Mining genomes of marine cyanobacteria for elements of zinc homeostasis

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Zinc is a recognized essential element for the majority of organisms, and is indispensable for the correct function of hundreds of enzymes and thousands of regulatory proteins. In aquatic photoautotrophs including cyanobacteria, zinc is thought to be required for carbonic anhydrase and alkaline phosphatase, although there is evidence that at least some carbonic anhydrases can be cambialistic, i.e., are able to acquire *in vivo* and function with different metal cofactors such as Co^{2+} and Cd^{2+}. Given the global importance of marine phytoplankton, zinc availability in the oceans is likely to have an impact on both carbon and phosphorus cycles. Zinc concentrations in seawater vary over several orders of magnitude, and in the open oceans adopt a nutrient-like profile. Most studies on zinc handling by cyanobacteria have focused on freshwater strains and zinc toxicity; much less information is available on marine strains and zinc limitation. Several systems for zinc homeostasis have been characterized in the freshwater species *Synechococcus* sp. PCC 7942 and *Synechocystis* sp. PCC 6803, but little is known about zinc requirements or zinc handling by marine species. Comparative metallo-genomics has begun to explore not only the putative zinc proteome, but also specific protein families predicted to have an involvement in zinc homeostasis, including sensors for excess and limitation (SmtB and its homologs as well as Zur), uptake systems (ZnuABC), putative intracellular zinc chaperones (COG0523) and metallothioneins (BmtA), and efflux pumps (ZiaA and its homologs).

Keywords: zinc limitation, Zur, COG0523, SmtA, BmtA

INTRODUCTION

Zinc might be considered as one of the most inconspicuous trace elements. To some extent, this is due to its "boring" (Levi, 1984) chemistry – in its only biologically relevant oxidation state, Zn(II), it is colorless and does not display any redox chemistry of its own. Because Zn^{2+} is not redox-active, many authors tend to consider it as less important than iron or copper in terms of both essentiality and toxicity, even though between 5 and 9% of the predicted proteomes of most organisms correspond to zinc-requiring proteins – in most cases more than either the predicted iron or copper sub-proteomes (Andreini et al., 2009).

This is even true for prokaryotes which once were thought to "avoid the hidden cost of zinc homeostasis" (Luisi, 1992). Several recent bioinformatic approaches aimed at predicting metalloproteomes (Andreini et al., 2006, 2008; Dupont et al., 2010) found that although overall zinc utilization in bacteria is undoubtedly lower than in eukaryotes, Zn-binding domains are yet highly abundant in predicted bacterial proteomes. For the case of currently existing prokaryotes, there is clear evidence for widespread zinc utilization, in particular in hydrolytic enzymes (Decaria et al., 2010). A major reason for lower zinc utilization by bacteria is likely the much lower abundance of zinc finger domains in their proteomes. Furthermore, at least in heterotrophs, the cellular quotas for zinc and iron tend to be similar (Outten and O'Halloran, 2001), although

it should be emphasized that metal quotas do not necessarily bear a direct relationship to metal requirements.

Virtually all organisms have elaborate mechanisms to control zinc levels and distribution (Hantke, 2005; Eide, 2006; Fukada and Kambe, 2011). Total cellular concentrations typically are in the high micromolar range; however, various lines of evidence have indicated that "free" cytosolic zinc concentrations are extremely low. Values given in the literature vary between nanomolar and femtomolar – with the true regulated value probably somewhere in the picomolar range (Krezel and Maret, 2006). The apparent need for the narrow range of tolerable zinc concentrations had initially puzzled some researchers, as there seems to be a widespread belief that zinc is not particularly toxic to cells. It could be argued that this is only true for cells that have efficient mechanisms to deal with zinc; otherwise free zinc concentrations as low as nanomolar can be toxic (Bozym et al., 2010). Deleterious effects of Zn^{2+} may, at least to some extent, be due to its high position in the Irving–Williams series (Irving and Williams, 1953), meaning that it outcompetes less competitive metal ions such as Fe^{2+} and Mn^{2+} for their protein binding sites, as demonstrated recently for the Mn-binding protein MncA (Tottey et al., 2008). This latter study illustrated why it is important to limit the amount of exchangeable zinc in the cytosol of cells, by the demonstration that the major periplasmic Mn-binding protein MncA

of *Synechocystis* sp. PCC 6803 (a freshwater cyanobacterium) can only incorporate the essential cofactor Mn^{2+} to a significant extent if this is present in 100000-fold molar excess over Zn^{2+}. Since MncA folds and is loaded with Mn^{2+} in the cytosol, this finding implies the need for a very low free cytosolic zinc concentration in *Synechocystis* sp. PCC 6803. Little information is available on zinc toxicity to marine cyanobacteria. A study on *Synechococcus* strains in the strait of Gibraltar noted that even micromolar concentrations of zinc had only a moderate effect on growth (Debelius et al., 2011), but zinc sensitivity may differ considerably depending on the natural habitat of a given cyanobacterium.

There are indications for an impact of zinc on major global biogeochemical cycles. A "zinc hypothesis" was put forward in 1994 in a study that demonstrated zinc and carbon co-limitation in marine phytoplankton (Morel et al., 1994). A link between zinc and carbon fixation is also reflected in the arctic ice-core record (Hong et al., 1996): during periods of glaciation, zinc levels were at least 10 times higher, whilst CO_2 levels were significantly lower than in the intervening periods. Changes in zinc levels due to increased deposition of dust into the oceans are thought to have had an effect on marine microbial community structure, and the decrease in CO_2 levels could be attributed to increased $CaCO_3$ production by coccolithophores and a resulting decrease in atmospheric CO_2 (Schulz et al., 2004). The amount of data on geochemical zinc fluxes is limited, and it is not clear whether dust deposition today does (Thuroczy et al., 2010) or does not (Bruland et al., 1994) significantly contribute. In terms of a direct biochemical link between zinc and organic CO_2 fixation, there are of course the carbonic anhydrases, which operate in all marine phytoplankton, including cyanobacteria (Cannon et al., 2010), although substitution with either Co or Cd has been demonstrated for eukaryotic phytoplankton (Xu et al., 2008). There are also strong indications for links between Zn and phosphorus cycles (Jakuba et al., 2008), thought to be due to the requirement of Zn for alkaline phosphatase.

An absolute requirement for zinc has been clearly demonstrated for marine eukaryotic phytoplankton (Sunda and Huntsman, 1995, 2005), but the situation is less clear for marine cyanobacteria, as discussed below. With this review, we aim to make a case for intensifying studies into the relevance of zinc for marine cyanobacteria.

MARINE CYANOBACTERIA

Cyanobacteria are a group of phototrophic prokaryotes that all have the ability to perform oxygenic photosynthesis. In the marine environment a large diversity of both unicellular (e.g., *Synechococcus*, *Prochlorococcus*, *Cyanobium*, *Acaryochloris*, and *Crocosphaera*) and filamentous (e.g., *Trichodesmium*, *Lyngbya*, *Oscillatoria*, *Nodularia*, and *Microcoleus*) genera exist, occupying habitats ranging from intertidal microbial mats through to oligotrophic open-ocean waters (see Whitton and Potts, 2000).

The numerically dominant cyanobacteria in open-ocean waters are the unicellular genera *Synechococcus* and *Prochlorococcus* which contribute significantly to marine CO_2 fixation (Li, 1994; Jardillier et al., 2010). *Synechococcus* are the more widely distributed, being found in waters covering a broad temperature range, from ca. 2–3°C to >30°C (Shapiro and Haugen, 1988; Waterbury et al., 1996; Fuller et al., 2006; Zwirglmaier et al., 2008), and including

open-ocean, coastal and estuarine environments (Partensky et al., 1999; Scanlan, 2003). *Prochlorococcus* appears to be more constrained in its distribution occupying waters roughly between 45°N and 40°S but within these latitudes it is extremely abundant, routinely reaching concentrations of 10^5 cells per ml or higher (Partensky et al., 1999; Partensky and Garczarek, 2010). *Prochlorococcus* can be distinguished from *Synechococcus* by its lack of a phycobilisome light-harvesting antenna complex. Instead, it possesses thylakoid membrane proteins binding unique divinyl derivatives of chlorophyll *a* and *b* (Goericke and Repeta, 1992; Partensky and Garczarek, 2003). In stratified tropical and subtropical waters *Prochlorococcus* cells undergo vertical partitioning between distinct high light- and low light-adapted ecotypes (Moore et al., 1998; West and Scanlan, 1999). Both *Synechococcus* and *Prochlorococcus* have relatively small genomes ranging in size between 1.64 and 2.7 Mb in *Prochlorococcus* and from 2.2 to 2.86 Mb in *Synechococcus* (Kettler et al., 2007; Dufresne et al., 2008; Scanlan et al., 2009). In the case of *Prochlorococcus*, significant genome reduction has occurred during evolution of the genus, likely an adaptation to the oligotrophic gyre systems they inhabit, providing significant economies in energy and nutrients (Dufresne et al., 2005).

As well as contributing to marine carbon cycling, some cyanobacteria are also capable of nitrogen fixation (Zehr, 2011). *Trichodesmium*, a filamentous non-heterocystous genus, is ubiquitous in tropical and subtropical environments (Capone et al., 1997) and until recently was thought to be the dominant marine nitrogen-fixer. However, it is now clear that the unicellular UCYN-A and UCYN-B lineages, the latter encompassing the genera *Crocosphaera* and *Cyanothece*, also contribute significantly to this process (Zehr, 2011). Surprisingly, despite its global distribution in the Atlantic and Pacific Oceans *Crocosphaera* appears to have limited genetic diversity with high identity and synteny of the cultured genome sequence to environmental metagenomic datasets for this genus (Zehr et al., 2007). Interestingly, metabolic insights from the genome of the UCYN-A lineage reveals a cyanobacterium lacking photosystem II, RuBisCO, and a tricarboxylic acid cycle (Tripp et al., 2010) suggesting it requires a symbiotic partner.

The high diversity of marine cyanobacteria is epitomized by *Acaryochloris marina*, a cyanobacterium that uniquely utilizes chlorophyll *d* as its main photosynthetic pigment (Kuhl et al., 2005) trapping the far-red light that penetrates beneath the didemnid ascidians (sea squirts) upon which these organisms are found (Ohkubo et al., 2006). Curiously, the *A. marina* genome is considerably larger than other sequenced unicellular strains (Swingley et al., 2008) comprising a circular chromosome of 6.5 Mb and nine distinct plasmids giving a total DNA content of 8.3 Mb. Over 10% of the protein families contain duplicated copies in *A. marina* and this information, together with its utilization of far-red light that is not absorbed by other aerobic photoautotrophs, suggests that *Acaryochloris* species fill a non-competitive niche where they are apparently free to specialize their metabolic library, and potentially explains their expansive genome size (Swingley et al., 2008).

BIOLOGICAL AND CHEMICAL CO-EVOLUTION, AND CYANOBACTERIAL METAL REQUIREMENTS

It has been hypothesized that metal ion bioavailability presented an evolutionary selection pressure on the "choice" of metals

within metalloenzymes (Williams and Da Silva, 2003). Conversely, biological evolution and the emergence of life has changed the chemical composition, or more precisely, the speciation of the atmosphere, the lithosphere, and of course the hydrosphere. Arguably, cyanobacteria might be deemed responsible for the greatest change of all by inventing oxygenic photosynthesis (Raymond and Blankenship, 2004). Consequently, they were amongst the first organisms that encountered, and had to cope with, the changes in the chemical composition of their environment that oxygenation brought about (Cavet et al., 2003; Saito et al., 2003). For metal ion speciation, both fundamental considerations (Williams and Da Silva, 2001) as well as detailed studies (Saito et al., 2003) agree that the presence of oxygen meant a drastic reduction in iron, cobalt, nickel, and manganese availability, and a significant increase in the concentrations of zinc, copper, and cadmium (Williams and Da Silva, 2001; Williams, 2011). It is reasonable to accept that these changes in chemistry and bioavailability directed biological evolution, including that of metal-binding biomolecules (Williams and Da Silva, 2000). Indeed, even though it has been suggested that microbial metalloproteomes are still largely uncharacterized (Cvetkovic et al., 2010), bioinformatic genome analyzes of known metal-binding protein domains (Dupont et al., 2010) as well as elemental analysis experiments on marine phytoplankton (Bertilsson et al., 2003; Heldal et al., 2003; Ho et al., 2003; Quigg et al., 2003, 2011; Morel, 2008) give a picture that is consistent with this idea. Thus, the "co-evolution of biology and chemistry" is imprinted on both the metallome and the metalloproteome. The interested reader is directed to a recent debate regarding the evolution of zinc-binding domains (Mulkidjanian and Galperin, 2009; Dupont and Caetano-Anolles, 2010).

In the case of cyanobacteria, metal ion requirements and sensitivities, as far as they have been experimentally determined, agree with the notion that they evolved in an environment with metal ion concentrations typical of a sulfidic or a ferrous ocean (Saito et al., 2003): both marine *Synechococcus* (Sunda and Huntsman, 1995) and *Prochlorococcus* (Saito et al., 2002) strains have been shown to be cobalt-limited, whereas the requirements for zinc are so low (Saito et al., 2003) that only mild reductions in growth rates were observed at the lowest possible free zinc concentrations (Saito et al., 2002).

Our previous genome-mining approaches have identified strong candidate genes for potentially zinc-requiring carboxysomal carbonic anhydrases, ABC-type zinc uptake systems, as well as for proteins involved in the intracellular handling of zinc (Blindauer, 2008b). Several other enzymes in cyanobacteria are also predicted to require zinc for function, including for example DNA ligase and alkaline phosphatase (Palenik et al., 2003), the latter leading to the suggestion that cyanobacteria may be Zn–P co-limited. Indeed, in certain cyanobacterial strains, alkaline phosphatase activity is elicited by phosphorus limitation (Moore et al., 2005), and direct crosstalk between P and Zn, mediated by the regulatory protein PtrA, has been found in *Synechococcus* sp. WH8102 (Ostrowski et al., 2010). PtrA responds to phosphorus depletion and its expression up-regulates not only the expression of phosphatases, but also that of proteins predicted to be involved in zinc acquisition and distribution – including ZnuABC and a member of the COG0523 family (see below). However, it has to

be noted that the true metal requirements of each of these proteins has yet to be experimentally verified, and there is reason to be cautious, with some evidence for the *in vivo* replacement of zinc with cobalt (Sunda and Huntsman, 1995) and cadmium (Lee and Morel, 1995) in marine phytoplankton. Very recently though, utilization of an alternative calcium-requiring phosphatase (PhoX) has been shown for uncultured *Prochlorococcus* (Kathuria and Martiny, 2011), suggesting a further mechanism for reducing zinc requirements.

In conclusion, although cyanobacteria are at the root of what life and marine trace metal chemistry are like today, their metal requirements require further study; none of the predicted major destinations for zinc are experimentally confirmed, and information about if, and how, zinc requirements can be alleviated by either Co or Cd substitution, is limited.

ZINC SPECIATION IN SEA WATER

In order to understand how cyanobacteria might acquire zinc from the marine environment, we must first understand its chemical nature in seawater. The concentration of zinc in oceanic waters follows typical nutrient-like depth profiles (Bruland, 1980; Butler, 1998), with the lowest concentrations found in the euphotic zone with dissolved zinc rapidly removed to lower depths as a constituent of colloidal particles (Bruland, 1989; Wells et al., 1998). Total dissolved zinc concentrations in surface waters of the North Atlantic Ocean range from just 0.1 to 0.3 nM (Ellwood and van den Berg, 2000), similar to values obtained from measurements taken in the North Pacific Ocean (Bruland, 1980). The vast majority of this zinc (∼98%) is found complexed to uncharacterized organic ligands (Bruland, 1989; Donat and Bruland, 1990; Ellwood and van den Berg, 2000) with conditional stability constants, $\log K'_{ZnL}$, of between 10.0 and 10.5 (Wells et al., 1998; Ellwood and van den Berg, 2000). This results in a concentration of free Zn^{2+} of 1–20 pM. The distribution of these metal complexing ligands suggests a surface source (Bruland, 1980) that may include phytoplankton including cyanobacteria. This contribution could be through the direct secretion of zinc-binding ligands into the ocean. There is already some evidence that cyanobacteria actively secrete ligands that complex other biologically important trace metals including copper, iron, and cobalt. The marine *Synechococcus* sp. strains WH8101 and WH7805 have both been found to produce siderophores (Wilhelm and Trick, 1994) for scavenging iron from nutrient-depleted environments, whilst Cu-complexing ligands are produced to mitigate the toxic effects of copper (Wiramanaden et al., 2008), with cyanobacteria particularly sensitive to this metal (Mann et al., 2002). Significant quantities of strong cobalt-binding ligands were produced by a *Synechococcus*-dominated microbial community in the Costa Rica upwelling dome (Saito et al., 2005).

Alternatively the Zn complexing ligands could be released from cyanobacterial cells indirectly, perhaps as a consequence of cell lysis by marine phages (Wells et al., 1998). Despite the fact that the vast majority of zinc in ocean waters is present in the form of organic metal complexes, there is evidence that free Zn^{2+} is the major form of this nutrient taken up by phytoplankton (Anderson et al., 1978; Sunda and Huntsman, 1992; Sunda et al., 2005). In the Pacific Ocean the concentration of free Zn^{2+} in surface waters

ranges from only 1 to 14 pM (Bruland, 1980; Donat and Bruland, 1990), and in the Atlantic Ocean ranges from 6.8 to 20 pM (Brand et al., 1983). The concentration of free Zn^{2+} in surface waters is thought to be sufficiently low to limit the growth of some marine phytoplankton (Brand et al., 1983; Sunda and Huntsman, 1992) although one study found that phytoplankton growth was not limited by low Zn^{2+} concentrations even after iron limitation was alleviated (Coale et al., 1996). Conversely, productivity in the subtropical Atlantic was further boosted by addition of Zn or Co to water that had also been enriched with Fe and P (Dixon, 2008), providing evidence for Zn/Fe/P co-limitation. Similarly, the iron-depleted waters of the Southern Ocean and the sub-arctic Pacific are also extremely zinc-depleted (Sunda and Huntsman, 2000) which appears to induce high levels of Cd uptake and high Cd:P ratios in phytoplankton. It is thus clear that interactions between the cycles of different metal ions exist, and that co-limitation needs to be studied (Saito et al., 2008). How important such crosstalk is in cyanobacteria is not yet well understood, and further work is required to determine if mechanisms exist for the active uptake of zinc, and if so, in what form zinc is acquired by cyanobacteria, and to determine the impact that cyanobacteria have on trace metal speciation in ocean waters (Leao et al., 2007).

SYSTEMS FOR ZINC HOMEOSTASIS

Zinc homeostasis in bacteria is largely achieved through a balance of active uptake and efflux by specific membrane transporters (Hantke, 2005), plus proteins mediating intracellular zinc handling (**Figure 1**). In the freshwater cyanobacterium *Synechocystis* sp. PCC 6803, a zinc-specific high affinity ABC transporter termed ZnuABC has been identified for the active uptake of zinc from the periplasm (Cavet et al., 2003). Putative ZnuABC systems have also been identified in most strains of marine cyanobacteria (Blindauer, 2008b; Scanlan et al., 2009). The putative *znuABC* gene cluster of many of the marine strains also contains a putative *zur* gene (Blindauer, 2008b). Zur proteins (for zinc uptake regulator) are low-zinc sensors; their zinc-loaded forms repress the expression of *znuABC* under zinc-replete conditions (Patzer and Hantke, 2000).

To date no specific mechanisms for the active uptake of zinc across the outer cell membrane of cyanobacteria has been identified. It is generally considered that metal ions are small enough to diffuse freely through porins in the outer-membrane of Gram-negative bacteria; however, as described above, the concentration of free Zn^{2+} in surface layers of the world's oceans is extremely low, with the vast majority of zinc complexed to as yet uncharacterized ligands of unknown structure and origin (Bruland, 1989). Whether at least some of these ligands are actively secreted to aid in zinc acquisition remains an open question; there is also the possibility that at least in coastal environments, ligands are synthesized in response to zinc excess and hence to avoid toxicity (Lohan et al., 2005; Leao et al., 2007).

In order to deal with excess zinc, *Synechocystis* sp. PCC 6803 has a zinc-specific efflux pump, ZiaA (Thelwell et al., 1998), similar to other P1-type ATPase metal ion transporters, that transports Zn^{2+} from the cytoplasm to the periplasmic space (**Figure 1**). Expression of this efflux system is induced by zinc and is regulated by ZiaR (Thelwell et al., 1998), a zinc-specific repressor protein.

With the exception of *Lyngbya* sp. and *Nodularia* sp., most marine strains of cyanobacteria appear to lack zinc-specific efflux pumps (Blindauer, 2008b; Scanlan et al., 2009) reflecting the nutrient poor environments they occupy, with free Zn^{2+} concentrations being in the picomolar range in ocean waters (Hunter and Boyd, 1999). Instead, at least some marine cyanobacteria seem to rely on a mechanism of zinc sequestration by bacterial metallothioneins (BmtAs; Blindauer, 2008b) to deal with any eventual excess. Metallothioneins are small cytosolic proteins rich in cysteine residues that bind and sequester metal ions and thereby prevent any deleterious interactions (Blindauer and Leszczyszyn, 2010). The metallothionein SmtA of the freshwater cyanobacterium *Synechococcus* sp. PCC 7942 is induced by several metal ions but most prominently by zinc (Huckle et al., 1993) and its expression is controlled by the zinc sensor SmtB (Osman and Cavet, 2010) that is highly similar to ZiaR. Several marine strains of cyanobacteria appear to lack an SmtB/ZiaR type regulator (Blindauer, 2008b) despite the presence of one or more genes for a BmtA (**Table 1**). Furthermore, many also appear to lack any established mechanism for dealing with zinc excess, with all *Prochlorococcus* and some *Synechococcus* strains apparently lacking both a ZiaA efflux pump and a metallothionein encoding gene (Blindauer, 2008b). Presumably these bacteria never encounter toxic levels of zinc in

FIGURE 1 | Known and proposed elements of zinc homeostasis in cyanobacteria. A requirement for zinc is sensed by the transcriptional regulator Zur, leading to the upregulation of the components of the ZnuABC uptake system. Some members of the putative metallochaperone family COG0523 are also regulated by Zur, and have, in other bacteria, been shown to be expressed in response to zinc deficiency. In most freshwater cyanobacteria, excessive levels of zinc are sensed by SmtB and its homologs (ZiaR, AztR, BxmR), and these sensors regulate either the expression of efflux pumps (ZiaA) or metallothioneins (SmtA and homologs).
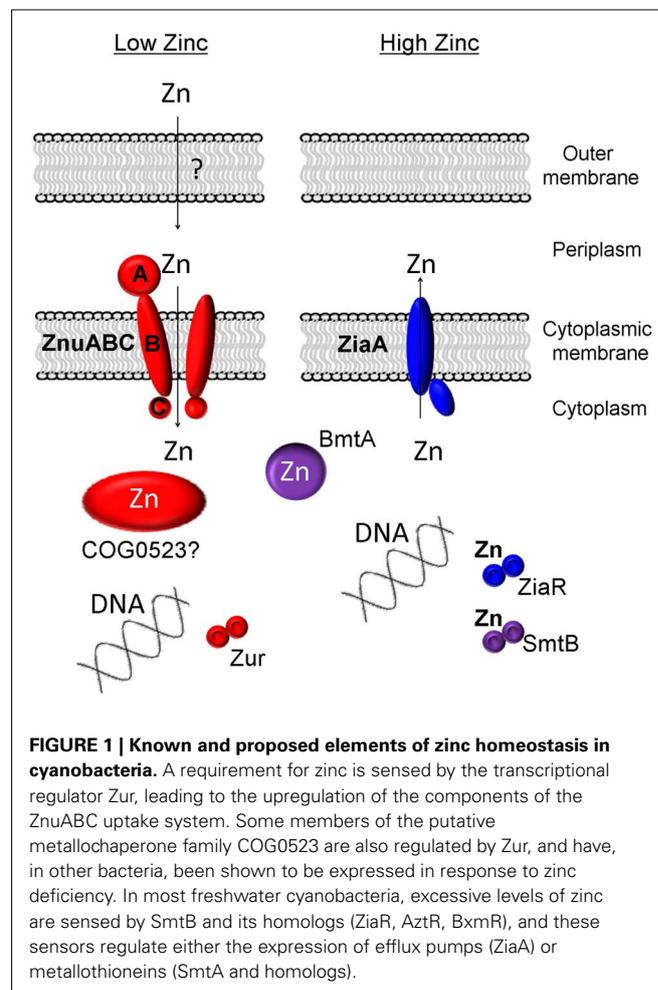
Table 1 | Presence of selected genes predicted to be involved in zinc homeostasis in marine cyanobacterial genomes.

Species/strain	Zur	Zinc-related COG0523?[a]	BmtA	SmtB	In gene cluster?
Acaryochloris marina MBIC11017	✓	✓✓	✓	✓	yes
Crocosphaera watsonii WH 8501	✓	?	✓	?	
Cyanobium sp. PCC 7001 [D][b]	✓	✓	✓	?	
Lyngbya sp. PCC 8106	✓	✓✓	✓	?	
Microcoleus chthonoplastes sp. PCC 7420 [D]	✓	✓	✓	✓	
Nodularia spumigena sp. CCY9414	✓	✓	✓	✓	
Oscillatoria sp. PCC 6506	✓	?	✓	✓	yes
Synechococcus sp. CC9311	✓	?	✓		
Synechococcus sp. CC9605	✓	?	✓		
Synechococcus sp. WH 7803	✓	?	✓		
Synechococcus sp. WH8102	✓	✓	✓		
Synechococcus sp. PCC 7002	✓	✓	✓	✓	yes
Synechococcus sp. WH 8109 [D]	✓	?	✓		
Synechococcus sp. WH5701	✓	?	✓		
Synechococcus sp. CC9902	✓	?			
Synechococcus sp. BL107 [D]	✓	?			
Synechococcus sp. PCC 7335 [D]	✓	?		✓	
Synechococcus sp. RCC307 [D]	✓	?			
Synechococcus sp. RS9916 [D]	✓	?			
Synechococcus sp. RS9917 [D]	✓	?			
Synechococcus sp. WH7805 [D]	✓	?			
Trichodesmium erythraeum IMS101	✓	✓			
Prochlorococcus marinus sp. AS9601	✓	✓			
P. marinus sp. MIT 9211	✓	✓			
P. marinus sp. MIT 9215	✓	✓			
P. marinus sp. MIT 9301	✓	✓			
P. marinus sp. MIT 9303	✓	✓			
P. marinus sp. MIT 9312	✓	✓			
P. marinus sp. MIT 9313	✓	?			
P. marinus sp. MIT 9515	✓	✓			
P. marinus sp. MIT9202 [D]	✓	✓			
P. marinus NATL1A	✓	✓			
P. marinus NATL2A	✓	✓			
P. marinus marinus CCMP1375[c]	✓	✓			
P. marinus pastoris CCMP1986[c]	✓	✓			

Empty cells indicate absence of a recognizable zinc-related homolog; "?" indicate presence of homologs with unclear metal specificity.

[a]According to analysis of syntenic genome regions or presence of upstream Zur box.

[b][D] Draft genome.

[c]CCMP1375 also known as SS120; CCMP1986 also known as MED4.

the environment, although it also remains possible that they may employ novel mechanisms for zinc homeostasis that have yet to be discovered.

SENSING A REQUIREMENT FOR ZINC: Zur TRANSCRIPTION FACTORS

In bacteria, the expression of proteins that deal with metal ion homeostasis is predominantly regulated at the transcriptional level (Finney and O'Halloran, 2003; Giedroc and Arunkumar, 2007; Waldron et al., 2009), and is mediated by sensor proteins for zinc excess (e.g., SmtB and its relatives; Giedroc and Arunkumar, 2007; Osman and Cavet, 2010) and zinc depletion (Zur and others). Seven major groups of bacterial metalloregulatory proteins have so far been defined: the Fur-family (for "ferric uptake regulator")

is one of them (COG0735; Bagg and Neilands, 1987), and also comprises paralogous sensors for zinc (Zur), nickel (Nur), manganese (Mur; Lee and Helmann, 2007), and hydrogen peroxide (PerR; Jacquamet et al., 2009).

Metal sensing in bacteria occurs overwhelmingly in the cytosol (Waldron and Robinson, 2009). Ultimately, the sensor proteins are the proteins that need to be the "most specific" – they should ideally either not bind any other metal ion, or if that is not possible, they should not respond to other metal ions in the same way as to their cognate metal. The key concepts of access and allostery, as well as the importance of relative affinities of different metalloproteins for different metal ions, have been highlighted by Waldron et al., 2009.

The dissociation constants of Zn^{2+}–Zur complexes are in the femtomolar range; similar data are also available for zinc excess sensors (Outten and O'Halloran, 2001; Giedroc and Arunkumar, 2007) – these data also support the idea that the free Zn^{2+} concentration in the cytosol is extremely low. Several crystal structures of representatives of the COG0735 family have been determined (Pohl et al., 2003; Lucarelli et al., 2007; Jacquamet et al., 2009; Sheikh and Taylor, 2009; Shin et al., 2011). All members studied adopt a "winged-helix" fold (**Figures 2A,C**) and all assemblies are homo-dimeric, as are many other proteins that specifically recognize DNA sequences.

All "urs" are thought to bind their cognate DNA in the presence of the entity to be sensed. Although no structures in the presence of DNA are available, it is thought that DNA-binding is mediated by the first ca. 80 residues, whilst dimerization, also a prerequisite of DNA-binding, is mediated by the C-terminal half, in particular by the formation of a six-stranded β-sheet formed by both monomers. The overall shape of the dimeric assembly can be described as an "arch" (**Figure 2C**), and the two DNA-binding domains are thought to "grip" the DNA using their DNA-recognition helices. It is likely that the interaction between protein and DNA requires a particular conformation that is stabilized by the presence of the sensed metal. The sensing appears to be mediated by two inter-domain hinges that are likely to be stabilized by metal-binding. In contrast to metal sensors of the SmtB family in which complete metal sites form *between* monomers, each metal site in Fur-family proteins is formed from residues from one monomer only. If the respective metal is absent, a different conformation may become more favorable, and DNA-binding no longer occurs, leading to the de-repression of gene transcription. Despite this general mechanistic idea, there is considerable ambiguity about the molecular detail of metal-binding, and how the binding of the "correct" metal mediates DNA-recognition. Despite the availability of X-ray structure for no less than seven Fur-family proteins, the identity of the residues involved in binding the metal ion to be sensed is unclear, in particular in those "urs" that contain three metal sites per monomer.

A structural zinc site formed by four conserved Cys residues is present in the majority of Fur-family members, independent of the sensed metal. One or two further sites participate in sensing. An inspection of various X-ray structures of Fur-family proteins suggested that sample preparation for such studies seems to be quite challenging; in particular, appropriate population with the correct complement of metal ions appears to be less than straightforward, and in several cases, workers appear to have resorted to populating all sites with Zn^{2+}. Whilst this is certainly appropriate for Zurs, in other cases this may lead to ambiguous conclusions, as the coordination preferences of Zn^{2+} are rather different to those of Fe^{2+} or Ni^{2+}. It has been demonstrated experimentally for SmtB/ArsR sensors that coordination geometry (Cavet et al., 2002) is an important discriminator in metal sensor proteins (via allostery). For *Pseudomonas aeruginosa* Fur, it has been shown *in vitro* that Zn^{2+}-loaded Fur interacted with a Fur-binding DNA sequence in a different manner to that observed with Fe^{2+} (Ochsner et al., 1995). It should also be noted that despite full conservation of the respective residues, the crystallographically observed metal-binding sites in the Fur proteins from *Pseudomonas aeruginosa*

on the one hand and *Vibrio cholerae* and *Helicobacter pylori* on the other (all structures contain only Zn^{2+} ions) are not identical (see **Figure 2B**), and that the domain orientations in the dimeric assemblies also differ significantly – likely as a consequence of the different coordination modes. It is conceivable that the binding mode for Fe^{2+} differs from both experimentally observed sites, with likely consequences for the structure that is competent to bind to Fur boxes.

Even in the case of the Zur sensors, metal population seems to be problematic, and as a consequence, there is some controversy over stoichiometry as well as the role of the various sites. For the Zurs from *Streptomyces coelicolor* (Shin et al., 2011) and *Bacillus subtilis* (Ma et al., 2011), there is agreement that site 2 (**Figure 2A**) is the major sensory site. Variations of site 2 are present in Fur, Nur, and PerR (**Figure 2B**), and in each case, this site has been identified as the sensory site. Site 3 was only partially occupied in the *M. tuberculosis* structure (Lucarelli et al., 2007), and the dimer displayed an open conformation, probably with reduced DNA-binding ability. In contrast, site 3 (site D) was fully occupied in the structure of *S. coelicolor* Zur, and the dimer showed a closed conformation, thought to be DNA-binding competent. This led to suggestions that site 3 fine-tunes the response to zinc (Shin et al., 2011). This assessment has been contested based on studies of mutants of *B. subtilis* Zur (Ma et al., 2011), which suggested that site 3 is not populated under physiological conditions at all, but that the two site 2s in the dimer have different affinities and show negative cooperativity. In either case, it was suggested that different metal affinities of the various sites allow the broadening of the operating range of the Zur proteins.

We believe that the preceding discussion demonstrates the challenges encountered in the study of metal-binding and -sensing proteins, but also highlights how important continued studies of metalloproteins are. Unfortunately, so far, no structure for any "ur" from a cyanobacterium has been elucidated, but in the following, we will explore what can be achieved using theoretical approaches.

BLAST searches in the genomes of marine cyanobacteria retrieved members of the COG0735 family as summarized in **Table 1**; **Figure 3** and Figures S1 and S2 in Supplementary Material. They cluster into four distinct groups. Although it should be recognized that sequence similarity and metal specificity of sensors (or indeed other metalloproteins) need not be congruent (Campbell et al., 2007), comparisons with other "urs" of known specificity, first and foremost *Synechocystis* Zur, suggest that the branch highlighted in light purple corresponds to Zurs. Furthermore, an analysis of the genome environments of the Zurs from the majority of *Prochlorococcus* strains supports this idea (**Figure 4**).

Intriguingly, sequence comparisons (**Figure 2B**) show that an equivalent of site 2 cannot be identified in the cyanobacterial Zur homologs, and neither are all residues of site 3 the same as in structurally characterized Zur proteins. We have constructed a homology model of the representative from *Prochlorococcus* sp. SS120 (CCMP1375) to test whether likely alternative binding sites might be predicted. The model obtained (**Figure 2C**) suggests that the cyanobacterial Zurs may contain a variation of site 3, comprising D80, H82, C98, and H118. Thus, the predicted zinc-sensing site is in a location corresponding to site 3, but has a ligand set that
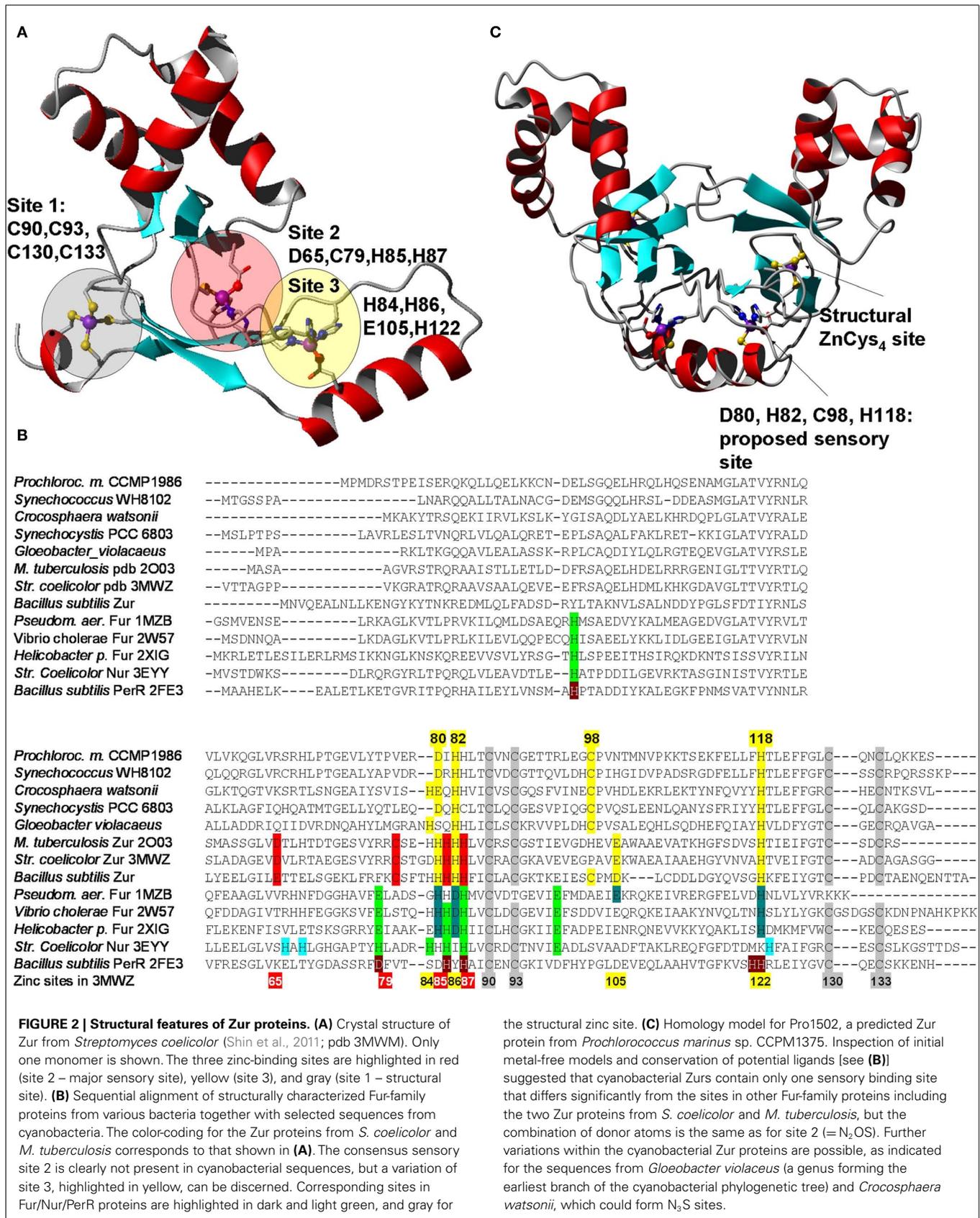
FIGURE 2 | Structural features of Zur proteins. (A) Crystal structure of Zur from *Streptomyces coelicolor* (Shin et al., 2011; pdb 3MWM). Only one monomer is shown. The three zinc-binding sites are highlighted in red (site 2 – major sensory site), yellow (site 3), and gray (site 1 – structural site). (B) Sequential alignment of structurally characterized Fur-family proteins from various bacteria together with selected sequences from cyanobacteria. The color-coding for the Zur proteins from *S. coelicolor* and *M. tuberculosis* corresponds to that shown in (A). The consensus sensory site 2 is clearly not present in cyanobacterial sequences, but a variation of site 3, highlighted in yellow, can be discerned. Corresponding sites in Fur/Nur/PerR proteins are highlighted in dark and light green, and gray for

the structural zinc site. (C) Homology model for Pro1502, a predicted Zur protein from *Prochlorococcus marinus* sp. CCPM1375. Inspection of initial metal-free models and conservation of potential ligands [see (B)] suggested that cyanobacterial Zurs contain only one sensory binding site that differs significantly from the sites in other Fur-family proteins including the two Zur proteins from *S. coelicolor* and *M. tuberculosis*, but the combination of donor atoms is the same as for site 2 (= N_2OS). Further variations within the cyanobacterial Zur proteins are possible, as indicated for the sequences from *Gloeobacter violaceus* (a genus forming the earliest branch of the cyanobacterial phylogenetic tree) and *Crocosphaera watsonii*, which could form N_3S sites.
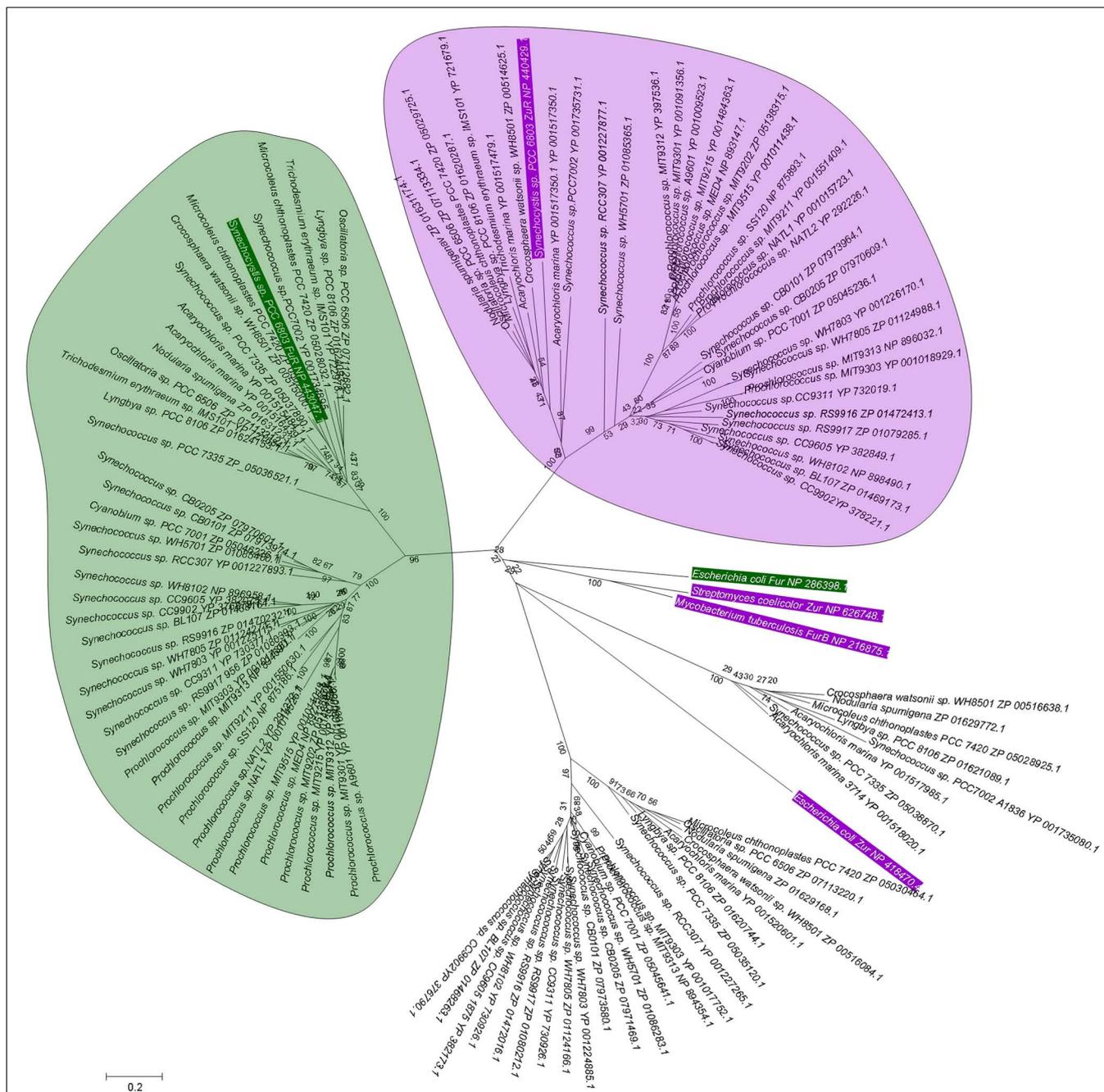
FIGURE 3 | Phylogenetic relationship among Fur-like proteins from marine cyanobacteria. Sequences are labeled with species and protein accession number; a rectangular tree including bootstrap statistics can be found in Figure S1 in Supplementary Material, and the actual sequences are documented in Figure S2 in Supplementary Material. Additional proteins for the experimentally verified Fur from *Escherichia coli* and *Synechocystis* sp. PCC 6803 are also included (green boxes), along with the Zur proteins from *Streptomyces coelicolor*, *Mycobacterium tuberculosis*, and *Synechocystis* sp. PCC 6803 (purple boxes). Amino acid sequences (see Figure S2 in Supplementary Material) were aligned using CLUSTALW (Larkin et al., 2007),

and manually edited prior to import into MEGA5 (Tamura et al., 2011). Phylogeny was inferred using the minimal evolution method. Bootstrap values are the result of 1000 replications. Evolutionary distance was estimated using the JTT model of substitution. There are four clear branches. Inclusion of *Synechocystis* Zur and Fur allows the suggestion that the branches containing these sequences correspond to zinc-responsive (light purple bubble) and iron-responsive (light green bubble) regulators. The Zur and Fur sequences from other bacteria do not cluster with any of the four branches, indicating that similarity between cyanobacterial sequences and those from other bacteria do not allow to infer metal specificity.

is similar to that of site 2, which may suggest that the zinc-binding affinity of this site is also closer to that of site 2. The four residues

identified are almost fully conserved in putative Zur proteins from all cyanobacteria (Figure S2 in Supplementary Material). A notable
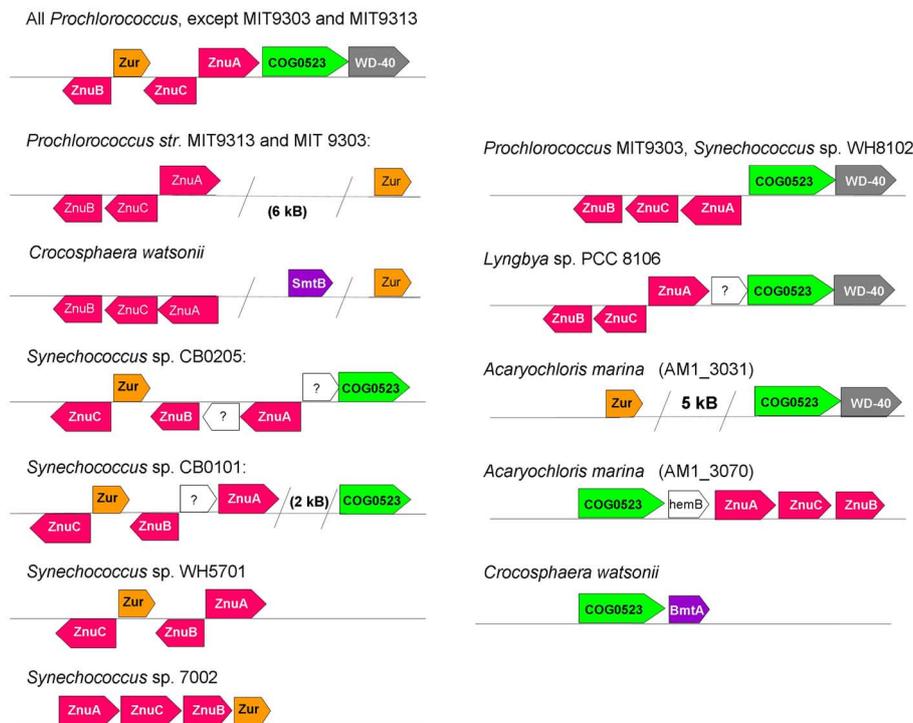
FIGURE 4 | Selected variations in genome neighborhoods of elements of zinc homeostasis in marine cyanobacteria, with a focus on putative Zur (left) and COG0523 (right) proteins. ZnuA is the periplasmic binding protein, ZnuB the permease, and ZnuC the ATPase component of the ZnuABC uptake transporter (also see **Figure 1**). In other strains and species (e.g., other *Synechococcus* strains, *Lyngbya* sp. PCC 8106, *Microcoleus chthonoplastes*), the gene for Zur is not co-localized with recognizable elements of zinc homeostasis. Note that there appear to be two ZnuABC-type systems in *Prochlorococcus marinus* sp. MIT 9303. The *Acaryochloris marina* genome and plasmids harbor at least nine COG0523 family members; AM1_3070 (corresponding to UniProt entry B0CDJ7_ACAM1) and AM1_3031 (corresponding to UniProt entry B0CCJ8_ACAM1) are directly or indirectly associated with zinc homeostasis.

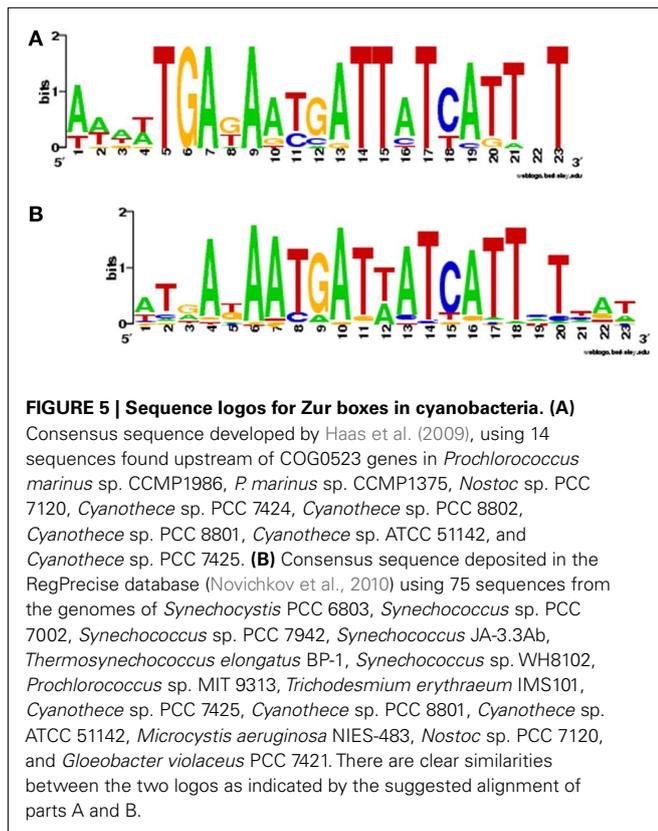exception is the homolog from *Trichodesmium erythraeum*, in which H82 is replaced by a Tyr residue.

From this analysis, we would predict that the molecular mechanism for sensing in cyanobacterial Zurs differs to some extent from that of other bacterial Zurs with three metal sites, but the general idea of zinc stabilizing domain orientation still holds. Notably, the recognition motifs (Zur boxes) for cyanobacterial Zurs differ somewhat from those of other bacteria (Haas et al., 2009), which may require an adapted mode of operation of the protein.

Using the Zur box motif developed by Haas et al. (2009) we have interrogated the genomes of *Synechococcus* sp. CC9311, *Prochlorococcus* sp. CCMP1375, and *Prochlorococcus* sp. CCMP1986. We have also examined the entries for cyanobacterial Zur regulons in the RegPrecise database (Novichkov et al., 2010). The motifs developed by Haas and by RegPrecise differ (**Figure 5**), but common features can still be discerned, and it is likely that the consensus Zur box in cyanobacteria corresponds to an (imperfect) inverted repeat, analogous to Zur and Fur boxes from other bacteria (Gabriel et al., 2008).

In the *Prochlorococcus* strains examined, a putative Zur box was found in the intergenic region between a putative *znuA* and *znuC*. All three components of the ABC transporter are arranged into one gene cluster that also comprises the respective putative *zur* gene (**Figure 4**). All other strains inspected (*Trichodesmium*

erythraeum, *Synechococcus* sp. WH8102, *Synechococcus* sp. PCC 7002) also contain one or more Zur boxes in their *znuABC* gene cluster.

In the genome of *Prochlorococcus* sp. CCMP1986, Zur boxes were also identified upstream of several genes encoding ribosomal proteins (S7, S12, and S14p/S29e). This is of potential interest, because in other bacteria several ribosomal proteins, e.g., S14, L31, L33, and L36, occur in two versions, one requiring a zinc ion to stabilize a zinc-ribbon fold, and one version not requiring zinc (Makarova et al., 2001). The latter versions have been shown to be regulated by Zur in several bacterial species (Panina et al., 2003; Owen et al., 2007; Gabriel and Helmann, 2009). It has been suggested that the ribosome is, under zinc-replete conditions, a substantial store for cellular zinc, and that the "alternative" versions are expressed in response to zinc deprivation (Owen et al., 2007), operating as a backup for growth in Zn-poor environments, thus helping to reduce the overall requirement for zinc and the cellular zinc quota. The transcriptional response of *E. coli* to extreme zinc limitation (Graham et al., 2009) highlights that zinc limitation not only affects the transcription of genes encoding proteins involved in zinc homeostasis, and that are zinc-requiring, but also, importantly, zinc-independent proteins. Similarly, in other bacterial species it has been demonstrated that a variety of zinc-independent proteins including an alternative version of the global

FIGURE 5 | Sequence logos for Zur boxes in cyanobacteria. (A) Consensus sequence developed by Haas et al. (2009), using 14 sequences found upstream of COG0523 genes in *Prochlorococcus marinus* sp. CCMP1986, *P. marinus* sp. CCMP1375, *Nostoc* sp. PCC 7120, *Cyanothece* sp. PCC 7424, *Cyanothece* sp. PCC 8802, *Cyanothece* sp. PCC 8801, *Cyanothece* sp. ATCC 51142, and *Cyanothece* sp. PCC 7425. **(B)** Consensus sequence deposited in the RegPrecise database (Novichkov et al., 2010) using 75 sequences from the genomes of *Synechocystis* PCC 6803, *Synechococcus* sp. PCC 7002, *Synechococcus* sp. PCC 7942, *Synechococcus* JA-3.3Ab, *Thermosynechococcus elongatus* BP-1, *Synechococcus* sp. WH8102, *Prochlorococcus* sp. MIT 9313, *Trichodesmium erythraeum* IMS101, *Cyanothece* sp. PCC 7425, *Cyanothece* sp. PCC 8801, *Cyanothece* sp. ATCC 51142, *Microcystis aeruginosa* NIES-483, *Nostoc* sp. PCC 7120, and *Gloeobacter violaceus* PCC 7421. There are clear similarities between the two logos as indicated by the suggested alignment of parts A and B.

transcription factor DksA in *Pseudomonas aeruginosa* (Blaby-Haas et al., 2011), are under the control of Zur, and genome analyses of Zur regulons suggest that this is a widespread phenomenon (Haas et al., 2009). However, neither of the two versions of S14 present in the genome of *Prochlorococcus* sp. MED4 displays any salient signatures for zinc-binding, and neither S7 nor S12 are known to bind zinc or occur in duplicate, so the significance of their vicinity to Zur boxes is in need of further investigation.

Another set of potential Zur boxes were found in a cluster that comprises FutC, ferritin, and a Rieske iron–sulfur protein. According to the manually curated database of bacterial regulons RegPrecise, the cognate sequences of Furs and Zurs differ significantly (Novichkov et al., 2010). Hence, the detection of potential Zur boxes within a cluster related to iron homeostasis suggests that there is some crosstalk between the homeostasis of these two metal ions; this has been observed in other bacteria, e.g., in *S. coelicolor*, where the gene cluster responsible for the production of the siderophore coelibactin is regulated by Zur (Kallifidas et al., 2010).

The only Zur box that we were able to identify in the genome of *Synechococcus* sp. CC9311 was upstream of a predicted ZIP transporter protein (Sync_2443). ZIP (for "zinc–iron permeases") proteins are involved in zinc uptake in a variety of organisms including plants, animals, fungi, and bacteria. However, ZIP proteins have as yet not been reported for cyanobacteria, and no recognizable homologs of Sync_2443 were found in any other cyanobacterium; the closest match in a BLAST search was a zinc transporter from the γ-proteobacterium *Francisella novicida*. If Sync_2443 really is a zinc transporter, this coastal strain has an

even more remarkable repertoire for dealing with fluctuations in zinc concentrations than previously thought (Palenik et al., 2006). Two further notable RegPrecise entries were found for the genomes of *Trichodesmium erythraeum*, indicating Zur boxes upstream of a putative metallochaperone of the COG0523 family (Tery_4617), and *Synechococcus* sp. WH8102, which contains two Zur boxes upstream of a gene encoding a potential bacterial metallothionein (SYNW0359) – both groups of proteins are discussed below.

Very recently, Napolitano et al. (2012) studied the response of *Anabaena* sp. PCC7120 to zinc starvation. Aided by gel shift assays and a deletion mutant, the product of the *all2473* gene, previously designated FurB and thought to be involved in the response to oxidative stress (López-Gomollón et al., 2009), was identified as a true Zur. Several gene clusters that contain putative Zur boxes were shown to be regulated by changes in zinc levels in this organism. The expression of four categories of proteins was regulated by Zur: (i) zinc-free paralogs of zinc proteins, (ii) putative metallochaperones of the COG0523 family, (iii) ABC transporters including a predicted ZnuABC system, and (iv) outer-membrane proteins, particularly TonB-dependent receptors. A 7-1-7 palindromic DNA sequence to which Zur bound with high specificity was also determined, and agrees well with the Zur box consensus motifs shown in **Figure 5**. The protein sequence of the *all2473* product clusters with those predicted by us to be Zurs (**Figure 3**), and the four residues predicted to be involved in zinc-sensing are also conserved in the *all2473* protein.

MEMBERS OF THE COG0523 FAMILY AND ZINC

In our earlier work (Blindauer, 2008b), we noted that the genome neighborhood of putative *zur* and *znuABC* genes in numerous genomes from cyanobacteria harbored genes that were annotated as "Putative GTPase, G3E family", "Cobalamin synthesis protein/P47K", or "CobW". The latter protein is a cobalt chaperone that is part of the machinery for Vitamin B12 synthesis, and at the time, this raised the question whether our putative zinc-related genes might actually be involved in the regulation and transport of cobalt. In the meantime however, Haas et al. (2009) have conducted a thorough analysis of the COG0523 family (Leipe et al., 2002) to which CobW belongs. All members of COG0523 have a P-loop GTPase domain, and are thus also related to nickel-chaperones of the G3E family with the same domain that are involved in the maturation and assembly of urease (UreG), and hydrogenase (HypB), as well as to the iron-chaperone Nha3 required for maturation of nitrile hydratase. G3E family proteins, and by inference, COG0523 proteins, function as either insertases – proteins that perform energy-requiring metal insertion into target proteins – or as cytosolic storage and transport devices for metals (=metallochaperones), or both.

The COG0523 members are composed of two domains, a well-conserved N-terminal GTPase domain, and a more variable C-terminal domain. They have been categorized as segmentally variable genes (Haas et al., 2009), and this points toward a role in adaptation to environmental stresses and/or variability, and indicates development of binding specificity for other proteins or small molecules. Haas et al. note that "the COG0523 family is a striking example of systematic homology-based misannotation" – a specific function (in this case a role in cobalamin

biosynthesis) being assigned even though the level of sequence similarity does not support this conclusion. We would add that such mis-annotation is certainly rife in the case of gene annotation for metal-binding proteins. The fact that many Zur proteins are annotated as Fur is another example of this problem, and issues surrounding the annotation of ABC-type transporters and metal-transporting ATPases have been discussed elsewhere (Blindauer, 2008b).

Importantly, the theoretical and experimental studies of Haas et al. as well as the work of other groups (Gabriel et al., 2008) strongly suggested that a subset of COG0523 proteins is involved in zinc homeostasis. The most prominent member of zinc-related COG0523 is probably *Bacillus subtilis* YciC (Gabriel et al., 2008); it has been established that its expression is under the control of Zur, leading to upregulation when zinc is scarce. It is suggested that the expression of zinc-related COG0523 proteins may provide advantages under conditions of poor zinc nutrition – obviously, these are conditions always present for open-ocean cyanobacteria. Unfortunately, no suitable studies at the protein level seem to be available for any COG0523 member, so although a link to zinc deprivation is solidly established at the transcriptional level, it is not known whether and which metal ion these proteins bind either *in vitro* or *in vivo*. Whilst the requirement of metallochaperones for copper, nickel, and cobalt can be easily understood, as the target proteins for whose assembly they are required are few and well identified, a similar scenario for zinc has been deemed unlikely, as it is thought that even in bacteria, there are too many destinations for zinc, so the tenet that there is one chaperone per metalloprotein is inconceivable. Hence, two hypotheses have been put forward regarding the role of the zinc-related COG0523 members (Haas et al., 2009): (i) they are up-regulated to function in the recruitment and supply of a metal ion that is not zinc to metalloproteins – the latter may be either normally zinc-requiring or a different paralog – (ii) these COG0523 proteins may be involved in the (re-)allocation of zinc when this becomes necessary.

Sequence analysis of cyanobacterial COG0523 proteins (**Figure 7**; Figures S3 and S4 in Supplementary Material) illustrates characteristic features, for example a well-conserved GCxCC motif which has been suggested as potential metal-binding site previously (Haas et al., 2009); however, its location in a β strand makes this less likely (see below and **Figure 8**). Another intriguing feature is the insertion of stretches with repetitive HHX, and HXH motifs of up to 70 amino acid residues in total. These His-rich stretches have been suggested to be hallmarks for metallochaperone activity of G3E GTPases. In the COG0523 subset we have analyzed 115 out of 150 proteins (77%) display such stretches. The phylogenetic tree for cyanobacterial COG0523 proteins (**Figure 6**) is roughly split into three major branches, each of which is divided into two sub-branches. Phylogenetic analysis, together with analyses of genome contexts (**Figure 4**), suggests that phylogeny and metal specificity are not congruent – in agreement with the findings of Haas et al. who defined 15 phylogenetic sub-groups for COG0523 members, with links to zinc homeostasis found in several sub-groups, and various sub-groups containing representatives with links to several different metal ions. A strong link to *znuABC*- and *zur*-containing gene clusters is present in one branch (1a) of the phylogenetic tree (**Figure 6**) for sequences from
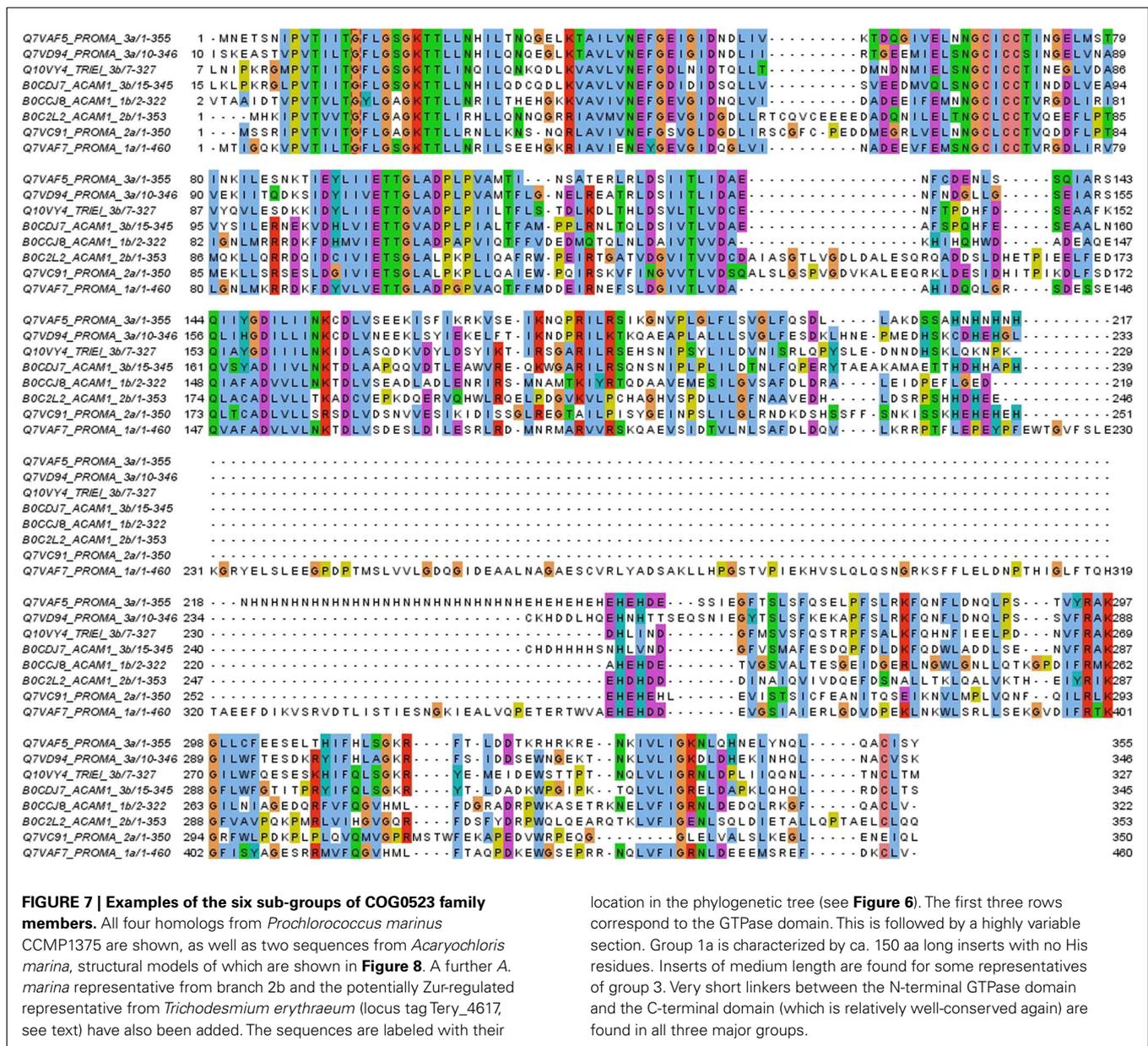
Prochlorococcus and *Synechococcus* sp. WH8102. Analysis of the genome environment for these sequences reveals co-localization of COG0523 with a WD-40 repeat gene (**Figure 4**). This association is also strongly conserved for other members in this branch, although the functional significance of the WD-40 repeat protein is unclear. Interestingly, COG0523 sequences of branch 1a contain long inserts (ca. 150 aa), distinct from those described above, as they are not particularly rich in His residues (**Figure 7**). Secondary structure prediction for these inserts suggests the presence of four to six β-strands and one or two short α-helices. It is conceivable that this insert forms an additional domain in its own right, though no similarity to any known domains was found in an InterPro-scan search.

There is currently only one X-ray structure available for a COG0523 protein – the YjiA protein from *E. coli* (Khil et al., 2004). According to the analysis by Haas et al. (2009) YjiA belongs to their subgroup 9, for which no clear association with any particular metal ion was reported. The crystal structure (pdb 1NJI) also does not contain any metal ions, but considering that the structure is a product of a structural genomics effort, this may not be surprising. Analysis of potential metal sites in the structure using the CHED server (Levy et al., 2009) revealed the presence of two surface-exposed sites with potential for metal-binding; these are both in the GTPase domain and are composed of H23, E27, and H29, and D52, D79, and D82.

We were interested to see whether it would be possible to pin-point distinguishing elements that would indicate metal-binding capacity, with the help of homology models for COG0523 proteins. We therefore chose B0CCJ8_ACAM1 from *Acaryochloris marina* that is present in branch 1 as a target for comparative modeling. The respective AM1_3031 gene is next to a WD-40 repeat gene and also in the neighborhood of a *zur* gene (**Figure 4**). In addition, we modeled B0CDJ7_ACAM1, since the gene for this protein (AM1_3070) is co-localized with a putative *znuABC* system (**Figure 4**). The sequence for this protein is located in branch 3b of the phylogenetic tree and is highlighted in **Figure 6**. Several other closely related sequences from the same organism (including some plasmid-encoded sequences) are present in all three branches, but none of these occurs in a zinc-related genomic context. The two models are shown in **Figure 8**. Beside a His-rich loop between the two domains that also contains two Asp and a Cys residue, there are no further recognizable metal sites in B0CDJ7_ACAM1. In contrast, B0CCJ8_ACAM1 has a much shorter loop comprising only two His and several Asp and Glu residues that might bind a metal. Other branch 1b members also contain this short loop, but so do some representatives from the other sub-branches. The model also displays two further potential metal sites composed of D132, H135, and H139, and E210, D212, and E300, as identified using the CHED server, but only D132 and H135 are conserved within the branch, questioning the significance of these sites. The quest for the true metal-binding sites and the mode of action of the zinc-related COG0523 proteins remains open.
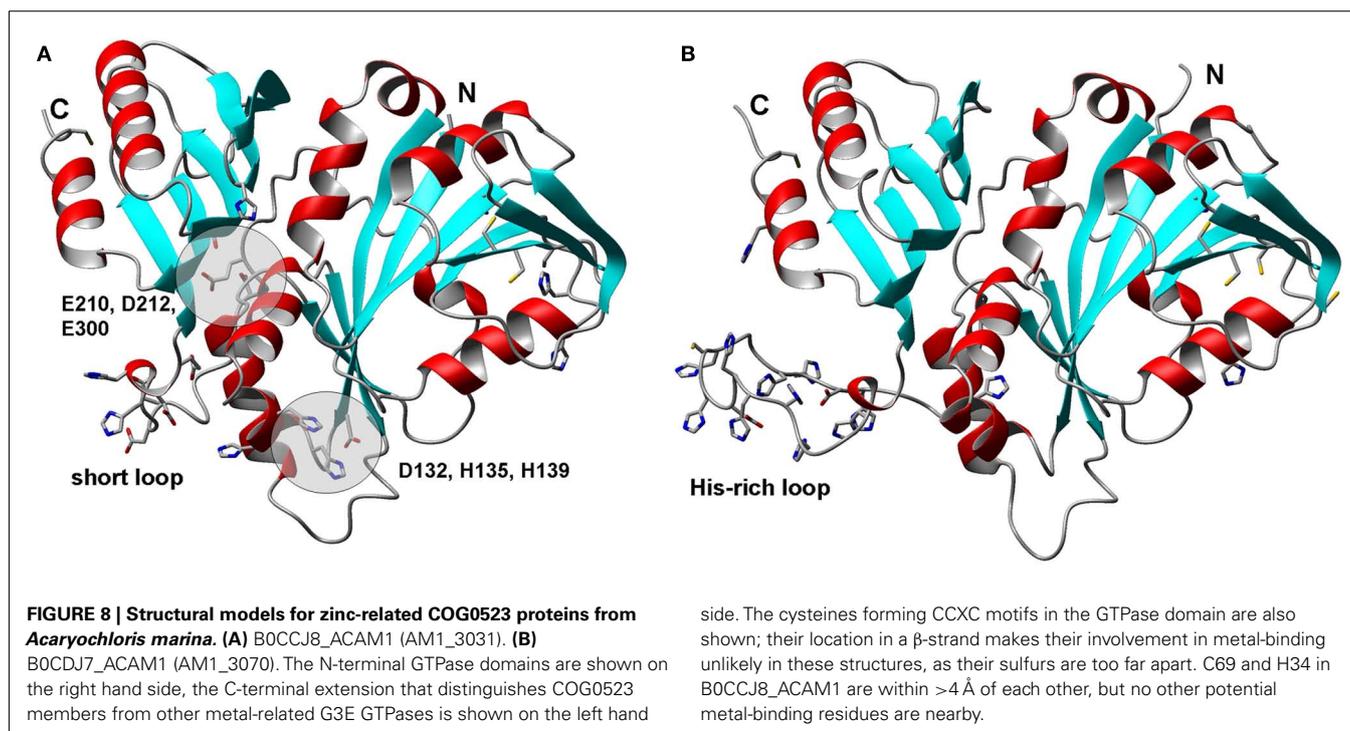
INTRACELLULAR HANDLING: METALLOTHIONEINS (BmtAs)

Metallothioneins, small proteins with a high content of cysteine with the capability to bind multiple metal ions in metal–sulfur

FIGURE 6 | Phylogenetic tree for COG0523 family members. The phylogenetic tree for cyanobacterial COG0523 family members was generated in MEGA5 using the maximum likelihood method with the JJT substitution model. Bootstrap values are the result of 1000 replications. Sequences are labeled with UniProt accession numbers; the actual sequences are documented Figure S3 in Supplementary Material, and a rectangular tree including bootstrap statistics can be found Figure S4 in Supplementary Material. The sequence of *E. coli* YjiA, the only COG0523 member for which an experimentally determined structure is available, was also included in the tree. Entries with a genomic context linked to zinc homeostasis are highlighted in purple (also see **Table 1**). Sub-branches are labeled and discussed in the main text.

clusters, were initially reported as cadmium-binding proteins in the livers and kidneys of mammals (Kägi, 1991). In the five decades since their discovery (Margoshes and Vallee, 1957), genes encoding metallothioneins have been identified in virtually all

FIGURE 7 | Examples of the six sub-groups of COG0523 family members. All four homologs from *Prochlorococcus marinus* CCMP1375 are shown, as well as two sequences from *Acaryochloris marina*, structural models of which are shown in **Figure 8**. A further *A. marina* representative from branch 2b and the potentially Zur-regulated representative from *Trichodesmium erythraeum* (locus tag Tery_4617, see text) have also been added. The sequences are labeled with their location in the phylogenetic tree (see **Figure 6**). The first three rows correspond to the GTPase domain. This is followed by a highly variable section. Group 1a is characterized by ca. 150 aa long inserts with no His residues. Inserts of medium length are found for some representatives of group 3. Very short linkers between the N-terminal GTPase domain and the C-terminal domain (which is relatively well-conserved again) are found in all three major groups.

phyla, and numerous studies regarding their biophysical properties (Blindauer and Leszczyszyn, 2010) and biological functions (Davis and Cousins, 2000; Cobbett and Goldsbrough, 2002; Klaassen et al., 2009) have been carried out. It has become clear that their main function is not restricted to cadmium detoxification nor to responses to other chemical and physical stresses. At least for vertebrates, it is now accepted that they also play a more general and essential role in zinc homeostasis (Maret, 2009; Colvin et al., 2010), and that they constitute an important link between cellular redox state and zinc signaling networks (Maret, 2011).

The presence of metallothionein-like proteins in bacteria (for recent reviews see (Blindauer, 2009, 2011) was first indicated in 1979, namely in the marine cyanobacterium *Synechococcus* RRIMP N1 (Olafson et al., 1979). So-called "pseudo-thioneins" were also discovered in cadmium-adapted *Pseudomonas putida* (Higham et al., 1984). The first gene for a bacterial metallothionein, *smtA*, was isolated from *Synechococcus* sp. PCC 7942 (Robinson et al., 1990), and was shown to be regulated by the zinc-sensing transcriptional repressor SmtB. Phenotypically, *smtA* knock-out mutants are hypersensitive to Zn^{2+}, and to a lesser extent to Cd^{2+}, with no effect on tolerance to other metal ions (Turner et al., 1993), even though *smtA* transcription is stimulated not only by Zn^{2+} (which is by far the strongest inducer) and Cd^{2+}, but also Hg^{2+}, Cu^{2+}, Co^{2+}, Cr^{3+}, and Ni^{2+} (Huckle et al., 1993). Moreover, SmtA expressed in *E. coli* was shown to bind not only zinc and cadmium, but also copper and mercury (Shi et al., 1992). Considering that SmtB is

FIGURE 8 | Structural models for zinc-related COG0523 proteins from *Acaryochloris marina.* **(A)** B0CCJ8_ACAM1 (AM1_3031). **(B)** B0CDJ7_ACAM1 (AM1_3070). The N-terminal GTPase domains are shown on the right hand side, the C-terminal extension that distinguishes COG0523 members from other metal-related G3E GTPases is shown on the left hand side. The cysteines forming CCXC motifs in the GTPase domain are also shown; their location in a β-strand makes their involvement in metal-binding unlikely in these structures, as their sulfurs are too far apart. C69 and H34 in B0CCJ8_ACAM1 are within >4 Å of each other, but no other potential metal-binding residues are nearby.

clearly a Zn^{2+}-responsive metal sensor (Turner et al., 1996), the documented responses to other metal ions may be mediated indirectly, by displacement of Zn from proteins by these metal ions.

As soon as the protein sequence of what was later to be called SmtA was available, it was clear that apart from the high cysteine content, there was very little sequence similarity between previously characterized metallothioneins and their bacterial counterparts (Olafson et al., 1988). However, it should be made clear that this statement is essentially true for all MTs from different phyla (Blindauer and Leszczyszyn, 2010). For example, in the animal kingdom, the sequences from MTs from nematodes, snails, earthworms, and vertebrates are so divergent that it is not possible to demonstrate a clear evolutionary relationship. To some extent, this is due to their small size, their low level of complexity, and the absence of a defined protein fold. The latter feature is also reflected in the fact that the folding of MTs is dominated by the formation of the metal–sulfur clusters, and unless the "correct" complement of metal ions is bound, MTs do not adopt well-defined conformations.

In that sense, bacterial metallothioneins of the BmtA type are an exception – they contain a clearly identifiable zinc finger fold (Blindauer et al., 2001, 2002; Blindauer and Sadler, 2005; **Figure 9A**), and it has been demonstrated experimentally that the constituents of this fold (residues 7–38) form an ordered, folded structure, even if only one Zn^{2+} ion is bound to SmtA (Leszczyszyn et al., 2007a). Another "special feature" that is becoming less exceptional as more and more MTs from other phyla are being studied, is the presence of aromatic residues, including histidines, the latter often with a direct involvement in metal-binding (Blindauer et al., 2007; Leszczyszyn et al., 2007b; Blindauer, 2008a; Peroza et al., 2009; Zeitoun-Ghandour et al., 2010).

The high abundance of cysteine residues are the cause of the high thermodynamic stability of MT complexes with soft[1] metal ions such as Cu^+ and Cd^{2+}, and these tend to bind more strongly to MTs than Zn^{2+}, which is classified as a borderline metal ion. It should however be noted that thermodynamic stability is not necessarily a criterion to determine which metal ions are handled by a particular MT (or indeed a particular protein) *in vivo*. If the metallated protein has not been obtained in its natively metallated form from the natural source, it is important to take into account information on which metal ion(s) induce(s) MT gene transcription most strongly, whether the protein confers tolerance against a particular metal ion, and also on how well-folded the protein is in the presence of different metal ions. Bofill et al. (2009) have compiled large amounts of biophysical data on recombinantly expressed MTs from a variety of species, and have suggested that there are clear Cu-MTs and Zn/Cd-MTs, as well as MTs between these two extremes with less well-defined metal preferences.

SmtA is thought to be a prototypical Zn-MT. To some extent, this is indeed also reflected in the *in vitro* properties of the protein: the zinc finger site and fold require a four-coordinate metal ion – which excludes Cu^+ as it prefers trigonal or linear coordination modes with thiolate ligands. In addition, the two His-containing metal sites augment the relative affinity for Zn^{2+} compared to Cd^{2+}, although this is certainly not their sole purpose (Blindauer et al., 2007). Nevertheless, SmtA folds equally well in the presence of four Zn (Blindauer et al., 2001) or four Cd (Blindauer et al., 2008) ions, or any mixture thereof, a feature that greatly facilitated the determination of its 3D structure.

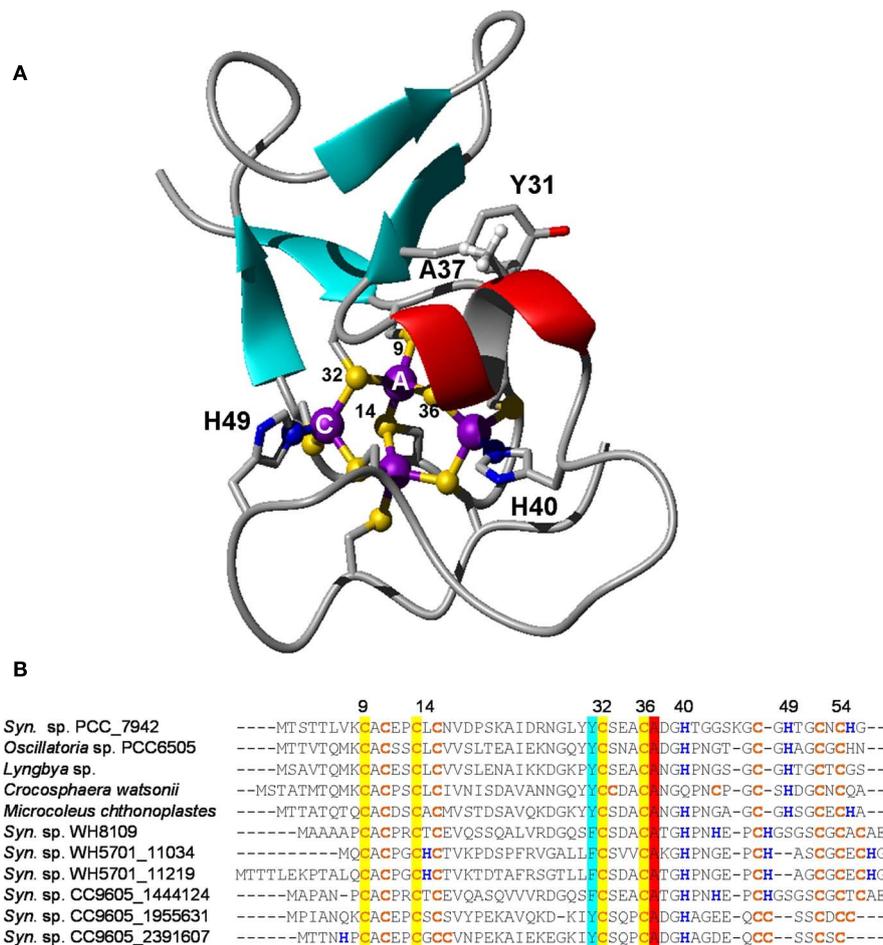[1]The terms "soft" and "borderline" refer to Pearson's HSAB principle: Pearson (1990).

FIGURE 9 | Structural features of bacterial metallothioneins. (A)
NMR solution structure of Zn$_4$SmtA. Residues important for the zinc
finger fold and metal ligands are highlighted. **(B)** Selected primary
sequences of BmtAs from marine cyanobacteria. The level of
conservation is different for different metal sites. Site A, the zinc finger
site, is fully conserved, as it is required for structural stability.

Conservation is also high for residues Y31 and A37; these mediate a
CH-π interaction between β hairpin and α-helix. The least conserved site
is site C, which is also the "business end" of BmtAs, i.e., the site that (in
SmtA) releases zinc most readily (Leszczyszyn et al., 2007a). It is
envisaged that the high variability of this site may allow to fine-tune the
metal-binding and -release properties of different SmtAs.

Although no in-depth study of SmtA (or any other BmtA) loaded
with copper ions has so far been carried out, recombinant expres-
sion of SmtA in the presence of added copper yielded samples
with only ca. 1.5 copper ions bound, whereas contents of Cd^{2+},
Hg^{2+}, and Zn^{2+} varied between ca. 4 and 6 when the respec-
tive metal ion was added to the culture medium (Shi et al.,
1992).

An analysis of the genomes of all marine cyanobacteria reveals
that 14 out of 33 sequenced genomes contain one or more genes for
a BmtA (**Table 1**; **Figures 9B**). The BmtA sequences were retrieved
using a protein vs. DNA TBLASTN search with the *Synechococcus*
sp. PCC 7942 sequence as query and the six-frame translations of
the genomic nucleotide databases. This is necessary to ensure that
all BmtAs are captured, as due to their small size, BmtA ORFs are
easy to overlook. This is illustrated by the fact that *bmtA* genes
are not annotated in the genomes of *Microcoleus*, *Cyanobium*, and
Crocosphaera watsonii, all of which are in their "Draft" stage, but

they have also been overlooked in the finished genome and plasmid
sequences of *Acaryochloris marina*.

In a select few genomes (*Synechococcus* sp. PCC 7002, *Oscil-
latoria* sp. PCC 6506, and the genome as well as pREB2 and
pREB6 plasmids of *Acaryochloris marina*) BmtAs occur in a clus-
ter together with SmtB. On the latter two plasmids, a COG0523
homolog is also nearby. In the *Synechococcus* sp. WH5701 genome,
both BmtAs are annotated, and WH5701_11219 (the latter num-
ber refers to the first base of the start codon) is located near a
CsbD-like protein, thought to be involved in the general stress
response, a Cd/Co/HG/Pg/Zn-transporting ATPase, and the per-
mease and ATPase components of an ABC transporter, but since
no periplasmic component is present in the vicinity, the specificity
for this transporter cannot be predicted.

Several of the benthic strains also have SmtB homologs, for
example *Microcoleus chthonoplastes* and *Lyngbya* (in a cluster with
ZiaA), and *Nodularia spumigena* (intriguingly in a gene cluster

with an ABC transporter), but even though several open-ocean and coastal marine *Synechococcus* (WH8102, WH8109, WH7803, CC9311, CC9605) have genes for BmtA, there are no identifiable SmtB or other known zinc excess sensors present. Therefore, it remains unclear how the expression of these metallothionein genes is regulated, although the discovery of two Zur boxes upstream of BmtA in *Synechococcus* sp. WH8102 gives rise to the suggestion that this strain might use its metallothionein in response to zinc limitation rather than excess.

We note that although the prototype *smtA* clearly responds most strongly to zinc, and the SmtA protein can be considered a "zinc-metallothionein," this may not necessarily hold true for all homologs from marine strains. Since the concentrations of Cd^{2+} can be significant in various natural seawaters, the marine BmtAs may also help with dealing with this metal ion – potentially both for preventing toxicity as well as in preparation for utilization as a cofactor. Cd also has a nutrient-like profile in stratified marine waters, and there are several precedents where marine phytoplankton have been found to utilize Cd instead of Zn (Lane et al., 2005). If this was also common in marine cyanobacteria, BmtAs may be part of this utilization network. Furthermore, it can also be envisaged that some BmtAs may play a role in defense against oxidative stress, as shown for metallothioneins from other species (Zeitoun-Ghandour et al., 2011).

Finally, it should be highlighted that cyanobacteria are not the only marine microorganisms with metallothioneins. Several γ-proteobacteria of the genus *Nitrosococcus* also harbor *bmtA* genes in their genomes, and additional *bmtA* sequences can also be retrieved from marine metagenomes (Blindauer, 2008b), suggesting that many more marine bacterial species utilize these proteins.

CONCLUSION

At the time of writing this report (December 2011), there were 64 cyanobacterial genomes available, 35 of them from marine species. Genome annotation must, ultimately, be based on experimental evidence, and we have seen that there is a persistent shortfall of reliable information regarding function of predicted proteins. This is a general problem, witnessed by the large number of hypothetical proteins even in finished genomes, but even in the case of well-defined protein families, annotations regarding metal specificity have to be approached with caution. Our discussions emphasize that sequence similarity alone does not hold the key to determine metal specificity, but we believe that, amongst other approaches, the generation of more sound data at the protein level is central to refine our ideas about how metal homeostasis works.

With this caveat in mind, we are highlighting in the following some salient points from our analyses. The ubiquitous presence of bona-fide Zur and ZnuABC systems – both thought to be involved in responding to a lack of cellular zinc – in all strains we have studied suggests that these cyanobacteria are at the very least capable of utilizing zinc. The identification of putative Zur boxes in several genomes we and others have inspected also suggests that zinc levels are an integral part of the metabolic network of marine cyanobacteria. Nevertheless, an absolute requirement for zinc has not been demonstrated experimentally for any cyanobacterium – in the few instances where this has been studied, only small reductions in growth rates were observed. This may indicate that marine cyanobacteria have either very low zinc requirements, or extremely efficient uptake mechanisms, or both, enabling them to thrive at extremely low free zinc concentrations. It would hence be interesting to study whether cyanobacteria that are growing at such extremely low zinc concentrations actually contain any zinc, and if so, what the ratio of bio-accumulation is.

Genes for zinc excess sensors appear to be absent from the genomes of open-ocean strains (Blindauer, 2008b), but other strains including *Microcoleus chthonoplastes*, *Synechococcus* sp. PCC 7002, and *Oscillatoria* sp. PCC 6506 have clear SmtB homologs. Although the coastal strain *Synechococcus* sp. CC9311 does not appear to have SmtB, it not only has four BmtA homologs, but also a putative CzrA efflux pump that is thought to be able to transport zinc (and cobalt). Together with at least one ZnuABC system and a putative ZIP transporter for uptake, as well as at least one zinc-related COG0523 protein, this strain is expected to cope well with both zinc excess and scarcity.

The suggestion that metallothioneins in marine *Synechococcus* (e.g., WH8102) may be under the control of Zur is exciting. Whilst this finding may at first seem counterintuitive, it suggests that, also in (some) bacteria, metallothioneins are not just devices to combat metal toxicity, but may play a more central role in essential zinc homeostasis. This hypothesis has been raised before (Robinson et al., 2001; Blindauer, 2009), and this newly identified association between Zur and BmtA provides support.

METHODS

Sequences of Fur-family proteins were retrieved from cyanobacterial genomes using BLAST (Altschul et al., 1997). Briefly, GenBank files were retrieved for each genome and all protein sequences were extracted using BioPerl (Stajich et al., 2002) and a custom BLAST database was created for each genome. Fur-like proteins were identified using the three Fur-like proteins previously identified in *Synechococcus* sp. CC9311 as queries (accessions YP_730377.1, YP_732019.1, and YP_730926.1) with an e value cutoff $<10^{-5}$. Any duplications were removed and amino acid sequences were aligned using CLUSTALW (Larkin et al., 2007). The Fur amino acid sequence from *Escherichia coli* and *Synechocystis* sp. PCC 6803, along with Zur from *Streptomyces coelicolor*, *Mycobacterium tuberculosis*, and *Synechocystis* sp. PCC 6803 were extracted from the Protein Data Bank[2]. Phylogenetic analysis was carried out in MEGA version 5 (Tamura et al., 2011).

Sequences for COG0523 family proteins were collected by searching the fully sequenced and annotated cyanobacterial proteomes plus all sequences from *Oscillatoria* sp. PCC 6506, *Lyngbya* sp. PCC 8106, *Microcoleus chthonoplastes* sp. PCC 7420, *Nodularia spumigena* sp. CCY9414, *Crocosphaera watsonii* WH 8501, and *Trichodesmium erythraeum* IMS101 from UniProt with the Pfam PF07683 Hidden Markov Model using HMMER4 with standard cutoffs. The collated sequences were filtered for fragments and aligned in JALVIEW and MUSCLE v.3.8 (Edgar, 2004), and were manually adjusted where necessary.

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[2]http://www.rcsb.org/

Gene Ortholog Neighborhood analyses were performed using the respective resource on the Integrated Microbial Genome Server (Markowitz et al., 2010).

Comparative modeling was carried out using Modeller version 9.7 (Eswar et al., 2008). A dimeric assembly of *Streptomyces coelicolor* Zur was generated from the two B chains in the original pdb file (3MWM; Shin et al., 2011) using PISA[3] (Krissinel and Henrick, 2007). This dimeric structure was used as a template for modeling the putative Zur from *Prochlorococcus marinus* CCMP1375 (locus tag Pro1502). Template and model sequences were aligned manually. Sidechains and improper dihedrals were optimized with SCWRL 3.0 (Wang et al., 2008). Inspection of initial models and sequence alignments suggested that Cys98 may be a zinc ligand; this residue is highly conserved in all putative Zur sequences from cyanobacteria (See Figure S2 in Supplementary Material). Hydrogens, Zn ions, and metal–ligand bonds were added in MOE v. 2004.03. Energy minimization of the final model was performed in MOE using a customized Amber94 force-field. Final structures were validated using the WHATIF server[4]. Structural images (**Figures 2**, **8**, and **9**) were generated with MOLMOL v.2K.2 (Koradi et al., 1996). A similar strategy was applied for modeling two putatively zinc-binding representatives of the COG0523 family based on the *E. coli* protein as a template (pdb-entry 1NIJ). The target template sequence alignment made use of the HHPRED (Soding et al., 2005) fold recognition server.

[3]http://www.ebi.ac.uk/msd-srv/prot_int/cgi-bin/piserver
[4]http://swift.cmbi.ru.nl/servers/html/index.html

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Microbiological_Chemistry/10.3389/fmicb.2012.00142/abstract

Figure S1 | Phylogenetic tree for Fur/Zur-like proteins extracted from genomes of marine cyanobacteria.

Figure S2 | Sequence alignment of Fur/Zur-like proteins.

Figure S3 | Phylogenetic tree for COG0523 family members.

Figure S4 | Sequence alignment of members of the COG0523 protein family from the genomes of cyanobacteria.

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