest that in the multicellular organism (Ccr), these channel proteins serve signaling functions analogous to those that are well documented in multicellular plants and animals. Such a signaling function may not be necessary in single-celled organisms such as Gsu and Cme.

The MIP family of aquaporins and glycerol facilitators has three representatives in Ccr, five in Gsu, and one in Cme. However, the paralogues in these three organisms hit different TC subfamilies. In Ccr, the three paralogues hit proteins in three different subfamilies, while for the five paralogues in Gsu, three are most similar to members of one subfamily, while the other two resemble members of another subfamily. Only one of these subfamilies is shared with that of Ccr. The Cme homologue resembles a subfamily that was not hit by any of the other red algal proteins. Three of the Gsu paralogues show the greatest sequence similarity to the *E. coli* glycerol facilitator, which, in addition to polyols, is known to be capable of exporting arsenite and antimonite, negatively charged anions (Meng et al. 2004). By contrast, the three MIP family members in Ccr have different ranges of specificity. For example, two dissimilar homologues respectively, probably transport water, urea, glycerol,

CO_2, NH_3, H_2O_2 and formamide (Loque et al. 2005, Saparov et al. 2007, Bienert et al. 2008, Soria et al. 2010, Gattolin et al. 2011) and most of these compounds are not known to be transported by the *E. coli* glycerol facilitator. The one Cme homologue most closely resembles aquaporin-1 of *Bemisia tabaci* (Sweet potato whitefly; TC# 1.A.8.8.15) which has specificity for water and perhaps small neutral solutes such as urea (Herraiz et al. 2011, Mathew et al. 2011).

Gsu has eight members of the CorA Metal Ion Transporter (MIT) Family (TC# 1.A.35), while Cme and Ccr have six and one, respectively. Five of the Gsu paralogues hit different eukaryotic CorA entries, but three show greatest sequence similarity to a single CorA entry in TCDB of the bacterium *Thermotoga maritima. Thermotoga maritima* CorA recognizes both Mg^{2+} and Co^{2+} as substrates and plays roles in Co^{2+} and Mg^{2+} homeostasis (Nordin et al. 2013). In contrast, the six Cme paralogues and the one Ccr paralogue only hit the eukaryotic CorA entries. As eukaryotic paralogues can catalyze high affinity Mg^{2+} uptake and efflux (Schindl et al. 2007), Gsu and Cme may have greater versatility than Ccr over their intracellular Mg^{2+} concentrations, and Gsu may have greater control over its Co^{2+} concentrations than Cme. Although not suggested by Schonknecht et al. (2013), it is possible that the bacterial-like Gsu paralogues were from a bacterium and underwent two single gene duplication events after transfer to this thermophilic alga to generate the three close paralogues.

A few channel protein families are represented only in Ccr or Gsu. For example, the Nucleotide-sensitive Anion-selective Channel (ICln) Family (TC# 1.A.47) transporters are present only in Gsu, while intracellular chloride channels of the CLIC Family (TC# 1.A.12) and the large mechanosensitive ion channels of the MscL Family (TC# 1.A.22) are present only in Ccr. No channel protein family appears to be represented solely by Cme. Interestingly, Gsu shares five families exclusively with Ccr while sharing four with Cme. The five Gsu-Ccr shared families include the Calcium-dependent Chloride Channel (Ca-ClC) Family (TC# 1.A.17), the Golgi pH Regulator (GPHR) Family (TC# 1.A.38), the Homotrimeric Cation Channel (TRIC) Family (TC# 1.A.62), the aforementioned Piezo Family, and the Mg^{2+}/Ca^{2+} Uniporter (MCU) Family (TC# 1.A.77). The four Gsu-Cme shared families include the Mg^{2+} Transporter-E (MgtE) Family (TC# 1.A.26), the Anion Channel-forming (Bestrophin) Family (TC# 1.A.46), the Magnesium Transporter1 (MagT1) Family (TC# 1.A.76), and the aforementioned OEP16 porin family (TC# 1.B.30).

Several beta barrel porin families were identified in the three red algae, and these include mitochondrial and plastid porins as well as the outer membrane protein insertion porin (OmpIP) constituents. It appears that Ccr lacks members of the Plastid Outer Envelope Porin of 16 kDa (OEP16) Family (TC# 1.B.30) found in the other two algae while exclusively featuring a Chloroplast Outer Membrane Porin (Oep23) Family (TC# 1.B.77) member. All three organisms possess multiple constituents of the Synaptosomal Vesicle Fusion Pore (SVF-Pore) Family (TC# 1.F.1).

Secondary carriers

Compared to Ccr's 20 and Cme's 15 representatives of the MFS Family, Gsu has 71. Most of the increases are accounted for by sugar porters (SP; TC# 2.A.1.1) and phosphate:H^+

symporters (PHS; TC# 2.A.1.9). Gsu has 37 paralogues within the SP family, some hitting prokaryotic homologues with highest scores in TCDB, while others hit eukaryotic homologues. Ccr and Cme have one homologue each, both of which hit different eukaryotic glucose transporters. Members of the SP family in TCDB to which Gsu proteins show greatest similarity are H^+ symporters, which can also transport arabinose, scyllo-, muco-, chiro-, and myoinositols, inositoltriphosphates and fructose (Hernandez-Montalvo et al. 2001, Aouameur et al. 2007, Schneider et al. 2008). The PHS family of phosphate symporters has 15 representatives in Gsu, four in Cme, and none in Ccr. The 15 Gsu paralogues, and two of the Cme paralogues, show greatest similarity to a single member of this family, GIT1 of *Saccharomyces cerevisiae*. This system has been shown to transport inorganic phosphate as well as glycerophosphoinositol and glycerophosphocholine (Almaguer et al. 2006). Finally, Gsu has six members of the Glycoside-Pentoside-Hexuronide (GPH):Cation Symporter Family (TC# 2.A.2), while Ccr has four and Cme has only one. The presence of multiple members of these MFS families corroborates previous studies which revealed that Gsu has a larger and more varied carbohydrate metabolic capability (Barbier et al. 2005, Weber et al. 2007) compared to Ccr and Cme. Interestingly, Ccr possesses MFS porters that can take up organic acids such as oxalate and tartrate, although these systems are lacking in the two Cyanidiophyceae algae. These observations suggest that while Gsu (and maybe Cme) predominately utilize sugars, Ccr may prefer organic acids. This suggestion is further substantiated by the observation that the Sweet Family (TC# 2.A.123) of sugar transporters is not represented in Ccr, but is present in Gsu and Cme.

Gsu has seven members of the Amino Acid-Polyamine-Organocation (APC) Family (TC# 2.A.3), while Cme has two and Ccr has only one. Three of the Gsu paralogues hit eukaryotic APC entries, but four show greatest sequence similarity to a single APC homologue of the archaeon, *Thermoplasma acidophilum*. Two of the eukaryotic paralogues show maximal sequence similarity to APC homologue RMV1 of *Arabidopsis thaliana*, which takes up polyamines and paraquat (Fujita & Shinozaki 2014). The APC homologue in *Thermoplasma acidophilum* has not been characterized but shows similarity to a low-affinity putrescine importer of *E. coli*. While the role of APC family members in Gsu is unclear, it can be suggested that they mainly mediate uptake of polyamines. This contrasts with one of Cme's homologues and Ccr's single APC homologue which show greatest similarity to a vacuolar cationic amino acid transporter of *A. thaliana*. The other Cme paralogue brings up a polyamine symporter from the protozoan, *Leishmania major*.

Related to the APC Family, are the amino acid/auxin uptake permeases of the AAAP Family (TC# 2.A.18). 14 members of this family are represented in Gsu, one in Cme, and none is found in Ccr. 11 of the 14 paralogues in Gsu hit the same TC entry, which suggests that these paralogues were generated by gene duplication during evolution of Gsu. All 14 of these Gsu paralogues probably transport neutral amino acids. Since Gsu has the most APC family members, the combined results suggest that in addition to sugars, Gsu, and to a lesser extent, Cme, in contrast to Ccr, uses polyamines and amino acids as primary nutrients.

Divalent cations, particular Zn^{2+} and Fe^{2+}, are taken up via ZIP Family (TC# 2.A.5) members, while these same cations, as well as other divalent cations, are exported via

members of the CDF Family (TC# 2.A.4). Ccr and Gsu both have three paralogues of the ZIP Family, and Cme has four. Ccr has four members of the CDF Family, while Gsu and Cme have one and three, respectively. The NRAMP Family of divalent metal ion transporters (TC# 2.A.55) can also function in the uptake of divalent cations. Ccr, Gsu, and Cme have two, four, and three representatives, respectively. We presume that the members of the NRAMP and ZIP families can substitute for each other. Interestingly, Cme has four members of the Iron/Lead Transporter (ILT) Family (TC# 2.A.108), a family that handles Fe^{2+} uptake, but these systems are not represented in the other two red algae. We identified three homologues of a cholesterol-modified peptide exporter of the RND Family in Ccr, but no RND family member was found in Gsu or Cme.

The Drug/Metabolite Transporter (DMT) Superfamily (TC# 2.A.7) is well represented in all three algae with 20, 23, and 25 members in Ccr, Gsu, and Cme, respectively. It is interesting that Gsu, in contrast to Ccr, has sugar phosphate uptake porters since only the former organism transports sugars as a principle carbon source. These porters are also present in Cme. However, most members of this family in all three algae transport activated sugars, exporting them from the cytoplasm to the lumen of the endoplasmic reticulum or Golgi apparatus. Many such activated sugars are known including UDP-glucose, UDP-galactose, UDP-N-acetylglucosamine, UDP-glucuronic acid, UDP-xylose, GDP-mannose, and GDP-fucose. DMT porters also take up vitamins and their derivatives such as thiamine pyrophosphate as well as divalent cations such as Mg^{2+}. All of these types are represented in the three organisms.

Ccr and Cme both have two proteins of the Cytochrome Oxidase Biogenesis (Oxa1) Family (TC# 2.A.9), while Gsu has six. One of the Ccr proteins and one of Cme hit the same mitochondrial Oxa1 protein, and the others of the two algae hit a same plastidial homologue. In contrast, Gsu has five that hit the same mitochondrial Oxa1 protein, corresponding to the Ccr/Cme orthologue, and one that hits the same plastidial homologue. The proliferation of close Oxa1 paralogues in Gsu is unexplained.

The next large family of transporters found in all three algae is the Mitochondrial Carrier (MC) Family (TC# 2.A.29). Ccr, Gsu, and Cme have 19, 37, and 29 members of this family, respectively. Thus, Gsu has nearly twice as many as Ccr and about a quarter more than Cme. These numbers reflect the greater numbers of ATP/ADP exchangers, Fe^{2+} transporters, vitamin transporters, and S-adenosylmethionine/S-adenosylhomocysteine exchangers in Gsu compared to Ccr, although the numbers of S-adenosylmethionine/S-adenosylhomocysteine exchangers are similar in Gsu and Cme. In this regard, it is interesting that Gsu and Cme also have one member each of the ATP:ADP antiporter (AAA) Family (TC# 2.A.12), which is lacking in Ccr. These porters are not related to the mitochondrial carriers and instead are distant members of the MFS (Reddy et al. 2012).

Continuing the analysis of Table S1, we see that many of the secondary carrier families are represented in similar numbers in the three organisms. However, Ccr, Gsu, and Cme each have two families not represented in the two other algae. For example, the aforementioned RND Family, and the Equilibrative Nucleoside Transporter (ENT) Family (TC# 2.A.57) are only found in Ccr. The Sulfate Permease (SulP) Family (TC# 2.A.53) and the Mitochondrial

Pyruvate Carrier (MPC) Family (TC# 2.A.105) are found exclusively in Gsu, while the K^+ Transporter (Trk) Family (TC# 2.A.38), and the previously-mentioned ILT Family, are represented only in Cme. Gsu shares two transporter families exclusively with Ccr. These include the Nucleobase/Ascorbate Transporter (NAT) Family (TC# 2.A.40), and the Hydroxy/Aromatic Amino Acid Permease (HAAAP) Family (TC# 2.A.42). Interestingly, Ccr shares the Folate-Biopterin Transporter (FBT) Family (TC# 2.A.71) exclusively with Cme.

Gsu shares with Cme a total of thirteen families not found in the genome of Ccr. These include the aforementioned AAA and AAAP transporter families, the Nucleobase:Cation Symporter-1 (NCS1) Family (TC# 2.A.39), the Auxin Efflux Carrier (AEC) Family (TC# 2.A.69), the K^+ Uptake Permease (KUP) Family (TC# 2.A.72), the Organic Solute Transporter (OST) Family (TC# 2.A.82), the Aromatic Acid Exporter (ArAE) Family (2.A. 85), the Autoinducer-2 Exporter (AI-2E) Family (TC# 2.A.86), the Vacuolar Iron Transporter (VIT) Family (TC# 2.A.89), the Acetate Uptake Transporter (AceTr) Family (TC# 2.A.96), the Ferroportin (Fpn) Family (TC# 2.A.100), the Tellurium Ion Resistance (TerC) Family (TC# 2.A.109), and the previously-mentioned SWEET family. It should be noted that all homologues of Gsu's AceTr Family may have been horizontally transferred from the bacterium *Desulfotomaculum acetoxidans* (Schonknecht et al. 2013). The prevalence of aromatic acid uptake porters in Gsu, compared to Ccr, and even Cme, correlates with the greater prevalence of amino acid transporters belonging to other families as noted above. Thus, this result substantiates the conclusion that Gsu uses amino acids as nutrients to a much greater extent than Ccr and Cme. It is possible that the 10 acetate porters in Gsu function in acetate excretion as an end product of metabolism in addition to their role in acetate uptake.

The significance of the occurrence of transporters of diverse function in Gsu has yet to be established. However, the occurrence of much greater diversity in Gsu, and to a lesser extent for Cme, could be due to the fact that these organisms are unicellular, while Ccr is a multicellular organism. Single-celled microorganisms may have a much greater need to scavenge a diversity of compounds found in nature, while multicellular organisms create their own homeostatic environment with a limited number of nutrients serving as the primary sources of energy and metabolic intermediates (Li et al. 2014).

Primary Active Transporters

As presented in Table S1, Ccr and Gsu have similar numbers of integral membrane proteins identified as probable constituents of ABC transport systems (TC# 3.A.1; 31 Ccr versus 35 Gsu), while Cme has 20. Only nine proteins thought to be involved in solute uptake were identified, two in Ccr, four in Gsu, and three in Cme, all probably occurring in chloroplasts. The two proteins in Ccr are possibly involved in the uptake of 1) taurine and aromatic sulfonates, and 2) lipids. However, it should be noted that the score obtained for the first of the two, when the Ccr proteins were BLASTed against TCDB, was poor (e^{-10}), although a good score was obtained with the second substrate (e^{-45}). In the case of Gsu, the four proteins may be specific for 1) sulfate and 2) other inorganic anions such as bicarbonate, cyanate, nitrite, and nitrate. While the scores for the first three of these proteins were

excellent, suggesting that these are truly sulfate uptake systems, the score for the fourth was poor (e^{-10}), making this substrate classification tenuous. Lastly, in Cme, two of the proteins display high scores for a sulfate uptake system, while the third protein gave a likewise high score for a lipid transporter. All remaining ABC-type integral membrane proteins identified in these algae are almost certainly involved in solute or macromolecular efflux with confidence levels inversely proportional to the score (e-value). Only seven of these proteins resemble bacterial transport proteins more than eukaryotic proteins, and two are present in Ccr, three in Gsu, and two in Cme. Three of these seven paralogues/orthologues, that is, one in Ccr and two in Gsu, are specific for a variety of drugs, although one of the two found in Gsu may also be capable of exporting lipids. One of the Cme paralogues shows an excellent score to an α-hemolysin exporter. All three algae show top hits with an iron export protein, YbbM, from *E. coli*, which functions to enhance resistance to oxidative stress (Nicolaou et al. 2013). Because the algal proteins hit the *E. coli* protein with good scores, it is probable that they function in divalent metal ion export. The cellular locations of these proteins are unknown, but they appear to lack mitochondrial targeting sequences. While all three proteins exhibit 6 TMSs, only the Gsu homologue has an N-terminal ATPase domain of the CbiO family. It is interesting to note that this family includes homologues from plants, one of which has been reported to be a UDP-glucose exporter (Huang et al. 2009).

All remaining ABC transporters in these algae are most similar to eukaryotic type ABC export proteins. As summarized in Table S1, the exporters that they hit in TCDB are specific for 1) multiple drugs and hydrophobic/amphipathic substances, 2) divalent cations, and 3) lipids, sterols, and hydrophobic vitamin derivatives. Most of these systems are of broad specificity with a capacity to transport many substrates. The functions in red algae are likely to be similar.

All three organisms possess subunits of both F-type and V-type ATPases (TC# 3.A.2) involved in 1) energy interconversions in mitochondria and chloroplasts, and 2) intracellular vesicle acidification, respectively. However, in addition, these algae also contain H^+ or Na^+-translocating pyrophosphophatases of the M^+-PPase Family (TC# 3.A.10). While Ccr appears to have only one such system, catalyzing Na^+ efflux, the three Gsu systems and the one Cme system all probably export H^+. This observation correlates with the fact that Ccr is multicellular, maintaining intercellular homeostasis, while Gsu and Cme are unicellular.

P-type ATPases generally function in the uptake or efflux of mono-, di- and tri-valent cations although one family (TC# 3.A.3.8) consists of phospholipid flippases (Thever & Saier 2009, Chan et al. 2010). Two calcium ATPases (TC# 3.A.3.2) are found in both Ccr and Gsu, while three are found in Cme, all with exceptionally good scores to those in TCDB. These function to maintain low cytoplasmic calcium concentrations in all eukaryotes and many prokaryotes. Of particular interest is the fact that while the multicellular Ccr has a homologue of mammalian Na^+/K^+-ATPases (TC# 3.A.3.1), but lacks a H^+-ATPase of the plant/fungal type (TC# 3.A.3.3), the opposite is true of the two Cyanidiophyceae algae. This fact correlates with our observation that only Ccr possesses multiple members of the Voltage-gated Ion Channel (VIC) Family as well as a Na^+-pumping pyrophosphatase. This confirms our suggestion that these proteins in Ccr function in signal transduction, possibly by generating action potentials. In this capacity, a Na^+/K^+-ATPase and a Na^+-pyrophosphate

would be expected to maintain low cytoplasmic concentrations of Na^+ but high concentrations of K^+.

Ccr has three members of the copper ATPase family (TC# 3.A.3.5) while Gsu and Cme have two and one, respectively. Pumps of this family can catalyzes either copper uptake or efflux and can act on either Cu^+ or Cu^{2+}. Gsu and Cme have multiple heavy metal cation exporters which are absent in Ccr. These ATPases are thought to function in protection against heavy metal toxicity in most organisms (Thever & Saier 2009; Chan et al. 2010). Ccr may lack them because of its multicellularity, allowing for other mechanisms of protection. Finally, a total of four Mn^{2+} efflux systems are found in the three algae, Ccr and Gsu both having one and Cme having two. These are likely to be present in the endoplasmic reticulum (ER). Surprisingly, while the Ccr and Cme proteins are a single polypeptide chain, the Gsu system apparently consists of two polypeptide chains encoded by two different genes. One of these proteins corresponds to the N-terminal half of the Ccr protein, while the other codes for the C-terminal half. It could be a genuine split, it could be a pseudogene, or the split could have resulted from a sequencing error. Loss of the corresponding protein in yeast results in ER stress and lowered Mn^{2+} in the ER lumen (Cohen et al. 2013).

Gsu has two arsenite/antimonite efflux (ArsB) proteins that are lacking in Ccr and Cme. These proteins function in protection against these two oxyanions by catalyzing their efflux (Yang et al. 2012).

TC class 3 includes several protein secretion systems, components of which were identified in all three algae (Saier et al. 2008). These include the General Secretory Pathway (Sec) Family (TC# 3.A.5), the Mitochondrial Protein Translocase (MPT) Family (TC# 3.A.8), the Endoplasmic Reticular Retrotranslocon (ER-RT) Family (TC# 3.A.16), and the Peroxisomal Protein Importer (PPI) Family (TC# 3.A.20). Because all constituents of these systems were not always identified in the three organisms, we cannot claim that all of these systems are complete or functional. Additionally, members of the Chloroplast Envelope Translocase (CEPT or Tic-Toc) Family (TC# 3.A.9) have been found in Ccr and Gsu, yet were not identified in Cme, possibly due to insufficient similarity to known members.

Constituents of all three of the mitochondrial H^+-translocating electron transfer complexes were identified in Ccr, Gsu, and Cme, although surprisingly, only Ccr contains the H^+-translocating transhydrogenase. Constituents of the light absorption-driven photosynthetic reaction center (PRC) Family (TC# 3.E.2) were also identified in all three organisms.

Discussion

Galdieria sulphuraria (Gsu) is a thermo-acidophilic single-celled red alga that resides in acidic hot springs frequently containing high concentrations of toxic metals. The environment in which Gsu resides can reach temperatures as high as 55 °C, and have pH values as low as 1, with exposure to high steam pressures (Heilmann et al. 1999). In addition to the extreme conditions under which Gsu can survive, this organism has developed the ability to grow photoautotrophically, mixotrophically, and heterotrophically on over 50 carbon sources (Barbier et al. 2005, Schonknecht et al. 2013). *Cyanidioschyzon merolae*

(Cme) is also a thermo-acidophilic single-celled red alga that resides in sulphate-rich, acidic, hot springs. It was originally isolated from the solfatane fumaroles in a large volcanic area located west of Naples, Italy, known as Campi Flegrei (Yoon et al. 2006). These environments can also reach temperatures of up to 55° C and have pH values as low as 1.5 (Zenvirth et al. 1985). In addition to surviving in these extreme conditions, Cme is thought to be one of the most primitive photosynthetic eukaryotes (Cunningham et al. 2007). It may retain primitive features of cellular and genomic organization, and it has a mixed gene repertoire characteristic of plants and animals (Misumi et al. 2005). This suggests a possible relationship to prokaryotes, even though the alga contains photosynthetic components similar to those of other algal phototrophs. In contrast to the other two, *Chondrus crispus* (Ccr) is an intertidal, multicellular autotrophic red alga, found along the rocky shores of the Atlantic Ocean (Smith & Bidwell 1989, Collen et al. 2013). The differences between these three algae can, in part, be understood by comparing the transport proteins encoded within their genomes and by contrasting the substrates recognized by those transporters.

Despite the similarly extreme conditions under which Gsu and Cme have evolved, the former has much greater physiological diversity. Gsu is a facultative heterotroph with expanded metabolic flexibility, which contrasts with that of most of its rhodophytic relatives, usually obligate photoautotrophs (Barbier et al. 2005). It has been reported that more than 30% of Gsu's gene sequences do not relate to those of Cme, its closest characterized relative, and many of these genes include a large number of membrane transporters and enzymes of carbohydrate metabolism (Barbier et al. 2005). This could be responsible for the fact that Cme is considered more primitive. Its genome contains almost no introns (only 26 genes contain introns) and it has a relatively low degree of genetic redundancy (Matsuzaki et al. 2004). Interestingly, the genomes of both Gsu and Cme encode many different sugar kinases, including gluco-, galacto-, fructo-, xylulo-, ribo-, and glycero-kinases. These enzymes were reported to be essential for Gsu's ability to sense sugars in "feast and famine" responses, which can influence the expression of relevant transporter genes (Oesterhelt & Gross 2002).

Gsu's sugar and polyol uptake genes were not induced in the absence of light, and Gsu only switches to a heterotrophic state when the relevant substrates are present in the external environment in sufficient concentrations (Oesterhelt & Gross 2002). Indeed, Gsu can efficiently grow photoautotrophically even in a minimal light environment (Thangaraj et al. 2011). True mixotrophic growth, which involves the transport of electrons in both photosystems I and II, does not occur in Gsu; heterotrophy is Gsu's preferred mode of growth (Oesterhelt et al. 2007). For its part, Cme uses photosynthesis, yet makes little phycoerythrin, the primary red algal pigment. Instead, it produces the light blue (red-absorbing) pigment phycocyanin and the green pigment (blue- and red-absorbing) chlorophyll. It thus appears blue-green, even though it is classified as a rhodophyte (Ueno et al. 2015).

Gsu has the ability to take up sugars from its surroundings through the use of its many sugar-specific transporters. The most important families to which these transporters belong include the sugar porters of the Major Facilitator Superfamily (MFS), the related symporters of the GPH Family, and the transporters of the Sweet Family. Members of these families allow the

uptake of hexoses such as glucose and fructose, glucuronides, inositols and inositol derivatives. Gsu may use sugars to generate ATP via glycolysis to energize its multiple ATPases, some of which appear to derive from archaea. There is a positive correlation between the expression of archaeal ATPases and the extent of Gsu's heat tolerance, although it is unclear as to why this is so (Schonknecht et al. 2013). The use of inositol derivatives has been found to modulate intracellular Ca^{2+} concentrations and establish monospore polarity of the red alga *Porphyra yezoensis* (Li et al. 2009). Since Gsu also produces endospores, inositol phosphates may have a similar function for this organism (Barbier et al. 2005).

Some organophosphate esters, such as glycerophosphoinositol and glycerophosphocholine, may be taken up by Gsu's PHS transporters for purposes of energy and carbon generation (Almaguer et al. 2006, Oesterhelt et al. 2008). Activated sugars are probably transported into the lumen of the ER and golgi via Gsu's DMT transporters for glycolipid and glycoprotein synthesis. The diversity and multiplicity of sugar transporters in Gsu contrasts with the minimal representation of these systems in Ccr and Cme, a possible reflection of the need for Gsu to obtain energy for its protective transport functions (e.g., H^+ and heavy metal ion expulsion). This could correlate with Ccr's and Cme's ability to catalyze photosynthesis and carbon fixation in order to create their own sugar products (Smith & Bidwell 1989).

Gsu's extensive metabolic capabilities also extend to other compounds such as polyamines and amino acids (APC and AAAP families). Polyamines are widely distributed among many algal species, and some of these compounds, such as norspermidine and norspermine, are present in large concentrations in Gsu (Hamana & Matsuzaki 1985). The role that polyamines play is unclear, although these cationic compounds are known to associate with and stabilize nucleic acids (Nayvelt et al. 2010) and could therefore promote cell stability under extreme conditions. They have also been shown to enhance sporulation development in the red alga, *Hydropuntia cornea* (Guzman-Uriostegui et al. 2012), and they could have a similar function in Gsu. In contrast, Ccr and Cme have far fewer such transporters. It may be that the multicellular Ccr has the capability of providing an intercellular homeostatic environment, thereby requiring a less diverse set of these transporters, while the environment in which Gsu resides could be harsher than that of Cme.

In terms of Gsu's high resistance to heavy metal and oxyanion toxicity (Schonknecht et al. 2013), several of the systems that are likely to catalyze transport of these compounds include the members of the MIP Family of aquaporins and glycerol facilitators, some of which export arsenite and antimonite, and the divalent metal ion transporters of the CorA, MgtE, ZIP, NRAMP, and CDF families. Through this last mentioned family, Gsu is able to effectively export divalent cations, thereby maintaining homeostasis of heavy metal ions such as Mg^{2+}, Co^{2+}, Zn^{2+}, and Fe^{2+}, which are normally taken up via the CorA, MgtE, ZIP and NRAMP porters. Many of the cation transporters in Cme are also responsible for the transport of divalent heavy metal cations, and the vast majority of these also utilize H^+ as the co- or counter-transport cation. The genome of Cme even encodes members of the ILT Family not found in the other two algae, which could contribute to the fact that intracellular metal homeostasis is a characteristic of this organism. Though fewer in numbers, the metal ion transporters of Ccr are also able to control intracellular Mg^{2+}, Zn^{2+}, and Fe^{2+} concentrations, although the ability to which Ccr can maintain homeostasis relative to Gsu

and Cme is undoubtedly less extensive. The protection afforded by Gsu's, and to a slightly lesser extent, Cme's, many metal ion/oxyanion transporters reflects the immediate need of a single-celled organism to retrieve critical nutrients while regulating the internal environment, ensuring survival. This need would not be expected to be as great for Ccr, which does not grow under these extreme conditions, and by virtue of its multicellularity, can maintain internal homeostasis using different mechanisms.

A feature that all three algae share is their tolerance to salty environments (Schonknecht et al. 2013), yet Ccr has more monovalent cation transporters than Gsu or Cme. The fact that Ccr has seven members of the VIC Family compared to only one for Gsu and two for Cme suggests that these proteins have signaling functions relevant to multicellularity. The predicted substrate of the single Ccr member of the M^+-PPase and one of the P-type ATPase is Na^+, and Gsu and Cme both lack corresponding enzymes of this specificity. This observation substantiates our suggestion that Ccr utilizes monovalent ions for signal transduction, possibly involving action potentials, whereas Gsu's and Cme's corresponding family members are primarily concerned with H^+ transport, the concentration of which would be high in their acidic (pH 0 to 4) environments (Schonknecht et al. 2013). Thus the specificities of the transporters appear to be explained both by their environmental stress needs and the requirements for maintenance of their single- and multi- cellular states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

BLAST	Basic Local Alignment Search Tool
Ccr	*Chondrus crispus*
CDD	Conserved Domain Database
Cme	*Cyanidioschyzon merolae*
GSAT	Global Sequence Alignment Tool
Gsu	*Galdieria sulphuraria*
HGT	horizontal gene transfer
TC	transporter classification
TCDB	Transporter Classification Database
TMS	transmembrane segment

WHAT Web-based Hydropathy, Amphipathicity, and Topology program

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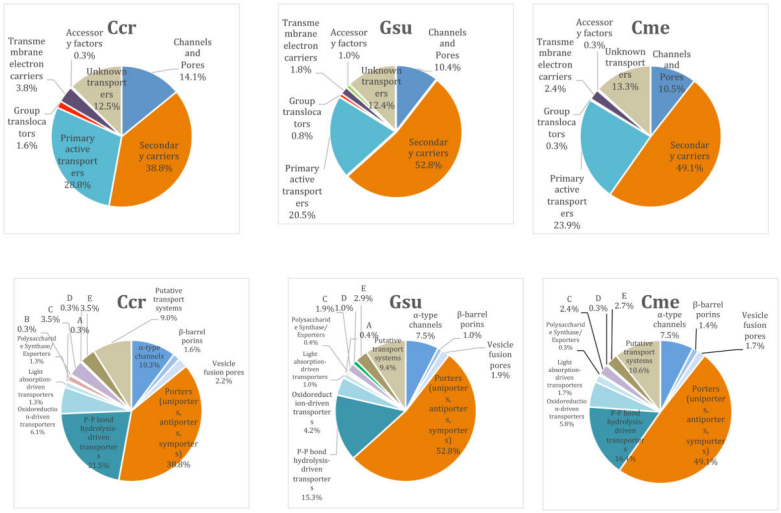

Figure 1.

Distribution of transporters based on TC (A) classes and (B) subclasses in C. crispus, G. sulphuraria, and C. merolae.

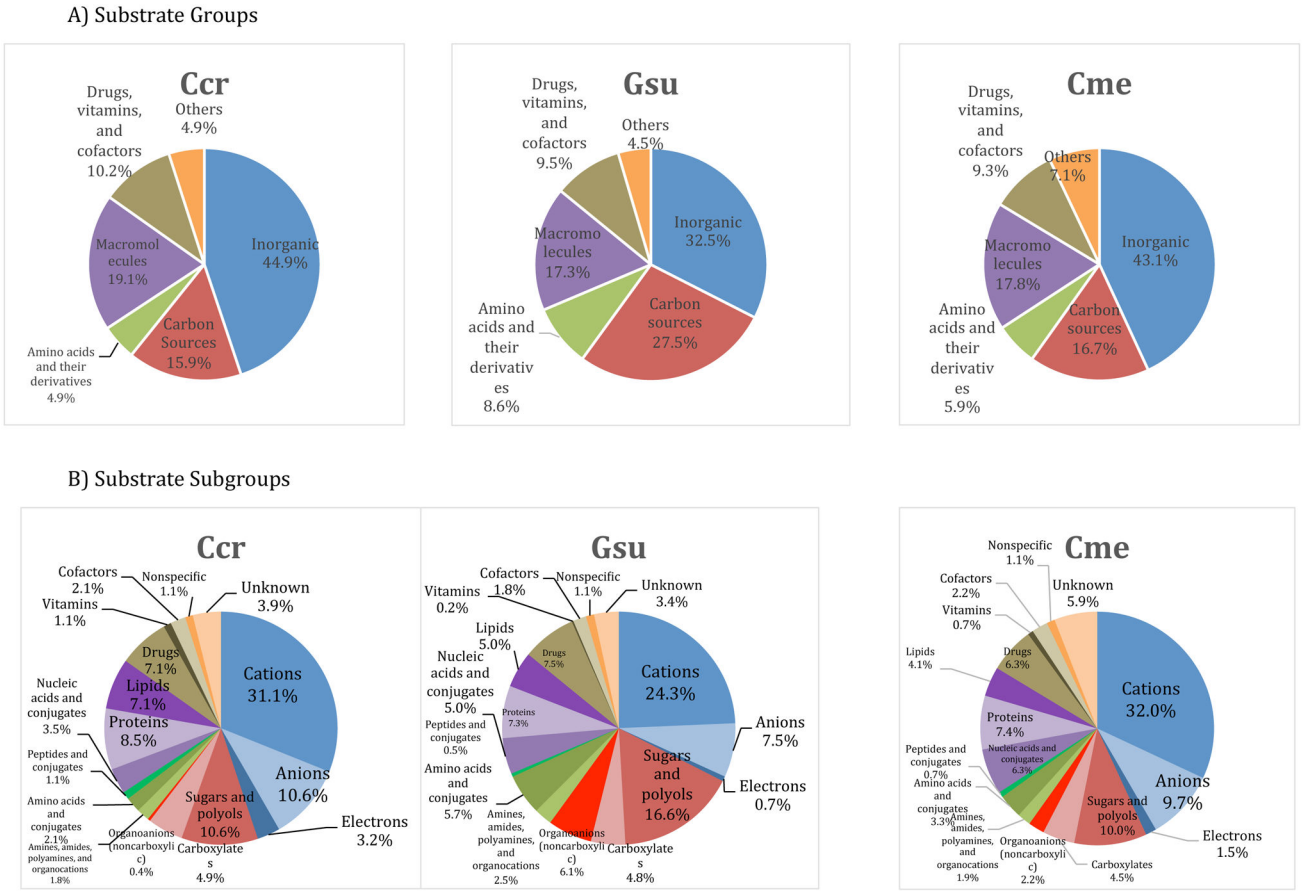

Figure 2.

Distribution of transporters based on TC (A) substrate groups and (B) substrate subgroups in C. crispus, G. sulphuraria, C. merolae.

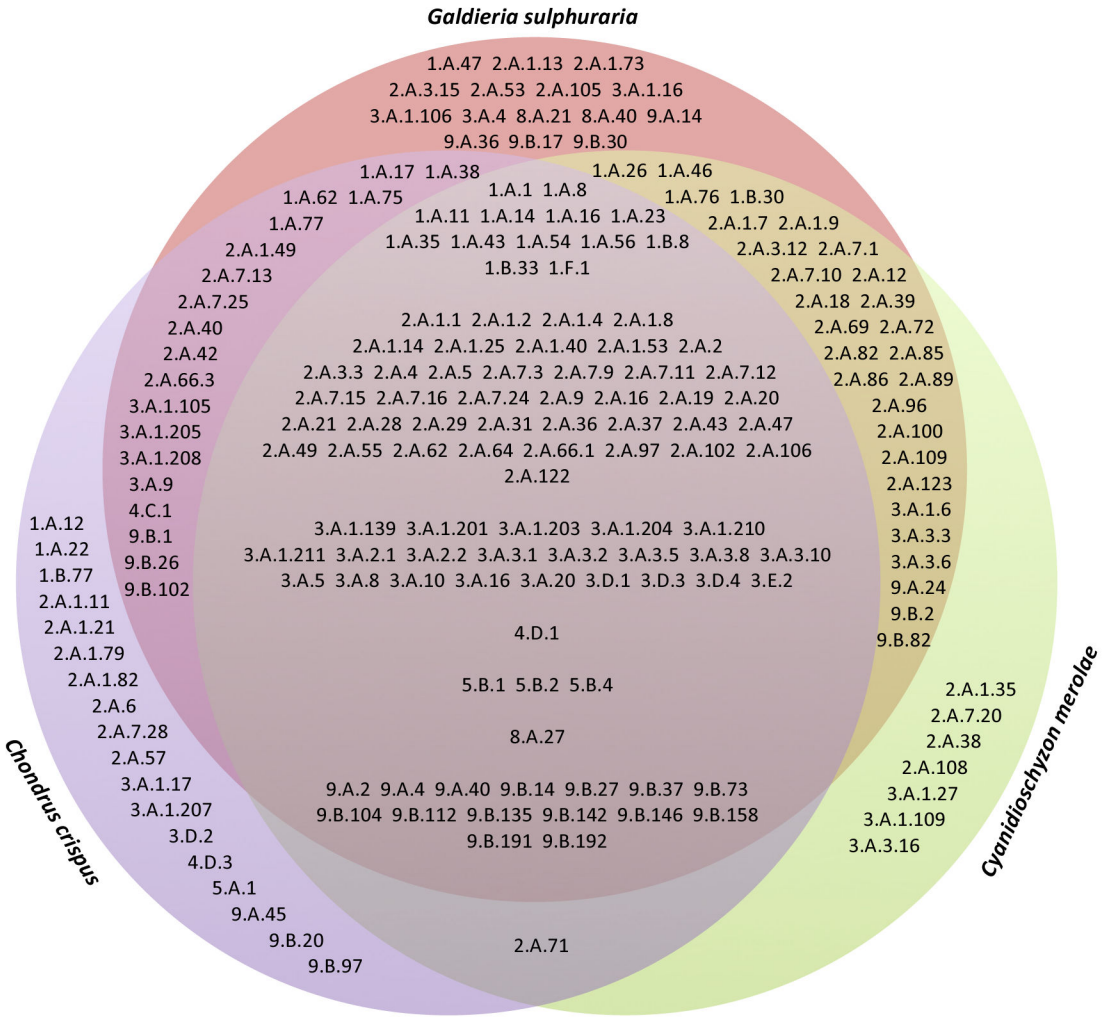

Figure 3.

Recognized transporter families found in the genomes of C. crispus, G. sulphuraria, and C. merolae.

Table 1

Overview of the *C. crispus*, *G. sulphuraria*, and *C. merolae* transporter distribution based on TC class and subclass.

TC Class and description	No. of transport proteins			TC subclass and description	No. of transport proteins		
	Ccr	Gsu	Cme		Ccr	Gsu	Cme
1) Channels and Pores	44	50	31	1.A) α-type channels	32	36	22
				1.B) β-barrel porins	5	5	4
				1.F) Vesicle fusion pores	7	9	5
2) Secondary carriers	121	252	144	2.A) Porters (uniporters, antiporters, symporters)	121	252	144
3) Primary active transporters	90	98	70	3.A) P-P bond hydrolysis-driven transporters	67	73	48
				3.D) Oxidoreduction-driven transporters	19	20	17
				3.E) Light absorption-driven transporters	4	5	5
4) Group translocators	5	4	1	4.C) Acyl-CoA ligase-coupled transporters	1	2	0
				4.D) Polysaccharide Synthase/Exporters	4	2	1
5) Transmembrane electron carriers	12	9	7	5.A) Transmembrane 2-electron transfer carrier	1	0	0
				5.B) Transmembrane 1-electron transfer carrier	11	9	7
8) Accessory factors	1	5	1	8.A) Auxiliary transport proteins	1	5	1
9) Unknown transporters	39	59	39	9.A) Recognized transporters of unknown biochemical function	11	14	8
				9.B) Putative transport systems	28	45	31
Total no. of transport proteins	312	477	293				
Total no. of proteins in genome	9836	7375	5044				
% transporters of genome	3.17	6.47	5.81				

Table 2

Substrates of transporter systems according to TC class identified in *C. crispus*, *G. sulphuraria*, and *C. merolae*. The total protein numbers that contribute to the transport systems for particular substrate subgroups are parenthesized.

Substrate Category	Number of proteins of indicated type acting on substrate type in *C. crispus*							
	Channels and Pores	Secondary carriers	Primary active transporters	Group translocators	TM electron carriers	Auxiliary proteins	Putative transporters	Total System #
Inorganic								
Cations	19	24	46 (28)				17	88
Anions	8	20					2	30
Electrons					12 (9)			9
Carbon sources								
Sugars and Polyols	1	21		4			4	30
Carboxylates	2	11					1	14
Organoanions (noncarboxylic)			1					1
Amino acids and derivatives								
Amines, amides, polyamines, and organocations	3	2						5
Amino acids and conjugates		6						6
Peptides and conjugates		3						3
Macromolecules								
Nucleic acids and conjugates		10						10
Proteins	2	4	13				5	24
Lipids		5	12 (11)	1		1	2	20
Drugs, vitamins, and cofactors								
Drugs		3	16				1	20
Vitamins		3						3
Cofactors		6 (5)					1	6
Others								

Substrate Category	Number of proteins of indicated type acting on substrate type in *C. crispus*							
	Channels and Pores	**Secondary carriers**	**Primary active transporters**	**Group translocators**	**TM electron carriers**	**Auxiliary proteins**	**Putative transporters**	**Total System #**
Nonspecific	9 (3)							3
Unknown		3	2				6	11
Total systems	38	120	71	5	9	1	39	**283**
Total proteins	44	121	90	5	12	1	39	**312**

Substrate Category	Number of proteins of indicated type acting on substrate type in *G. sulphuraria*							
	Channels and Pores	**Secondary carriers**	**Primary active transporters**	**Group translocators**	**TM electron carriers**	**Auxiliary proteins**	**Putative transporters**	**Total System #**
Inorganic								
Cations	19	38	52 (30)		(1)	4	15	107
Anions	7 (6)	20	6				1	33
Electrons					9 (3)			3
Carbon sources								
Sugars and Polyols	5	63 (62)		2			4	73
Carboxylates	2	19						21
Organoanions (noncarboxylic)		27						27
Amino acids and derivatives								
Amines, amides, polyamines, and organocations	2	9						11
Amino acids and conjugates	1	24						25
Peptides and conjugates		2						2
Macromolecules								
Nucleic acids and conjugates		22						22
Proteins	2	7	11				12	32
Lipids		3	7	2		1	9	22
Drugs, vitamins, and cofactors								

Substrate Category	Number of proteins of indicated type acting on substrate type in *G. sulphuraria*							
	Channels and Pores	**Secondary carriers**	**Primary active transporters**	**Group translocators**	**TM electron carriers**	**Auxiliary proteins**	**Putative transporters**	**Total System #**
Drugs		9	22				2	33
Vitamins		1						1
Cofactors		6					2	8
Others								
Nonspecific	12 (4)						1	5
Unknown		2					13	15
Total systems	41	251	76	4	4	5	59	**440**
Total proteins	50	252	98	4	9	5	59	**477**

Substrate Category	Number of proteins of indicated type acting on substrate type in *C. merolae*							
	Channels and Pores	**Secondary carriers**	**Primary active transporters**	**Group translocators**	**TM electron carriers**	**Auxiliary proteins**	**Putative transporters**	**Total System #**
Inorganic								
Cations	13	34	42 (24)		(1)		14	86
Anions	3	19	2				2	26
Electrons					7 (4)			4
Carbon sources								
Sugars and Polyols		22		1			4	27
Carboxylates	2	10						12
Organoanions (noncarboxylic)		6						6
Amino acids and derivatives								
Amines, amides, polyamines, and organocations	3	2						5
Amino acids and conjugates	1	8						9
Peptides and conjugates		2						2
Macromolecules								
Nucleic acids and conjugates		17						17

Substrate Category	Number of proteins of indicated type acting on substrate type in *C. merolae*							
	Channels and Pores	**Secondary carriers**	**Primary active transporters**	**Group translocators**	**TM electron carriers**	**Auxiliary proteins**	**Putative transporters**	**Total System #**
Proteins	1	4	10				5	20
Lipids		2	6			1	2	11
Drugs, vitamins, and cofactors								
Drugs		7	10					17
Vitamins		2						2
Cofactors		3					3	6
Others								
Nonspecific	7 (3)							3
Unknown	1	6					9	16
Total systems	27	144	52	1	5	1	39	**269**
Total proteins	31	144	70	1	7	1	39	**293**