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Characterization of the Response to Zinc Deficiency in the Cyanobacterium *Anabaena* sp. Strain PCC 7120

Mauro Napolitano,[a] Miguel Ángel Rubio,[a] Javier Santamaría-Gómez,[a] Elvira Olmedo-Verd,[a]* Nigel J. Robinson,[b] and Ignacio Luque[a]

Instituto de Bioquímica Vegetal y Fotosíntesis, CSIC, and Universidad de Sevilla, Seville, Spain,[a] and Biophysical Sciences Institute, Durham University, Durham, United Kingdom[b]

Zur regulators control zinc homeostasis by repressing target genes under zinc-sufficient conditions in a wide variety of bacteria. This paper describes how part of a survey of duplicated genes led to the identification of the open reading frame *all2473* as the gene encoding the Zur regulator of the cyanobacterium *Anabaena* sp. strain PCC 7120. All2473 binds to DNA in a zinc-dependent manner, and its DNA-binding sequence was characterized, which allowed us to determine the relative contribution of particular nucleotides to Zur binding. A *zur* mutant was found to be impaired in the regulation of zinc homeostasis, showing sensitivity to elevated concentrations of zinc but not other metals. In an effort to characterize the Zur regulon in *Anabaena*, 23 genes containing upstream putative Zur-binding sequences were identified and found to be regulated by Zur. These genes are organized in six single transcriptional units and six operons, some of them containing multiple Zur-regulated promoters. The identities of genes of the Zur regulon indicate that *Anabaena* adapts to conditions of zinc deficiency by replacing zinc metalloproteins with paralogues that fulfill the same function but presumably with a lower zinc demand, and with inducing putative metallochaperones and membrane transport systems likely being involved in the scavenging of extracellular zinc, including plasma membrane ABC transport systems and outer membrane TonB-dependent receptors. Among the Zur-regulated genes, the ones showing the highest induction level encode proteins of the outer membrane, suggesting a primary role for components of this cell compartment in the capture of zinc cations from the extracellular medium.

In a changing environment, bacterial cells are continuously challenged by either insufficient, elevated, or even toxic concentrations of metals. However, both excess and deficiency of a particular metal in the cytoplasm are detrimental for cell growth. The maintenance of the cytoplasmic concentrations of metals within certain levels needs to be tightly controlled to avoid misincorporation of particular metals into noncognate proteins, and this is achieved primarily by regulating their flow into and out of the cell through the control of specific import systems or efflux pumps (61). Adaptation to the availability of metals in the environment may involve complex signaling systems and vast metabolic rearrangements (61).

Zinc is one of the most important divalent metals in biology. It may act as a structural element, helping to maintain the conformation of particular protein domains, or as a catalytic cofactor in the active site of a variety of enzymes (13). Zinc sensing in bacteria is carried out by regulators of different families, including SmtB/ArsR, MerR, TetR, MarR, and the Fur family (10, 26, 32, 37, 54). Zur (zinc uptake regulator) proteins belong to the Fur family. These proteins function as dimeric transcription factors that bind to palindromic DNA sequences in the promoters of regulated genes (33). In general, proteins of the Fur family work as repressors by binding to DNA targets that overlap promoter sequences, thus blocking the access of the RNA polymerase (17, 33). The Fur family includes proteins that, despite showing broad sequence similarity and a similar composition of structural domains, are diverse enough to respond to distinct stimuli. Thus, this family includes members like Zur, Fur, Nur, and Mur, which sense distinct divalent metals (Zn, Fe, Ni, and Mn, respectively), and PerR and Irr, which sense cytoplasmic peroxides and heme, respectively (33). Like other members of the Fur family, Zur proteins have two structural domains connected by a mobile hinge, an N-terminal winged-helix DNA-binding domain and a C-terminal dimerization domain (39, 55). Zur contains several coordination sites for zinc (39, 41, 55) and senses the cytoplasmic concentration of exchangeable zinc by binding to this metal, which in turn allows Zur binding to DNA (33). Effective sensing of zinc is presumed to require the concentration of the metal in the cytoplasm to approach the affinity of the regulatory coordination site(s) (41, 55), and zinc has been estimated to be present in subpicomolar concentrations in *Escherichia coli* (45). Occupancy of the regulatory site probably induces a conformational reorientation of the two domains so that, in the dimer, the DNA-binding domains adopt an optimal orientation for binding to DNA (39, 41, 55).

Cyanobacteria are oxygenic photosynthetic prokaryotes, distributed throughout a wide variety of environments ranging from oceanic and fresh waters to continental habitats at all latitudes (62). Although some may form symbiotic associations with fungi and plants, most species are free-living organisms. Their capacity to fix atmospheric CO_2 and release O_2 by photosynthesis together with their abundance on Earth make these organisms quantitatively important in some biogeochemical cycles. For instance, it is estimated that oceanic cyanobacteria account for a significant portion of global primary production (11, 23, 35, 47). Cyanobacteria have a strong dependency on zinc, since one of the most

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Address correspondence to Ignacio Luque, ignacio.luque@ibvf.csic.es.
* Present address: Institute for Cell and Molecular Biosciences, Newcastle University, Newcastle, United Kingdom.
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important and abundant enzymes for CO_2 fixation in these organisms, carbonic anhydrase, is most commonly a zinc metalloenzyme which generates a substrate for this reaction from bicarbonate (57). Although several aspects of detoxification of excess zinc have been exhaustively analyzed in cyanobacteria (7–9, 12, 30, 36, 49), the response of these organisms to zinc deficiency remains poorly investigated. Indeed, the Zur regulator has scarcely been characterized in this phylum (7, 12, 59).

In this study, the response of *Anabaena* sp. strain PCC 7120 (also known as *Nostoc* sp. strain PCC 7120) to zinc deficiency was analyzed. The Zur regulator was identified with *in vitro* and *in vivo* evidence of the specificity of this factor for zinc. An empirical characterization of the Zur-binding sequence is provided, and components of the Zur regulon are identified.

MATERIALS AND METHODS

Organisms and growth conditions. *Anabaena* sp. strain PCC 7120 was routinely grown in BG11 medium (48) at 30°C, illuminated with white fluorescent lamps at 75 μE m^{-2} s^{-1}, in Erlenmeyer flasks that were shaken or aerated by bubbling with a mixture of air enriched with 1% CO_2. Bubbled cultures were buffered with 10 mM $NaHCO_3$. Solid medium was prepared by the addition of 1% agar to BG11. The *zur* mutant was cultured in medium containing 2 to 5 μg ml^{-1} streptomycin and 2 to 5 μg ml^{-1} spectinomycin. When indicated, *N*,*N*,*N*′,*N*′-tetrakis(2-pyridilmethyl)ethylenediamine (TPEN) was added to the cultures at a final concentration of 20 μM. TPEN was prepared in dimethyl sulfoxide (DMSO) at a concentration of 20 mM. In experiments using TPEN, DMSO was added to control cultures not containing TPEN at the same final concentration as those containing it.

Escherichia coli was grown in Luria-Bertani (1) medium with antibiotics, when needed, at the following concentrations: ampicillin, 50 μg ml^{-1}; kanamycin, 25 μg ml^{-1}; chloramphenicol, 30 μg ml^{-1}; streptomycin, 25 μg ml^{-1}; spectinomycin, 100 μg ml^{-1}. *Escherichia coli* DH5α was used for routine cloning. XL1-Blue and BL21(DE3) were used for overexpression of genes under the control of the *trc* promoter and the T7 promoter, respectively.

Generation of an *Anabaena zur* mutant. A deletion-insertion mutant of the *all2473* gene (*furB*/*zur*) was generated as follows. A DNA fragment containing the *all2473* gene and flanking sequences was amplified by PCR using *Anabaena* genomic DNA as a template and primers 2473_DEL-1F and 2473_DEL_1R. The PCR product was cloned in plasmid pBluescript SK+ (Novagen). The resulting plasmid, pCMN34, was PCR amplified with divergent primers 2473_DEL-2F and 2473_DEL_2R and ligated to the C.S3 cassette (15) conferring resistance to streptomycin and spectinomycin. The insert was cloned into the pRL278 vector and transferred by triparental conjugation to *Anabaena* (14).

Overexpression and purification of proteins. *all1691* (*furA*), *all2473* (*furB* or *zur*), and *alr0957* (*furC*) were PCR amplified from *Anabaena* genomic DNA using the primer pairs furA-1F–furA-1R, 2473-CLN-1F–2473-CLN-1R, and 0957-CLN-1F–0957-CLN-1R, respectively, cloned in the pCMN28b expression vector (M. Napolitano and I. Luque, unpublished data) in frame with the N-terminal Strep-Tag II sequence tag, and introduced into BL21(DE3). Cells were incubated for 16 h at 16°C after the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cell extracts were prepared, and proteins were purified through 1-ml Strep-Tactin Superflow columns (IBA) by following the instructions of the manufacturer.

RNA extraction and Northern blotting. RNA preparation from cyanobacterial cells and Northern assays were carried out as described in reference 40.

5′-RACE. Promoter mapping by 5′ rapid amplification of cDNA ends (5′-RACE) was carried out as described in reference 4, with some modifications. Briefly, 50 μg of RNA from *Anabaena* cells was incubated with 20 U of tobacco alkaline phosphatase (TAP) (1) for 2 h at 37°C. A control reaction mixture was incubated in parallel without enzyme. Both reaction mixtures were ligated to 15 pmol of an RNA oligonucleotide named Bensing RNA (see Table S2 in the supplemental material) with T4 RNA ligase. Aliquots (5 to 7 μg of RNA) of both reaction mixtures were annealed to specific primers for each of the genes in the *all4729-all4721* cluster and subjected to retrotranscription with 100 U of Superscript II reverse transcriptase (Invitrogen) at 47°C. cDNAs were amplified by using a forward primer named Bensing DNA (see Table S2) partially overlapping the sequence of the RNA oligonucleotide mentioned above and reverse primers all4727 4R, all4727 5R, all4726 1R, all4726 3R, all4725 3R, all4725 4R, all4724_1R, all4724 2R, all4724 3R, all4724 4R, thrS2 5R, thrS2 6R, thrS2 7R, thrS2 8R, all4722_2R, all4722 3R, all4722 4R, all4722 5R, all4722 6R, all4722 7R, and all4721 1R annealing with the 5′ region of the corresponding open reading frames (ORFs) (see Table S2). The PCR products were resolved on agarose gels, and bands found exclusively in the lanes corresponding to TAP-treated samples were sequenced.

Electrophoresis mobility shift assay (EMSA). Unless otherwise stated, radioactive DNA fragments to be used in EMSAs were generated by annealing partially overlapping oligonucleotides (all4725-GS-1F and all4725-GS-1R for *all4725*, all4723-GS-1F and all4723-GS-1R for *all4723*, all4722-GS-1F and all4722-GS-1R for *all4722*, all4721-GS-1F and all4721-GS-1R for *all4721*; see Table S2 in the supplemental material) and filling in with Klenow DNA polymerase in the presence of [^{32}P]dCTP. Forward primer all4725-GS-1F and reverse primers all4725-GS-2R to all4725-GS-14R (see Table S2) were used to generate DNA fragments with mutated versions of the Zur-binding site at the *all4725* promoter. DNA was used at 0.1 to 0.5 fmol per reaction, incubated in a buffer containing 20 mM Tris-HCl (pH 8), 50 mM KCl, 1 mM dithiothreitol (DTT), and 20% glycerol for 15 min at room temperature in the absence or presence of 0.01 to 20 pmol of protein, and resolved on 4 to 5% acrylamide gels. For the determination of the K_d, retarded and nonretarded bands were quantified in a Cyclone Plus storage phosphor system (Perkin Elmer), and the percentage of retarded bands was plotted against the concentration of protein in the assay and fitted to a simplified version of the Hill equation (53).

Real-time PCR. RNAs from wild-type (WT) *Anabaena* and the *zur* mutant were treated with DNase and inspected for DNA contamination by conventional PCR. DNA-free RNA (5 μg) was used for cDNA synthesis with a Superscript First Strand cDNA synthesis kit (Invitrogen) using random hexamers as primers. cDNAs were used as templates to set up PCRs with the SensiMix SYBR kit (Bioline) in an iQ5 multicolor real-time PCR detection system (Bio-Rad). The *rnpB* gene (60) was used as a standard gene for normalization.

RESULTS

Description of a gene cluster encoding several metalloproteins in *Anabaena*. In a survey of duplicated genes in the genome of the cyanobacterium *Anabaena* sp. strain PCC 7120 (referred to here as *Anabaena*), a cluster of 9 genes (*all4729* to *all4721*), of which 7 had homologues at different locations in the genome, was observed (Fig. 1). Features of the genes in the cluster are as follows: (i) they appear to be not functionally related; (ii) 6 of their putative products are predicted to contain metal binding sites; (iii) their expression is very low or silent under standard laboratory growth conditions; and (iv) some genes (*all4725*, *all4723*, and *all4721*) encode proteins with putative essential housekeeping functions. In contrast, the respective paralogues (*alr4380*, *alr0335*, and *alr5287*) of the latter genes were highly expressed under standard growth conditions (data not shown).

Characterization of the expression profile of the cluster. The RNA level of some genes in the cluster was analyzed under several conditions, including diazotrophy, anaerobiosis, nitrogen stress, and high light, and very low mRNA levels were observed under all conditions. Given the presence of putative metal-binding sites in

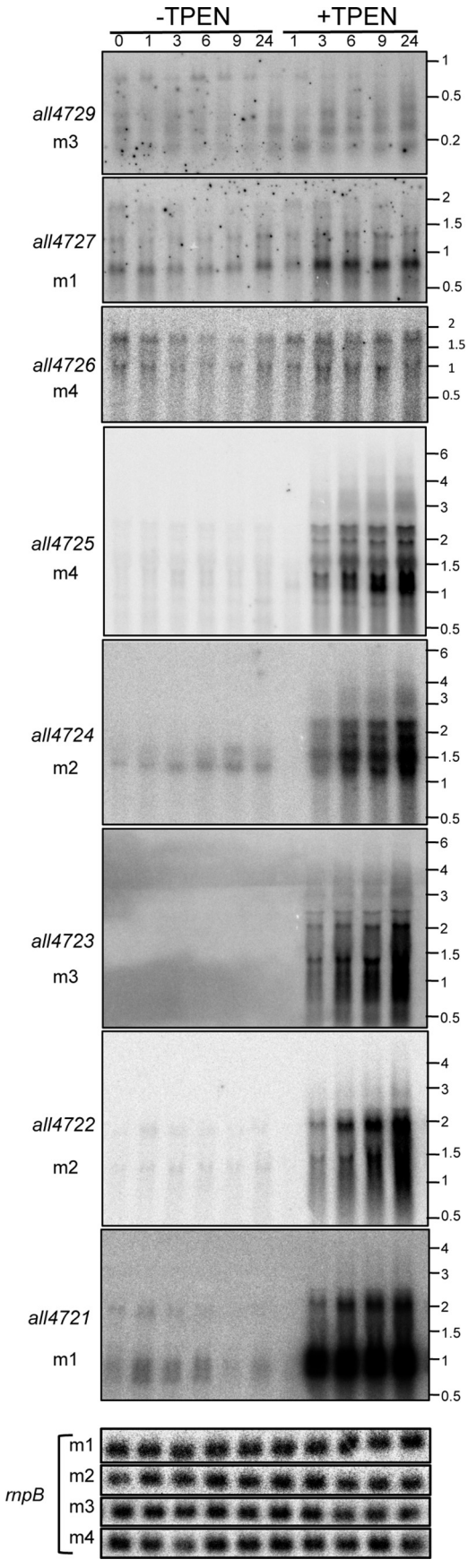
Gene	Synonym	Putative product	Predicted metal cofactor	Homologous genes in the *Anabaena* genome
all4729		Putative metalloprotein	+	*all0232, alr4839*
asl4728		Non-conserved 55 amino acid peptide		-
all4727		Two-component response regulator		*
all4726		Membrane-anchored histidine kinase		*
all4725	*hemE*	Phorphobilinogen synthase	+ (Mg or K)	*alr4380*
all4724		Putative FAD-dependent oxidoreductase		-
all4723	*thrS*	Threonyl-tRNA synthetase	+ (Zn)	*alr0335*
all4722		P-loop GTPase (COG0523-family)	+	*alr1093, all1751, all3392*
all4721	*folE*	GTP-cyclohydrolase	+ (Zn)	*alr5287*

FIG 1 Structure of a divalent metal-responsive gene cluster of *Anabaena*. A diagram of the gene cluster is at the top. +, presence of a putative metal cofactor (specified in parentheses in cases where it is predictable). *Anabaena* genes homologous to those in the cluster are indicated with their locus tags or with an asterisk when many paralogues are present in the genome.

several proteins encoded in the cluster, its expression was analyzed in cells grown for a 24-hour period in the absence or presence of TPEN, a divalent metal chelator. In time course experiments two different profiles were observed; on one side, *all4729*, *all4727*, and *all4726*, in the 5′ region of the cluster, showed discrete hybridization bands that slightly increased their intensity (around 2-fold) upon treatment with TPEN, whereas *all4725* to *all4721*, in the 3′ half of the cluster, showed wide-range (from 0.5 to 7-kb upon overexposure) smeared hybridization signals with some prominent discrete bands that, when quantified as a whole, dramatically increased in intensity (almost 100-fold) a few hours after the addition of TPEN to the cultures (Fig. 2). This indicated cotranscription of genes *all4725* to *all4721* in long unstable polycistronic transcripts. To check this possibility, the existence of transcripts overlapping contiguous ORFs in the cluster was analyzed by reverse transcription-PCR (RT-PCR). As shown in Fig. 3A, transcripts overlapping contiguous ORFs existed from *all4727* to *all4721*; however, no transcript overlapping *asl4728* and *all4727* could be observed. Therefore, *all4729* and *asl4728* are transcribed independently of the remainder of the cluster.

Promoter mapping. Transcription start points in the cluster were mapped by the 5′-RACE procedure described by Bensing et al. (4), suitable for the distinction of 5′ ends with a triphosphate group, corresponding to true transcription initiation sites, from those with a monophosphate group resulting from the processing of a longer transcript. Transcription start points were mapped upstream of *all4727*, *all4725*, *all4723*, *all4722*, and *all4721* at positions −68, −19, −20, −118, and −90 from their respective ATG codons (Fig. 3B and C). A single transcription start point upstream of *all4727* was detected when primers annealing with either *all4727* or *all4726* were used, indicating that these two ORFs cotranscribe from a single promoter. It is worth noting that the 5′-RACE products obtained with primers for *all4725*, *all4723*, *all4722*, and *all4721* correlated in intensity with the signals observed by Northern blotting for samples that were not treated or treated with TPEN, suggesting that they correspond to regulated promoters (Fig. 3C).

Identification of regulatory factors. In the *Anabaena* genome, 3 ORFs (*all1691*, *all2473*, and *alr0957*, also known as *furA*, *furB*, and *furC*) encode proteins of the Fur family (27). To test the possible involvement of any of these three factors in the regulation of the operon, the binding of recombinant purified proteins to a DNA fragment from the promoter region of *all4725* was assayed by electrophoresis mobility shift assays (EMSA) (Fig. 4). Only FurB was able to produce a retarded band (Fig. 4A). Binding of FurB to this DNA fragment was demonstrated to be specific in competition assays: an excess of the unlabeled *all4725* promoter fragment but not an equivalent amount of an unrelated DNA fragment was able to abolish binding (Fig. 4B). Binding of FurB to other promoter regions in the *all4725-all4721* operon was also tested. FurB was able to bind in EMSAs to the promoter regions of *all4723* and *all4721* but not to that of *all4722* (Fig. 4C). To confirm regulation of the operon by FurB, an insertion-deletion mutant was generated by gene replacement. A segregated mutant (i.e., a strain carrying the mutated version in all chromosomes copies) was selected for further studies. In this mutant, expression of the genes in the operon was found to be high under noninducing conditions (e.g., in the absence of TPEN) (Fig. 5A), consistent with a repressor role for FurB in these genes. *all4727* was not upregulated in the *furB* mutant, suggesting that it does not belong to the same transcriptional unit as *all4725-all4721*. Maximal noninhibitory concentrations of several divalent metals (Cu, Zn, Ni, and Co) for WT *Anabaena* were determined, and growth of the WT and the *furB* mutant were tested under these conditions (Fig. 5B). Growth of the *furB* mutant was severely affected by the presence of zinc but not other metals (Fig. 5B), indicating a major role for FurB in the regulation of zinc homeostasis in *Anabaena.* To corroborate the specificity of FurB for zinc, EMSA reaction mixtures containing FurB were treated with increasing concentrations of the TPEN chelator, and it was observed that concentrations



above 500 μM mostly inhibited the binding of FurB to DNA (Fig. 5C, lane 3). DNA binding was restored when reaction mixtures were later supplemented with zinc (Fig. 5C). These results identified FurB as Zur, the zinc uptake regulator of *Anabaena* (referred to here as Zur).

Characterization of the Zur-binding sequence. The promoter sequences of *all4725*, *all4723*, and *all4721* were compared searching for the putative binding sequence for Zur. Sequences with a dyad symmetry axis with some similarity to 7-1-7 sequences reported as targets for proteins of the Fur family (33) were observed in the promoters of *all4725* and *all4723*. In the promoter of *all4721*, two overlapping imperfect sequences were observed (Fig. 6A). The affinity constant (K_d) of Zur calculated by EMSAs was estimated to be 2.5×10^{-9} and 7×10^{-9} M for the target sequences at *all4725* and *all4723*, respectively (the affinity for the sequences at the *all4721* promoter was very low, and the K_d could not be calculated). The affinity of Zur for the sequence at the *all4725* promoter was on the same order of magnitude as that of other Fur family factors for their consensus binding sequences (22, 33). To determine the relative contribution of the DNA nucleotides to the interaction with Zur, the effect of mutations in the sequence at the *all4725* promoter on the affinity for Zur was analyzed. Except for the mutation at position 1R (Fig. 6B), which makes the sequence perfectly symmetric and does not significantly alter the K_d, any other of the introduced changes had a severe effect on the K_d, indicating that Zur binding to DNA is highly sequence specific. It is be noticed, however, that not all changes had a similar effect, with the mutation of nucleotides 2 and 3 and nucleotides 4 and 5 giving a greater effect than the mutation of nucleotides 6 and 7. Changes at symmetric positions had additive effects in the case of nucleotides 2-3 and 4-5 but not in the case of 6-7. Sequences flanking the symmetric 7-1-7 motif also contribute to Zur binding, since their mutation decreases the affinity for Zur (Fig. 6B).

Insight into the Zur regulon in *Anabaena*. As a first approach to identify genes in the Zur regulon in *Anabaena*, the two sequences with lower K_ds in our affinity analyses (Fig. 6B) were used as a seed to search for similar sequences in the *Anabaena* genome using the Genolist Web server at the Pasteur Institute (http://genodb.pasteur.fr/cgi-bin/WebObjects/GenoList.woa/) allowing a maximum of 2 mismatches. Since only sequences properly located in the promoter (i.e., those overlapping the RNA polymerase binding region) are expected to affect transcription, the search was restricted to sequences located between positions −400 and +30 with respect to the translational start codon. Thirty-three putative Zur-binding sites were retrieved from this search. Downstream adjacent genes were tested for expression in the WT and the *zur* mutant by quantitative real-time PCR (Q-PCR) (*all4723* and *all4725* were retrieved and included in the analysis as controls). When one site was between two divergent ORFs, both were

FIG 2 Transcriptional response of the genes in the *all4729-all4721* cluster to the chelation of divalent metals. *Anabaena* cells growing in BG11 medium were split into two cultures; one of them was supplemented with TPEN at a final concentration of 20 μM, and both were further cultured for 24 h. Aliquots were extracted at the times indicated at the top of the panels; RNA was extracted and subjected to Northern hybridization with probes for the genes indicated at the left. m1, m2, m3, and m4 refer to the membranes used for the hybridization. Numbers on the right indicate the positions of RNA molecular weight markers. Hybridization of the four membranes (m1, m2, m3, and m4) with the *rnpB* probe, used as a control for RNA loading, is shown at the bottom.

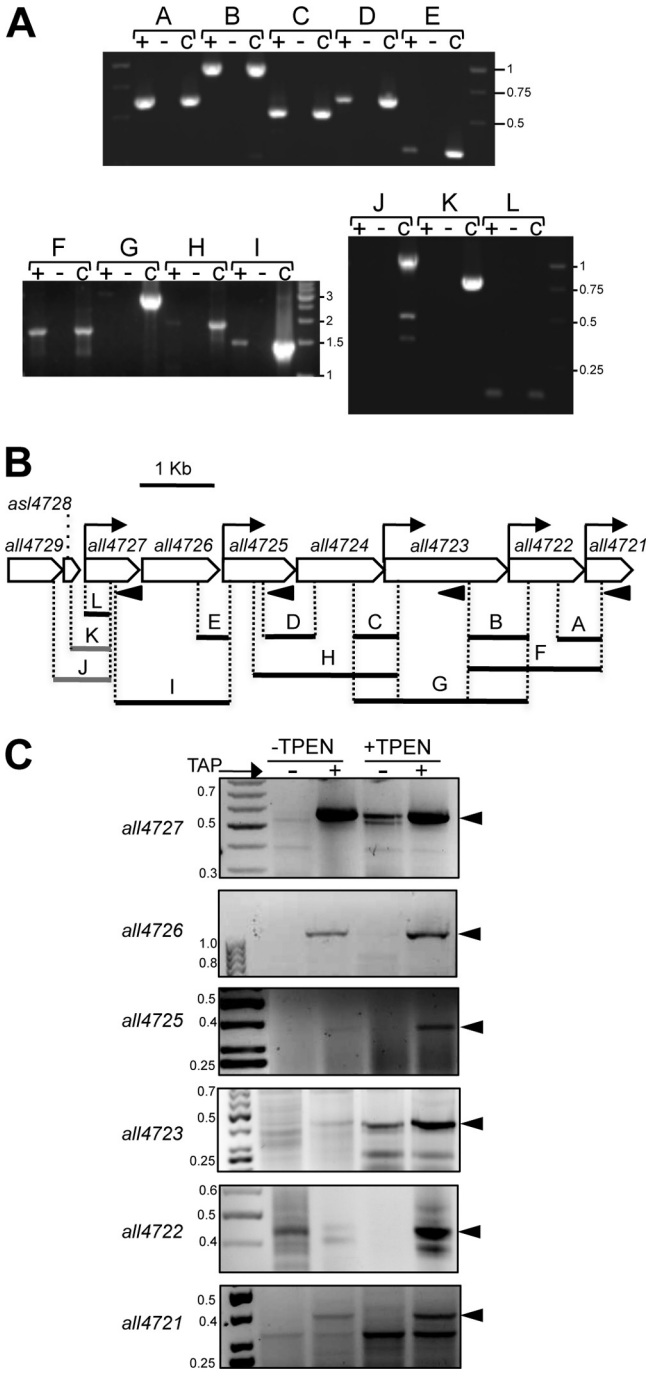


FIG 3 Determination of the limits of the operon by RT-PCR and promoter mapping. (A) Products of RT-PCRs. + and −, reactions in which retrotranscriptase was added or omitted, respectively. C, control PCRs using genomic DNA as the template. The letters A to L identify the fragments on the electrophoresis and correspond to the fragments in panel B. (B) Fragments that were amplified by RT-PCR are depicted with solid lines, and those that failed to be amplified are depicted with gray lines. The primers used for retrotranscription are indicated by arrowheads. Bent arrows indicate the position of transcription start points, mapped by 5′-RACE. (C) Promoter mapping by 5′-RACE. RNA samples from *Anabaena* cells cultured for 24 h in the presence or absence of TPEN were treated with TAP or left untreated and subjected to 5′-RACE as described in Materials and Methods. The reverse oligonucleotides used in the PCR step annealed with the 5′ region of the ORF indicated to the left of each panel. Numbers indicate the positions of DNA size markers. Arrowheads indicate the positions of major differential bands.

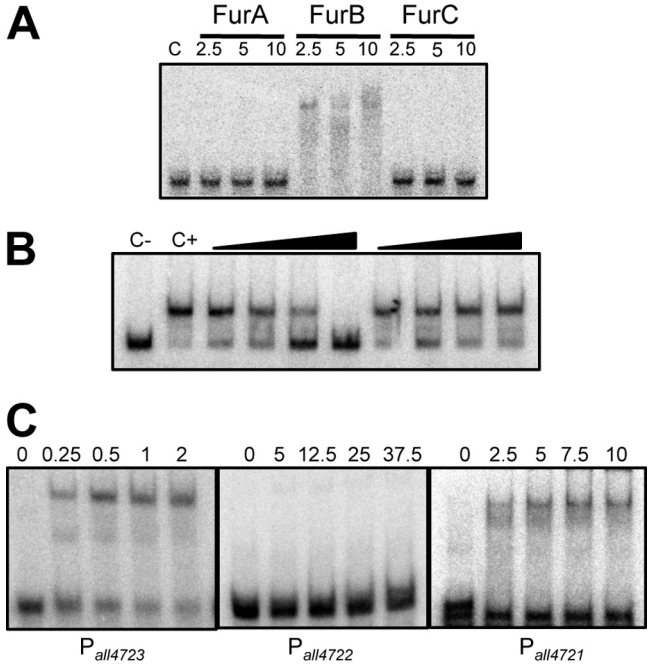


FIG 4 Binding of Fur proteins to the promoter regions of genes in the *all4725-all4721* operon. (A) EMSA of FurA, FurB, and FurC with a DNA fragment from the promoter region of *all4725*. C, control assay in which no protein was added to the reaction mixture. The amounts (pmol) of dimer protein in 15-μl assay mixtures are indicated at the top. (B) Binding to 0.25 fmol of a labeled fragment of the promoter region of *all4725* generated with primers all4725_2F and all4725_2R was assayed with no protein in the reaction mixture (C−) or with 0.1 pmol FurB, in the absence (C+) or presence (C+) of a 10-, 50-, 100-, or 200-fold excess of cold *all4725* promoter fragment (left) or cold unrelated competitor DNA (right) generated by PCR with primers all4726_2F and all4726_1R. (C) EMSA of FurB with DNA fragments of the promoter regions of *all4723*, *all4722*, and *all4721*. The amount (pmol) of FurB dimer in each reaction is indicated on top of each lane.

tested. The expression of genes not retrieved in our search but previously reported to be involved in zinc homeostasis in *Anabaena*, including *aztA*, *aztR*, *znuABC*, and *bmtA*, was also analyzed (8, 36). Of the 41 genes analyzed (see Table S1 in the supplemental material), 14 showed a higher level of expression in the *zur* mutant than in the WT, suggesting a direct regulation by Zur (Table 1; a 2-fold difference was established as the minimal threshold). No gene showed a higher level in the WT than in the mutant, and 27 had no change or showed a barely detectable expression level (see Table S1). Comparison using the WebLogo program of the putative Zur-binding sequences upstream of the regulated genes allowed the definition of a consensus binding sequence (TGATAATNATTATCA) for the *Anabaena* Zur protein (Table 1). Sequences flanking this 7-1-7 palindrome are not highly conserved, but there seems to be some preference for A or T.

The gene context of the Zur-regulated genes was analyzed, and 7 genes were found clustered with neighbor genes that could potentially be cotranscribed (Table 2). The expression of 15 additional genes clustered with Zur-regulated genes was analyzed by Q-PCR (see Table S1 in the supplemental material). Six were upregulated in the *zur* mutant, indicating that they likely form transcriptional units with Zur-regulated genes. Thus, in addition to the *all4725-all4721* operon (named operon 1), five new Zur-reg-

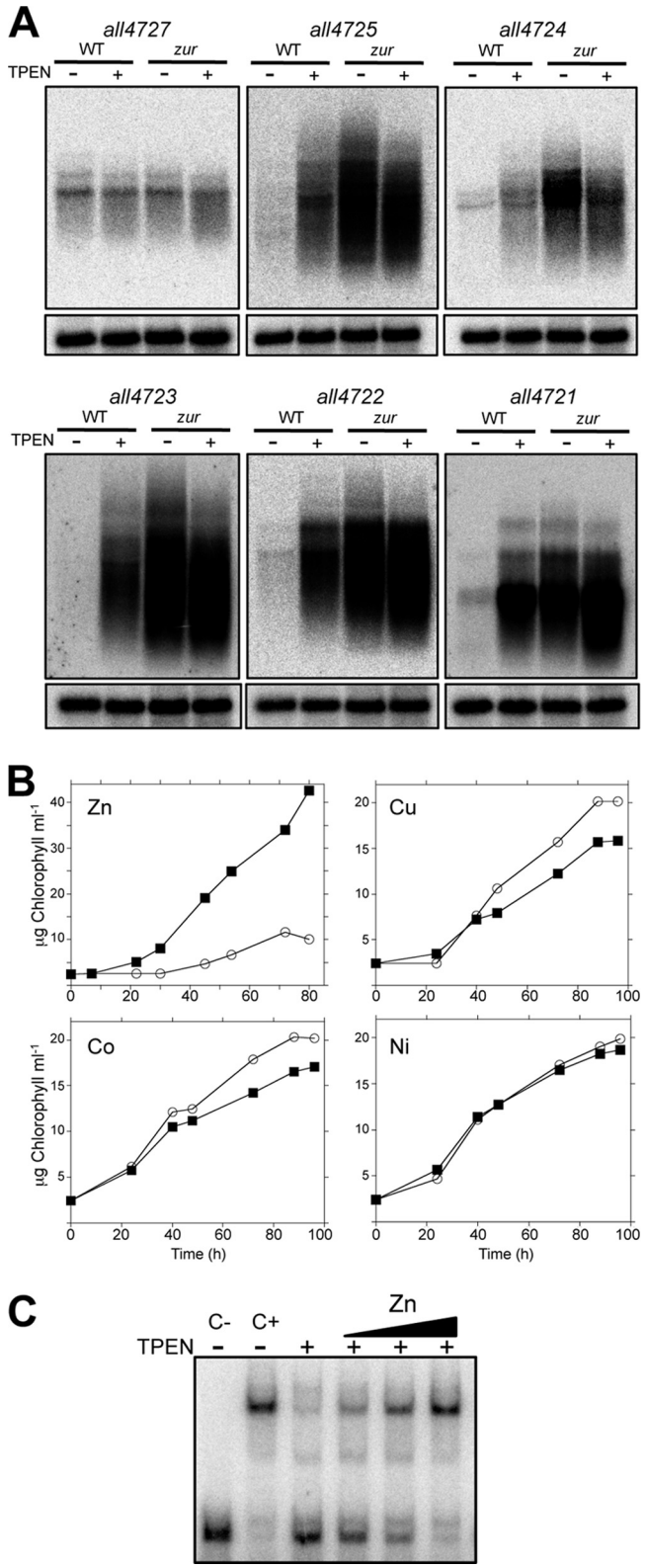


FIG 5 Phenotype of the *zur* mutant. (A) Northern hybridizations of RNA from cells of the WT or the *zur* mutant treated with TPEN for 24 h or left untreated. Probes used for hybridization are shown above the panels. Hybridizations of the membranes with the *rnpB* gene used as a RNA loading control are shown at the bottom of each panel. (B) Growth curves of the WT (squares) and the *zur* mutant (circles) in the presence of 25 μM $ZnSO_4$, 25 μM $CuSO_4$, 25 μM $CoCl_2$, or 25 μM $NiSO_4$. The data are representative of three independent repeats. (C) EMSA reaction mixtures (15 μl) containing 0.1 pmol of FurB dimer were incubated in the presence (+) or absence (−) of 500 μM TPEN for 1 h at 25°C and subsequently supplemented with increasing concentrations (100, 250, or 500 μM) of $ZnSO_4$, incubated 30 min at 25°C, and resolved in a native acrylamide gel.

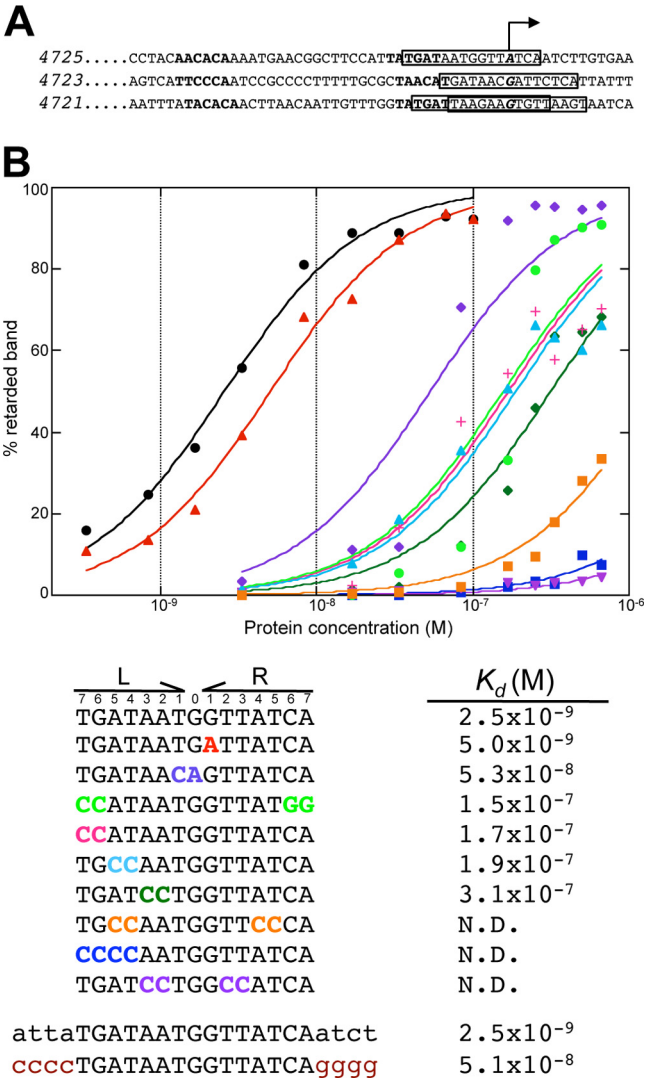
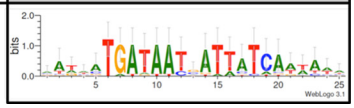


FIG 6 Mapping and characterization of Zur-binding sequences. (A) The sequences of the promoter regions of *all4725*, *all4723*, and *all4721* are depicted. The bent arrow indicates the position of the transcription start point, bold indicates putative −35 and −10 sequences, and putative Zur-binding sequences are boxed. (B) The binding of FurB to different DNA sequences was assayed by EMSA. The plot shows the curves resulting from the adjustment of the percentage of retarded band to a simplified version of the Hill equation. The colors correspond to the sequences at the bottom. The top sequence is that of the *all4725* promoter. Mutations introduced to generate the other DNA fragments are indicated by colors corresponding to the graph. The K_d of Zur for each fragment is indicated. The curve for the sequence at the very bottom is not shown.

ulated operons (named operons 2 to 6 [Table 2]) have been uncovered. In summary, our analyses have revealed 23 genes regulated by Zur, 17 organized in 6 operons and 6 in single transcriptional units.

TABLE 1 Genes derepressed in the *zur* mutant

Gene	Synonym	Fold induction[a]	Description	Putative Zur binding sequence[b]
alr1197		12.5±6.9	CobW-C superfamily	ctgcaTGAAAATGATTATCAtttaa
all1474		2.1±0.7	CRISPR-associated RAMP protein, SSO1426 family COG1337	tcttcTGATATTGATTCTCAatctt
all1751		3.1±1.0	Putative metallochaperone COG0523	ataggTGATAATGATTATCcgtatg
alr2866		3.1±2.3	Glycosyl transferase, family 2	agaaaTGTTAATCATTTTCAaaaaa
alr3242		117.6±31.2	TonB-dependent transporter (outer membrane)	aaacaTGATAATCATTATCAaaaaa
alr3495		2.8±1.4	Uncharacterized conserved protein COG1262	aataaTGATAACCTTTATCAaaaac
all3515		262.7±36.8	Putative outer membrane protein	gattaTGATAATCATTATCGgaagt cttgcTGATTATGATAATCAttatc
alr4028-4029		10.4±3.9	TonB-dependent transporter (outer membrane)	cagtgTGATAATAATAATCAttatc gataaTAATAATCATTATCTaaaca
all4723	*thrS2*	34.1±15.6	Threonyl-tRNA synthetase	taacaTGATAACGATTCTCAttatt
all4725	*hemE*	159.8±38.2	Porphobilinogen synthase	cattaTGATAATGGTTATCAatctt
all0833	*znuA*	51.0±8.8	Periplasmic solute binding protein	gagaaTGAGAATTATTATAAtataa
all0832	*znuB*	45.8±17.3	ABC-transporter, ATP binding protein	
all0830	*znuC*	5.3±1.9	ABC-transporter permease protein	aattcTGATAATAAAAATAAacacg
all7621	*aztR*	3.0±0.6	ArsR/SmtB-family transcriptional regulator	gagatTGATAACTATTGCTAagtaa



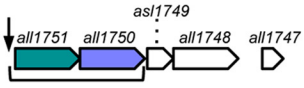
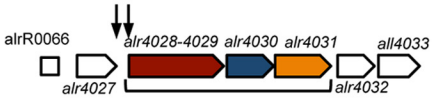
[a] Expressed as the ratio of the expression level in the *zur* mutant to that in the WT in Q-PCR experiments. Data are means ± standard deviations from three independent experiments.
[b] Putative Zur-binding sites on a gray background were not detected in our search at GenoList. A WebLogo sequence built with the sequences in the table is shown at the bottom.

DISCUSSION

Regulation of the *all4725-all4721* operon. Results obtained by RT-PCR (Fig. 3A) showed that transcripts encompassing two or three contiguous ORFS from *all4727* to *all4721* were detected; however, *all4727* and *all4726* seemed to be mostly transcribed independently, as deduced from their expression pattern, which is very different from downstream genes (Fig. 2). Indeed, *all4727* does not seem to be under the control of *zur* (Fig. 5A), unlike genes in the *all4725-all4721* operon. Four promoters were mapped in the operon, and three were shown to be controlled by *zur*. It is difficult to determine whether prominent bands hybridizing with probes of the operon in Fig. 2 were due to transcription initiation from internal promoters, to premature termination, or both. However, for some transcripts, the promoter from which they originate could have been ascertained with some precision. For instance, the 1-kb and 2-kb bands hybridizing with probes of *all4721* or *all4721* and *all4722*, respectively (Fig. 2), were still visible in a mutant with a polar insertion in *all4723* (M. Napolitano, unpublished observations), indicating that the corresponding transcripts originate from promoters located downstream of the insertion. The 2-kb band hybridizes with *all4722* (1,062 bp) and *all4721* (651 bp) and probably corresponds to a dicistronic transcript originating at $P_{all4722}$, and the 1-kb band probably corresponds to a monocistronic transcript originating at $P_{all4721}$. The size of the 2-kb band hybridizing to *all4723* is similar to the distance between the promoter of this gene and a transcriptional terminator located at the 5′ end of *all4722*, suggesting that it may correspond to a monocistronic transcript originating at $P_{all4723}$.

Although TPEN is often referred to as a zinc-specific chelator, it has been reported to bind other metals (6), and in *E. coli* it has been shown to alter the expression of genes responding not only to zinc but also to a variety of other divalent metals (56). In our experiments, TPEN altered the expression of the *isiA* and the *petE* genes of *Anabaena* that respond to iron and copper, respectively (see Fig. S2 in the supplemental material). Therefore, in

TABLE 2 Zur-regulated operons[a]

Operon	Gene	Description	Fold-induction
Operon 2 	*alr1194*	Two component regulator winged helix	
	alr1195	HP 90 Aas	
	alr1196	MscS mechanosensitive channel COG3264	
	alr1197	Putative metallochaperone COG0523	12.5±6.9
	alr1198	Metallophosphoesterase COG0622	14.6±4.2
	alr1199	Metallo-dependent phosphatase	6.2±4.5
Operon 3 	*all1751*	Putative metallochaperone COG0523	2.9±0.4
	all1750	WD40 repeat-containing protein	3.1±1.0
	asl1749	HP 90 Aas	
	all1748	Periplasmic protein of ABC-type metal ion transporter (COG0803)	
	all1747	HP 116 Aas	
Operon 4 	*alr3240*	ABC-transporter, permease protein COG0609	
	alr3241	ABC-transporter, ATP binding protein COG1120	
	alr3242	TonB-dependent receptor (outer membrane) (COG1629)	117.6±31.2
	alr3243	ABC transporter, periplasmic-binding protein COG0614	induced*
Operon 5 	alr_R0066	Cobalamin riboswitch	
	alr4027	Predicted metal binding protein COG5469 (thioredoxin fold)	
	alr4028-29	TonB-dependent receptor (outer membrane)	10.4±3.9
	alr4030	Putative ferredoxin (thioredoxin fold) COG 3411	7.2±0.4
	alr4031	ABC transporter, periplasmic-binding protein COG0614	9.0±0.6
	alr4032	ABC-transporter, permease protein COG0609	
	alr4033	ABC-transporter, ATP binding protein COG1120	
Operon 6 	*all0833*	Periplasmic solute binding protein (ZnuA)	51.0±8.8
	all0832	ABC-transporter, ATP binding protein (ZnuB)	45.8±17.3
	alr0831	ArsR/SmtB regulator	
	all0830	ABC-transporter, permease protein (ZnuC)	5.3±1.9

[a] Zur-regulated genes are depicted in arbitrary colors and indicated by a bracket. Other neighboring genes and genetic elements that encode putative metalloproteins or that may be functionally related to genes in the operon are in white. Other details are as in Table 1. An asterisk indicates that the induction of *alr3243* could not be calculated since its expression was not detected in the WT.

Anabaena, TPEN seems to induce a general divalent metal deficiency, which is consistent with the observations in *E. coli* (56).

The Zur regulator and the Zur-binding sequence in *Anabaena*. Zur is a major regulator of zinc homeostasis, which although well characterized in a variety of bacteria has received little attention in cyanobacteria. The high degree of conservation between different members of the Fur family and the presence of multiple homologues per genome hinders the determination of the metal specificity of these proteins from sequence data alone. Moreover, overall sequence similarity does not always correlate with metal specificity. In this study, *all2473* (also named *furB*) has been identified as the *zur* gene of *Anabaena* sp. strain PCC 7120 based on experimental data. All2473/FurB was previously proposed to be a protein that binds unspecifically to DNA and protects it from oxidative damage (38). In contrast to this, results presented in this study show that All2473/FurB binds specifically, with high affinity ($\sim 2.5 \times 10^{-9}$ M) and in a zinc-dependent manner, to palindromic sequences in the promoter of regulated genes. Furthermore, a deletion mutant is shown to be impaired in the regulation of zinc homeostasis, and the genes under its regulation

appear to be involved in the adaptation of *Anabaena* to zinc deficiency (see below). Altogether, these results strongly indicate that All2473/FurB is the Zur regulator that controls zinc homeostasis in *Anabaena*. If under particular circumstances *in vivo* this protein binds unspecifically to DNA, as proposed by López-Gomollón et al. (38), All4723/FurB/Zur would be a "moonlighting" protein with a dual role in the physiology of *Anabaena*.

Binding to 7-1-7 palindromic sequences has been previously reported for other Fur-family proteins from a variety of bacteria (2, 3, 33, 59). Nucleotides flanking the 7-1-7 sequences also contribute to the interaction with *Anabaena* Zur (Fig. 6B), and this is consistent with what has been reported for the Zur protein of *Bacillus subtilis* (22). Zur binding and dissociation from DNA are governed by the occupancy of a regulatory coordination site. Recent crystal structures have revealed three potential metal-coordinating sites in Zur from distinct bacteria (39, 55). The role of site 3 is controversial (41, 55), and it has been proposed that *in vivo*, zinc never binds to this site (41). Site 1 is a structural site, whereas site 2 is postulated to be the regulatory on-off switch (39, 41, 55). Consistently, the affinity of zinc for this site is in the predicted range of the buffered concentration of zinc in the cytoplasm (pico- to femtomolar) (41, 45). Importantly, regulatory site 2 is missing in the protein identified as the *Anabaena* Zur; only one (His78) of the four coordinating residues is conserved. In contrast, structural site 1 and site 3 are conserved (see Fig. S1 in the supplemental material), although amino acids at positions 75 and 96 of site 3 are permuted with respect to other Zur proteins (see Fig. S1). The absence of the regulatory site 2 in *Anabaena* Zur raises the question of which site functions as the on-off switch. Further work is required to elucidate the structural basis for the responsiveness of *Anabaena* Zur to zinc.

The Zur regulon. These data have revealed 23 genes regulated by Zur, organized in 6 operons and single transcriptional units. The presence of internal promoters in operon 1 indicates that distinct genes may require some degree of independent regulation. Distinct Zur-regulated promoters have also been observed in the *yciABC* operon of *Bacillus subtilis*, correlating with different expression levels of the genes (20). Most proteins upregulated in the *zur* mutant of *Anabaena* can be classified into 4 categories, described below, that outline the strategy of this cyanobacterium for adaptation to zinc limitation.

Category 1: paralogues of zinc metalloproteins. It has been shown that three genes in operon 1 encode proteins with essential housekeeping functions (All4725/HemE, All4723/ThrS and All4721/FolE). Their paralogues Alr4380/HemE, Alr0335/ThrS, and Alr5287/FolE, which are highly expressed under standard growth conditions, are all putative zinc metalloproteins based on the conservation of essential liganding residues. Very importantly, All4725/HemE is a porphobilinogen synthase with an aspartate-rich active site (DvALDpFtthGHDG) that fits the consensus sequence DxALDx(Y/F)xxxG(H/Q)DG for Mg- or K-dependent enzymes. In contrast, the active site of the paralogous Alr4380/HemE protein conforms to the consensus sequence of zinc-dependent enzymes [DxCxCx(Y/F)x3G(H/Q)CG] (31). Thus, the derepression of *all4725* under zinc limitation would probably lead to the replacement of a zinc-dependent porphobilinogen synthase (All4380) by a zinc-independent one (All4725), under conditions in which the former is most probably inactive. Replacement of zinc-dependent proteins by zinc-independent isoforms seems to be a common strategy in a variety of prokaryotes (5, 16, 21, 31, 50). Less intuitive is the model for All4723/ThrS and All4721/FolE, both of which do conserve the deduced coordination residues for zinc. Adaptation to zinc limitation via the replacement of a zinc-dependent enzyme by another which is also zinc dependent does not appear to be an effective strategy, unless the alternative enzyme is somehow more efficient at the recruitment of zinc cations. Another possibility is that All4723/ThrS and All4721/FolE could be functional with a metal cofactor other than zinc. In any case, All4723/ThrS (and probably All4721/FolE) must somehow better fulfill the housekeeping function under zinc-limiting conditions and is essential for survival under these conditions (Napolitano et al., unpublished).

Category 2: genes encoding putative metallochaperones. *all4722* and *all1751* encode G3E P-loop GTPases of the COG0523 family. Like other members of the family, they show a GTPase motif in the N terminus, a conserved putative metal-binding CXXC motif, and a C-terminal domain with a His-rich stretch. However, All4722 contains 19 His residues, whereas All1751 contains only 2. *all1197* is also in this category although its product has a deletion in the N-terminal domain and a single histidine in the C-terminal domain. A recent exhaustive bioinformatic analysis classified members of the COG0523 family into 15 subfamilies (24). That study proposed that several of these subfamilies have a role in survival under conditions of deficient zinc nutrition and are frequently associated with Zur-binding sites in diverse bacteria (24). Consistent with this, *Bacillus subtilis* mutants defective in a protein of the COG0523 family exhibit a growth defect in low-zinc medium (19). Although the exact role of All4722 is still to be determined, it seems important for the survival of *Anabaena* under metal limitation (Napolitano et al., unpublished). Thus, our results further support a role for proteins of the COG0523 family in the adaptation to zinc limitation.

Category 3: genes encoding components of plasma membrane ABC transport systems. Several genes regulated by Zur encode subunits of ABC transport systems of the plasma membrane. In general, these transporters are constituted by a substrate-binding periplasmic protein, an intrinsic membrane component, and an ATPase subunit in the cytoplasmic site (28). *all0833*, *all0832*, and *all0830* in operon 6 were previously identified as the genes encoding the ZnuABC zinc uptake system of *Anabaena* based on sequence similarity (36). Two other genes, *alr3243* in operon 4 and *alr4031* in operon 5, encode the periplasmic protein of ABC transporters. In both cases, neighboring genes not regulated by Zur (*alr3240* and *alr3241* in operon 4 and *alr4032* and *alr4033* in operon 5) encode the membrane-intrinsic and the ATPase subunits, suggesting that each cluster may encode an individual ABC transport system (Table 2). The substrate specificity of these transport systems will need to be determined by specific approaches.

Category 4: genes encoding proteins of the outer membrane. TonB-dependent receptors (TBDRs) are integral proteins of the outer membrane of Gram-negative bacteria which function as energy-dependent importers of extracellular molecules (44). Transport by TBDRs of iron complexes (siderophores, heme, and ferritin) and vitamin B_{12} (cobalamin) is well characterized (44). However, the involvement of the TBDR in the import of divalent metals other than iron was not reported until very recently (29, 51, 58). Twenty-two genes in the *Anabaena* genome encode putative TBDRs (43), suggesting that some of them may be involved in the transport of molecules other than iron complexes or vitamin B_{12}.

Genes encoding TBDRs have not been found in previous analyses of Zur regulons in Gram-negative bacteria (34, 46). Two genes, *alr3242* and *alr4028-4029* (the latter is a single ORF mistakenly annotated as two independent genes [43]), encoding putative TBDRs regulated by Zur in *Anabaena* are described here. *alr3242* is highly induced (~100-fold) and reaches a high level of expression in the *zur* mutant, suggesting that Alr3242 is probably a TBDR specific for the import of zinc complexes from the outer medium, which hitherto has been described only for human pathogens (58). The *alr4028-4029* gene is also induced in the *zur* mutant but to a lower extent (~10-fold) (Table 2). Interestingly, the two genes encoding TBDRs cotranscribe with genes for periplasmic proteins of ABC transporters (Table 2), suggesting the necessity of a coordinated regulation of transporters of the outer membrane and the plasma membrane.

The protein encoded by the *all3515* gene is predicted by the PSORT.3b program (63) to be located in the outer membrane. This gene shows the highest induction level of the Zur regulon (more than 250 times) and reaches a very high level of expression in the *zur* mutant. All3515 contains a putative signal peptide in the N terminus, a PEP-CTERM domain in the C terminus, which is proposed to mediate secretion through the plasma membrane (25), and two conserved His-rich regions in the N-terminal part that may be involved in metal coordination. Although the exact role of the All3515 protein is unknown, its regulation by Zur, its peripheral localization in the cell, and its expected abundance suggest a role in scavenging zinc or zinc complexes under limiting conditions.

Other global analyses of the Zur regulon performed in Gram-positive and Gram-negative bacteria have unveiled a variety of genes directly or indirectly regulated by Zur (18, 20, 34, 42, 46, 52). Although our analysis of the Zur regulon of *Anabaena* would need to be completed with further studies, genes uncovered in our work outline how *Anabaena* rearranges its physiology to adapt to zinc limitation: transporters of the outer membrane and plasma membrane are induced for scavenging extracellular zinc or zinc complexes; putative metallochaperones, which may mediate the delivery of zinc or alternative metals to target proteins, are expressed in the cytoplasm; and some zinc-specific metalloproteins are replaced by paralogues able to fulfill essential functions during zinc limitation.

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