



Comparative study of the different mechanisms for zinc ion stress sensing in two cyanobacterial strains, *Synechococcus* sp. PCC 7942 and *Synechocystis* sp. PCC 6803

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In response to an increased level of Zn^{2+} , *Synechococcus* sp. PCC 7942 expresses SmtA, a metallothionein-like metal-chelating protein, while *Synechocystis* sp. PCC 6803 expresses ZiaA, a transporter of Zn^{2+} . The gene expression of these proteins is regulated by repressor protein, SmtB and ZiaR, respectively. In spite of contributing to different response systems, both repressor proteins belong to the ArsR family and are highly homologous to each other. To understand the different systems responsible for dealing with excess Zn^{2+} , we examined the *cis*-elements in the promoter regions of *smtA* and *ziaA*, as well as the binding affinities of recombinant SmtB and ZiaR proteins. The operator/promoter region of *smtA* included two palindromic sequences and that of *ziaA* included one. Electrophoretic mobility shift assay revealed that SmtB formed four different complexes with the operator/promoter region of *smtA*, whereas it formed only two different complexes with the corresponding region of *ziaA*. For ZiaR, the corresponding results were quite the same as those for SmtB. Furthermore, the complex formation between SmtB and operator/promoter regions is inhibited in the presence of Zn^{2+} at higher concentrations than $16 \mu M$. On the other hand, the corresponding Zn^{2+} concentration is $128 \mu M$.

These results demonstrate that the degrees of protein-DNA complex formation between repressor proteins and the operator/promoter regions of regulated genes depend on the structures of the operator/promoter regions, and the effects of Zn^{2+} on the dissociation of these complexes are mainly associated with the structures of the repressors.

Key words: Electrophoretic mobility shift assay (EMSA), zinc ion binding, transcription factor, operator/promoter

All living organisms require trace metal ions to maintain the physiological roles of proteins including metalloenzymes, regulatory factors, membrane-associated transporters and so on. Among these proteins, zinc finger proteins that form protein-protein or protein-DNA interaction surfaces are also included¹. These trace metal ions are indispensable, however, in excess amounts, they are highly toxic. Therefore, in order to respond rapidly to changes in the environmental concentrations of trace metal ions around a cell, the regulatory systems that control the cytosolic concentrations of these metal ions are tight^{2,3}. Among these regulatory systems, in addition to gene regulatory proteins, metalloregulatory proteins³ and/or metal sensors⁴ are included.

In response to Zn^{2+} stress, freshwater cyanobacterium *Synechococcus* sp. PCC 7942 expresses SmtA, a class II metallothionein⁵, while freshwater cyanobacterium *Synecho-*

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cystis sp. PCC 6803 expresses ZiaA, a Zn²⁺ transporting CPx-ATPase⁶. The gene expression of these proteins is regulated by repressor proteins SmtB⁷ and ZiaR⁶, which also have function as sensors of the Zn²⁺ concentration, respectively.

In *Synechococcus* sp. PCC 7942, SmtA and SmtB are encoded in the *smt* locus (*smtA* and *smtB*), and they are transcribed divergently. Deletion of the *smt* locus causes a reduction of this Zn²⁺/Cd²⁺ tolerance⁸. In the absence of heavy metal ions, the transcription of *smtA* is repressed through the binding of SmtB to the operator/promoter region (about 100 bp long) of *smtA* located between the *smtA* and *smtB* coding regions⁹. The transcription of *smtA* is thought to be induced by trace amounts of heavy metal ions (especially Zn²⁺ and Cd²⁺) that cause inhibition of the complex formation between SmtB and the recognition DNA sequence, following heavy metal ion binding to SmtB⁹. In previous studies¹⁰⁻¹², we confirmed that Zn²⁺ specifically inhibits the SmtB-DNA complex formation with the operator/promote region of *smtA*. To clarify the molecular changes of SmtB following Zn²⁺ binding, Turner and co-workers analyzed the binding properties of mutated SmtB and suggested several candidates for the amino acid residues ligating to Zn²⁺. SmtB acts as a dimeric form¹⁴, and Cook and co-workers solved the crystal structures of the SmtB dimer¹⁵. They found that SmtB has a helix-turn-helix motif, which is thought to be responsible for its binding to the recognition DNA sequence¹⁵, and tried to identify the structure of the Zn²⁺ binding pocket by the mercury soaking technique¹⁵. In spite of these structural studies, the following molecular mechanisms remain to be elucidated.

- 1) The ion-specific sensing by SmtB
- 2) The loss of the affinity of SmtB to the recognition DNA sequence by Zn²⁺

On the other hand, an ORF, *slr0798*, gene of the fully sequenced genome of the cyanobacterium *Synechocystis* sp. PCC 6803⁶ encodes a putative CPx-type ATPase with a single metal binding motif (GMDCTSC) at its N terminus, and the ORF, *slr0792*, divergently transcribed from *slr0798* encodes a protein exhibiting the high similarity to SmtB, and the product of this gene regulates the expression of *slr0798*. The divergent organization (*zia* locus) and the functional correlation of these ORFs are reminiscent of those of *smtA* and *smtB* in *Synechococcus* sp. PCC 7942.

In spite of the analogy to the arrangement of two divergently transcribed genes, the structures of the operator/promoter regions of *smtA* and *ziaA* located between the two corresponding genes are different. In the operator/promoter region of *smtA*, there are two palindromic sequences that are the target binding sites for SmtB. Furthermore, one is followed by a direct repeat in the downstream region, and the other is not. In the case of *ziaA*, there is one palindromic sequence followed by a direct repeat in the downstream region.

In this study, to elucidate the structural and functional

meaning of the differences found in these systems responsible for sensing excess Zn²⁺, we analyzed the binding affinities of recombinant proteins of SmtB and ZiaR to the *cis*-element in the promoter regions of *smtA* and *ziaA* by the electrophoretic mobility shift assay (EMSA) technique, and found that the numbers of protein-DNA complexes formed between repressors and the operator/promoter regions are defined mainly by the structures of the operator/promoter regions, and the sensitivities to Zn²⁺ and the effect of excess Zn²⁺ on protein-DNA complex formation are defined mainly by the structures of the repressors.

Materials and Methods

SmtB and ZiaR were overexpressed in *E. coli* BL21/DE3 cells harboring pET-21d and pGEX-4T-3 based plasmids. In these expression systems, SmtB was overexpressed without any additional amino acid sequences at the N- and C-terminal ends, and ZiaR was overexpressed as a GST-tagged (at the N-terminal end) protein.

Cells harboring these plasmids were cultured in a baffled flask at 30°C at a rotating speed of 160 rpm. Protein overexpression was induced by the addition of 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when OD₆₅₀ of the culture reached 0.5. After further 4 hours culture at 30°C, cells were harvested by centrifugation at 6,000 g for 5 min and then disrupted by sonication (3 min × 7 times). The soluble materials including overexpressed proteins were purified with a HiTrap SP HP or GSTrap HP column (GE Healthcare). For SmtB, the adsorbed proteins were eluted with a KCl concentration gradient, from 50 mM to 1.0 M. The eluted fragments containing SmtB were further purified by gel filtration on Superdex 75 pg (GE Healthcare). For ZiaR, the adsorbed GST tagged ZiaR was digested with thrombin (50 unit-12 ml; 14 hours incubation at 4°C) and then the tag-free protein was eluted.

For the EMSA experiments, we used the DIG Gel Shift Kit 2nd generation (Roche Applied Science). As a probe DNA for EMSA experiments, the operator/promoter region of *smtA* (80 bp; *pr-smtA*) and that of *ziaA* (124 bp; *pr-ziaA*) were amplified by the PCR method with the genome DNA as the template. The amplified probe DNAs were labeled at the 3'-ends with digoxigenin and then used as probes. For the analyses in the absence of Zn²⁺, DNA binding was carried out at 25°C for 15 min in a 20 μl reaction mixture comprising 20 mM HEPES-KOH, pH 7.6, 30 mM KCl, 10 mM (NH₄)₂SO₄, 1.0 mM EDTA, 1.0 mM DTT, 50 ng μl⁻¹ poly[d(I-C)], 5 ng μl⁻¹ poly L-lysine, 0.2% Tween 20, 1.5 nM end-labeled *pr-smtA* and *pr-ziaA* sequences. For the analyses of Zn²⁺ inhibition, we used the binding buffer without EDTA. In both analyses, the buffers used were pre-treated with Chelex resin (Sigma-Aldrich) to eliminate the trace amount of divalent metal cations. The protein stock solutions contain 50 μM SmtB or ZiaR. The EDTA concentrations in these protein stock solutions are 1.0 mM for the

analyses in the absence of Zn²⁺, and 0.1 mM for the analyses of Zn²⁺ inhibition, to store the proteins stably without any non-specific binding divalent cations. The final concentrations of SmtB and ZiaR in DNA-binding experiments were adjusted in the range of 10⁻⁶ to 10⁻⁹ M.

DNA-protein complexes were separated from the free DNA probe by 8% PAGE in 0.25 × TBE running buffer (in the Zn²⁺ free condition) or in 0.25 × TB running buffer (without EDTA for the Zn²⁺-binding conditions). Zn²⁺ was prepared as a 10 mM Zn(CH₃COO)₂ stock solution (pH 4.5). To examine the effects of the presence of metal ions on the complex formation between SmtB (or ZiaR) and the corresponding probe DNA sequences, the stock solution of Zn²⁺ was added to the DNA binding solution, to give the final concentrations of 0 to 512 μM (pH 7.6).

Results and Discussion

In Figure 1(a), we compare the amino acid sequences of SmtB (122 a.a.) and ZiaR (132 a.a.), and we found that 62% of the whole sequences are identical (indicated by*). In particular, the helix-turn-helix motifs encompassed by a black box are highly conserved (17 a.a. of 21 a.a. (81%) are identi-

cal). In addition to this, L83 and L88 in SmtB are also conserved in ZiaR. We have estimated the three dimensional structure of ZiaR on the basis of PDB data for SmtB with the use of modeler Ver 9.10, and found that the relative positions of corresponding two leucine residues (L93 and L98) are quite similar to those for SmtB, as shown in Figure 1 (b). This result suggests that these two leucine residues must determine the relative positions of the two recognition helices of each SmtB monomer as speculated on the basis of the three-dimensional structure of SmtB¹⁵. This speculation is also supported with the preliminary results of the lower binding affinities of L83A and L88A SmtBs to the recognition DNA sequences than that of native SmtB (Data not shown). These two results mean that the structures around the DNA binding motifs are quite similar in SmtB and ZiaR. In spite of this, the residues that are expected to be the ligands for Zn²⁺ are mostly but not completely conserved. This difference must induce the different binding affinities of repressors to Zn²⁺. In other words, the differences in the lower limit of the concentrations inducing the dissociation of repressor-DNA complexes must be different.

In Figure 2, the sequence structures of the operator/promoter regions of *smtA* and *ziaA* are compared. In the *smtA*

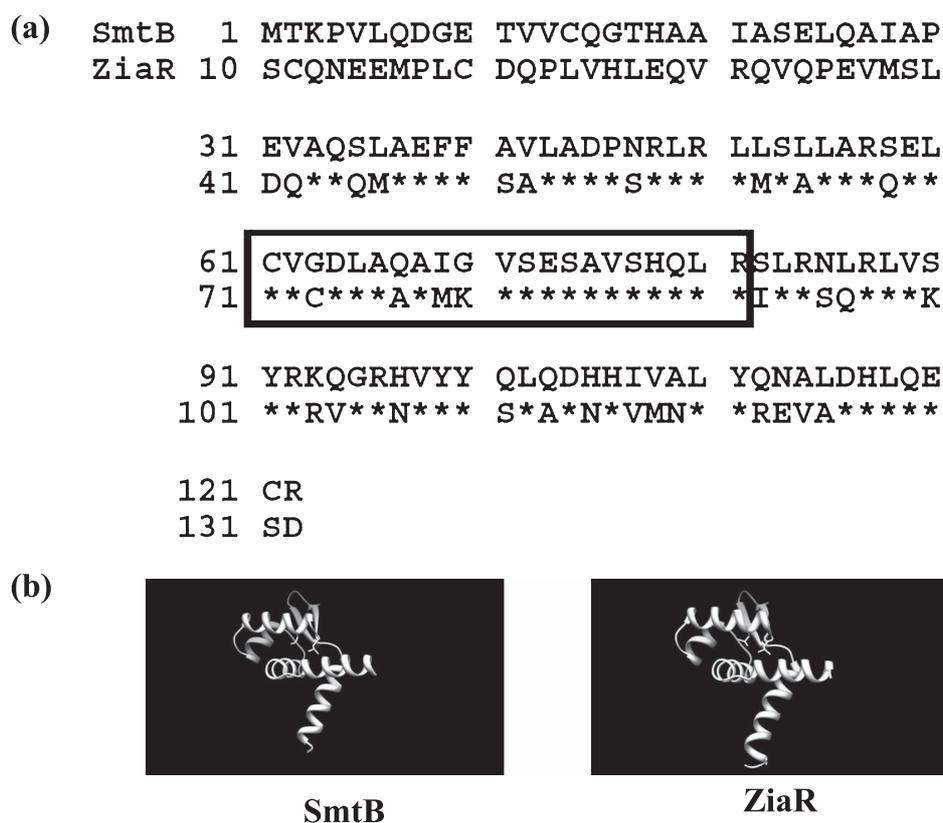


Figure 1 Comparison of the amino acid sequences and the three dimensional structures of SmtB (122 a.a.) and ZiaR (132 a.a.). a) Conserved amino acids are indicated by*, and the helix-turn-helix motifs encompassed by a black box. b) In the left, global folding of secondary structures of SmtB is shown as ribbon model on the basis of PDB datum for SmtB without Zn²⁺, and side chain atoms of L83 and L88 are shown in wireframe model. In the right, global folding of secondary structures of ZiaR speculated with the modeler Ver 9.10 program is shown in the same way as that of SmtB. In this, side chains of L93 and L98 corresponding to L83 and L88 in SmtB are shown.

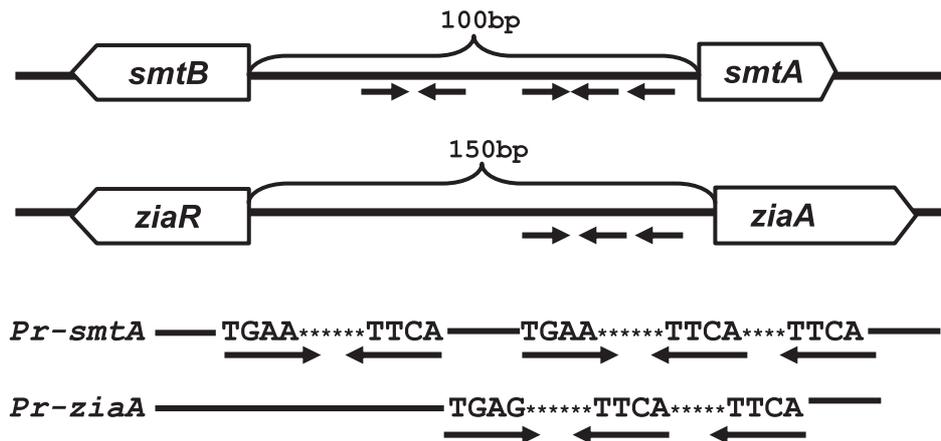


Figure 2 Structural comparison of the operator/promoter regions of *smtA* and *ziaA*. In the *smtA* operator/promoter region (*Pr-smtA*), there are two palindromic sequences ($\rightarrow\leftarrow$), and a direct repeat sequence ($\leftarrow\leftarrow$) following in the downstream region of the lower palindromic sequence. In the *ziaA* operator/promoter region (*Pr-ziaA*), there is only one pseudo palindromic sequence, followed by a direct repeat in the downstream region.

operator/promoter region (*Pr-smtA*), there are two palindromic sequences. In the upper region, there is only a palindromic sequence, and in the lower region, in addition to a palindromic sequence, there is also a direct repeat sequence four bases downstream region. On the other hand, there is only one palindromic (not perfect) sequence followed by a downstream direct repeat sequence in the operator/promoter region of *ziaA*.

In our previous study¹², we found that SmtB binds to each palindromic sequence, independently, with almost the same affinity. However, we did not examine the patterns of complex formation with both palindromic sequences at the same time. To address this and to compare the patterns of repres-

or-DNA (operator/promoter region) complex formation between two cyanobacterial species, we have amplified the operator/promoter regions of *smtA* (*Pr-smtA*) and *ziaA* (*Pr-ziaA*), using the PCR technique, and studied the patterns of protein-DNA complex formation by the EMSA technique.

As shown in Figure 3, in the presence of 1.5 nM probe DNA and 1.0 nM to 1.0 μM protein, four different sized protein-DNA complexes (C1~C4) are formed for SmtB, and two different sized protein-DNA complexes (C5 and C6) for ZiaR. To identify the factors that induce the difference in the number of complexes formed, we also studied the patterns of protein-DNA complex formation by changing the protein and DNA combination, *i.e.* the complex formation

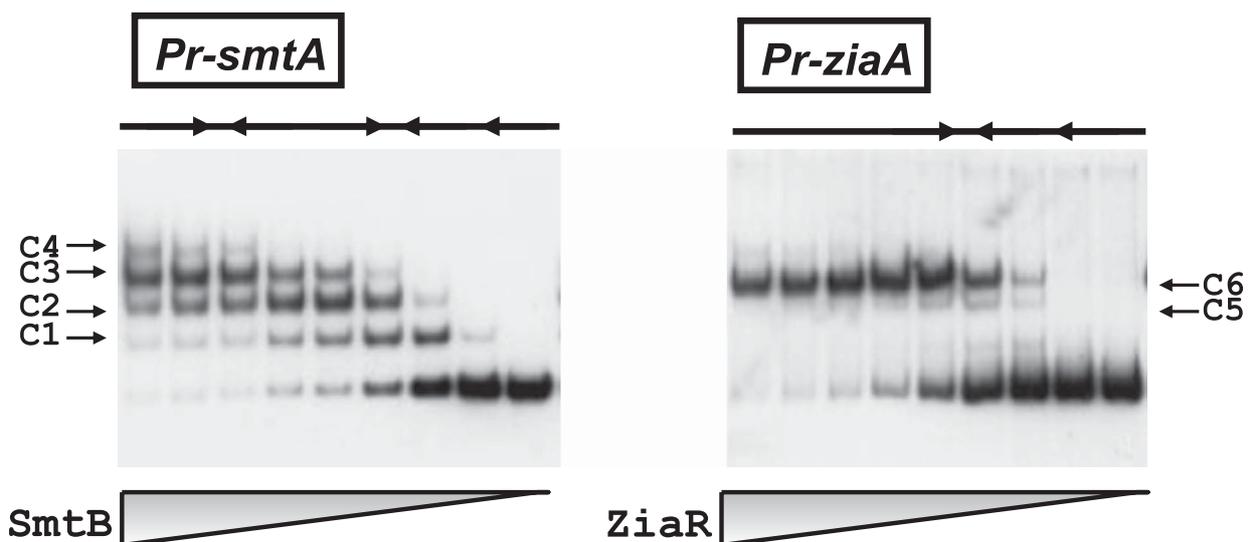


Figure 3 Protein-DNA complexes between zinc sensing transcription factors and their recognition sequences. In both cases, the probe DNA concentration is 1.5 nM, and the protein concentrations are 1.0 μM, 100 nM, 46 nM, 22 nM, 10 nM, 4.6 nM, 2.2 nM, 1.0 nM, and 0 M, from left to right. In the cases of SmtB and *Pr-smtA*, four different sized complexes (C1~C4) were formed. In the cases of ZiaR and *Pr-ZiaA*, two different sized complexes (C5 and C6) were formed.

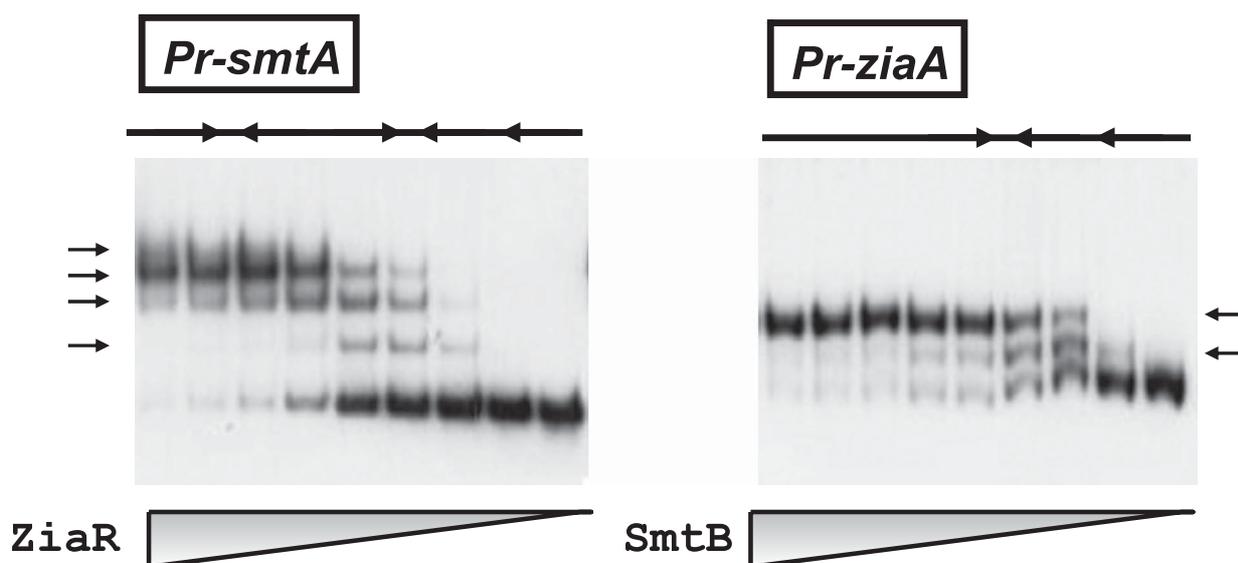


Figure 4 Protein-DNA complexes between SmtB and *Pr-ZiaA*, and between ZiaR and *Pr-smtA*. The EMSA conditions were completely the same as those in Figure 3. In the cases of SmtB and *Pr-ZiaA*, two different sized complexes were formed and in the cases of ZiaR and *Pr-smtA*, four different sized complexes were formed.

between SmtB and *Pr-ziaA*, and that between ZiaR and *Pr-smtA*, as shown in Figure 4. We also observed four complexes in the combination of ZiaR/*Pr-smtA* and two for SmtB/*Pr-ziaA*. These results strongly suggest that the number of species in complexes between repressors and operator/promoter DNA sequences depends on the structures of the operator/promoter DNA sequences (i.e., the number of palindromic sequences, the absence of a direct repeat sequence in the downstream region of the palindromic sequence, and so on). With the same DNA sequence, SmtB and ZiaR form the same number of complexes. In our experimental conditions, both SmtB and ZiaR form 4 complexes with *Pr-smtA*. However, the relative intensity of band attributed to the largest complex to the most intense band in SmtB (C4/C3) is clearly larger than the corresponding in ZiaR. In consideration of the previous stoichiometric analysis¹⁶, it can be concluded that the binding constants as K_{11} 's are small in both SmtB and ZiaR, and that as K_0 in ZiaR is smaller than that in SmtB. One of the reasons of this difference is thought to be the mutations in the DNA recognition helix of ZiaR to the corresponding amino acids in SmtB.

To study the inhibitory effects of Zn²⁺ on protein-DNA complex formation, we also analyzed the protein-DNA complex formation between SmtB, ZiaR (100 nM each), and *Pr-smtA*, *Pr-ziaA* (1.5 nM each) by the EMSA technique, in the presence of 0 to 512 μ M Zn²⁺. The results obtained were summarized in Figure 5.

In the case of SmtB, with higher zinc ion concentrations than 16 μ M, the size of the most preferential protein-DNA complex became smaller, as Zn²⁺ concentration became higher. In the case of ZiaR, with higher Zn²⁺ concentrations than 128 μ M, the protein-DNA complexes were all almost

completely dissociated. This tendency is the same for *Pr-smtA* and *Pr-ziaA*. The residual EDTA concentrations in the reaction mixtures were 0.2 μ M and the effects of the presence of EDTA are considered to be almost negligible comparing with the Zn²⁺ concentrations in the reaction mixtures. These results indicate that for SmtB and ZiaR, the lower limit concentrations of Zn²⁺ showing an inhibitory effect on protein-DNA complexes are different. Furthermore, in the presence of higher concentrations of Zn²⁺ than the lower limit one, the concentration dependency of the inhibitory effect of Zn²⁺ differs between SmtB and ZiaR. The corresponding amino acid in ZiaR to C14 in SmtB, functioning as the ligand for Zn²⁺, is mutated as Leu, and this structural difference between SmtB and ZiaR must be one of the reasons for the difference in minimum Zn²⁺ concentrations needed to inhibit the protein-DNA complex formation and for the different concentration dependencies of the inhibitory effects of Zn²⁺. The results obtained so far are summarized in Table 1.

In Figure 6, the correlations of the Zn²⁺ concentration and the size of the protein-DNA complex for SmtB and ZiaR are schematically illustrated. This figure shows that the responses to Zn²⁺ stress, in gene expression, are quite different between SmtB and ZiaR. After sensing Zn²⁺ stress, in the case for SmtB, the size of the protein-DNA complex gradually becomes smaller, however, in case of ZiaR, the protein-DNA complex is rapidly dissociated. This difference is strongly correlated with the difference in the regulation of gene expression. In spite of this, as to cellular resistance to Zn²⁺ stress, not so significant differences are observed between these two cyanobacteria. This means that to cancel out the differences in the physiological functions of metallo-

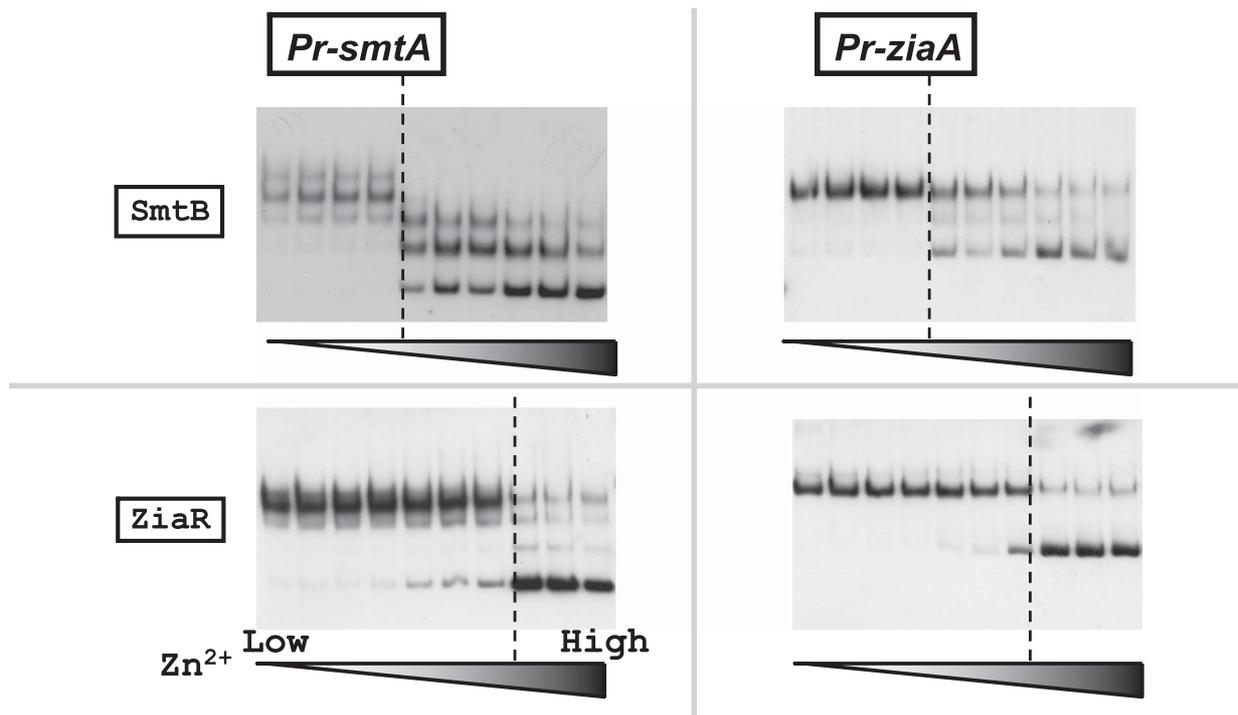


Figure 5 Inhibition of complex formation between SmtB, ZiaR and *Pr-smtA*, *Pr-ziaA* by zinc ions. For EMSA, the protein concentrations were 100 nM and the probe DNA concentrations were 1.5 nM. The zinc ion concentration was changed to 0 μM , 2.0 μM , 4.0 μM , 8.0 μM , 16 μM , 32 μM , 64 μM , 128 μM , 256 μM , or 512 μM , from left to right.

Table 1 Numbers of repressor-probe DNA complexes (upper column), and the zinc ion concentrations that dissociate them (lower column).

Repressor	Probe DNA	
	<i>Pr-smtA</i>	<i>Pr-ziaA</i>
SmtB	4 ~10 μM	2 ~10 μM
ZiaR	4 ~100 μM	2 ~100 μM

thionein and Cpx-ATPase in no- Zn^{2+} stress conditions, the regulatory systems for the expression of these proteins are delicately adjusted.

Conclusion

We found the followings, based on the EMSA for the protein-DNA complex formation between regulatory factors (SmtB, ZiaR) and operator/promoter DNA sequences (*Pr-smtA*, *Pr-ziaA*), and the effect of Zn^{2+} on the inhibition of their complex formation.

1) The numbers of different sized complexes formed between SmtB and *Pr-smtA* (4; C1, C2, C3 and C4), and between ZiaR and *Pr-ziaA* (2; C5 and C6) are different. The reason for this difference is mainly due to the difference in structures of *Pr-smtA* (two palindromic sequences) and *Pr-ziaA* (one palindromic sequence). Furthermore, the mutations in the DNA recognition helix of ZiaR to the cor-

responding amino acids in SmtB may induce the different stoichiometric equilibrium between complexes.

2) The minimum Zn^{2+} concentration inducing dissociation of the SmtB-DNA complex is quite different from that for ZiaR. Furthermore, the presence of excess Zn^{2+} induces different dissociation patterns for the different complexes between sensory proteins and their recognition DNA sequences. In the case of the SmtB-*Pr-smtA* complex, as the Zn^{2+} concentration exceeds the lower limit and becomes higher, the size of the main complex becomes smaller and the proportion of the higher sized complexes becomes lower. In spite of this, in the case of the ZiaR-*Pr-ziaA* complex, almost of all complexes dissociate immediately if the concentration of Zn^{2+} exceeds the lower limit. This is mainly because of the difference in the structures of SmtB and ZiaR, such as the mutation of C14 in SmtB to L24 in ZiaR.

The reason why the sensitivities to Zn^{2+} stress (~10 μM Zn^{2+}) of these two cyanobacteria are almost the same remains unclear, considering the presence of these physiochemical differences in protein and operator/promoter DNA sequence structures. However, on the basis of our results, we can speculate that the differences between the regulatory mechanisms for SmtA and ZiaA expression are strongly correlated with the functions of these proteins. SmtA is a metallothionein-like protein and the intrinsic expression level of this protein has to be kept low, because in the presence of Zn^{2+} stress, expression of SmtA is induced relatively rapidly, which directly reduces an excess level of cytosolic

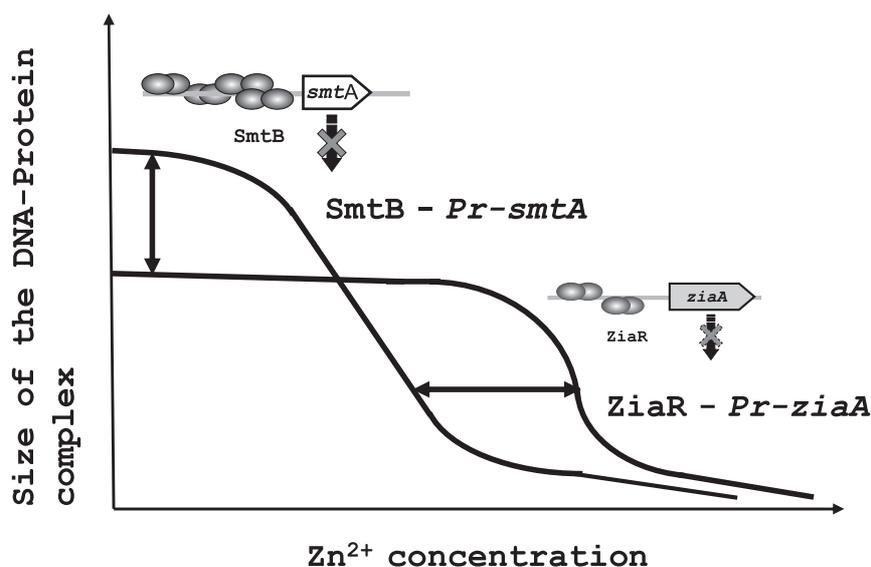


Figure 6 Correlations between the zinc ion concentration and the sizes of the complexes formed between SmtB and *Pr-smtA*, and between ZiaR and *Pr-ziaA*. The EMSA results shown in Figure 5 are summarized and illustrated.

Zn²⁺. On the other hand, ZiaA is a transporting P-type ATPase. It functions as a membrane-bound Zn²⁺ transporter and regulates the cytosolic Zn²⁺ concentration. Thus, the expression pattern of this protein must be quite different from that of SmtA. Considering the physiological and physicochemical differences between these proteins, to the Zn²⁺ tolerances of these cyanobacteria be almost the same, regulatory mechanisms for SmtA and ZiaA are delicately different.

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