A metallothionein and CPx-ATPase handle heavy-metal tolerance in the filamentous cyanobacterium *Oscillatoria brevis*[1]

Tong Liu, Susumu Nakashima*, Kazunobu Hirose, Yoshiko Uemura, Mineo Shibasaka, Maki Katsuhara, Kunihiro Kasamo

Research Institute for Bioresources, Okayama University, Kurashiki, Okayama 710-0046, Japan

Abstract A metallothionein (BmtA) and a CPx-ATPase (Bxa1) have been identified and characterized from the cyanobacterium *Oscillatoria brevis*. Both *bmtA* and *bxa1* expression can be markedly induced in vivo by Zn^{2+} or Cd^{2+}. Over-expression of *bmtA* or *bxa1* in *Escherichia coli* enhances Zn^{2+} and Cd^{2+} tolerance in the transformant. Dynamic studies on the expression of two genes showed that the maximum expression of *bxa1* induced by Zn^{2+} and Cd^{2+} was much quicker than that of *bmtA*, suggesting distinct physiological roles of metallothionein and CPx-ATPase in the handling of surplus metal.
© 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

1. Introduction

Organisms have a range of mechanisms involved in tolerance to heavy metals at the cellular level. Physiological studies show that the principal mechanisms involve the chelation of metal ions in the cytosol (metallothioneins (MTs), phytochelatins, etc.) or exclusion of specific metal ions out of the cytomembrane (ABC transporter, CPx-ATPases, CDF families, etc.) [1–7]. Research in microbial cells has shown that organisms tend to utilize only one of the mechanisms described above for basic heavy metal tolerance [8–11]. In cyanobacteria, a CPx-ATPase (ZiaA) which handles zinc tolerance by effluxing excess zinc out of the cytosol has been identified in *Synechocystis* PCC 6803 [12], but there is no putative MT-encoding gene in its genome [13]. In contrast, a MT (SmtA) has been isolated and characterized in *Synechococcus* PCC strains, and mutants lacking the SmtA locus are hypersensitive to elevated zinc levels; however, there has been no evidence so far that an efflux-type CPx-ATPase is involved in zinc tolerance in *Synechococcus* PCC [13–15]. How and why organisms utilize CPx-ATPase or MTs to regulate heavy metal ion homeostasis remains to be answered.

As we reported [16], the filamentous cyanobacterium *Oscillatoria brevis* shows high tolerance to multiple heavy metals. We have proposed that a novel CPx-ATPase (named Bxa1) is responsible for this tolerance. Bxa1 shares high homology with ZiaA except for a unique His-rich N-terminus [16]; the identity is even higher in the putative transmembrane region (Fig. 2). In this report, we describe for the first time that both a MT (BmtA) and a CPx-ATPase (Bxa1) related to zinc metabolism and heavy metal tolerance exist in a certain cyanobacterium. The expression of both *bmtA* and *bxa1* in *O. brevis* can be induced markedly in vivo by Zn^{2+} and Cd^{2+} and confers tolerance to Zn^{2+} and Cd^{2+} in *Escherichia coli* transformants. The maximum expression of *bxa1* mRNA in *O. brevis* was induced within 1 h, whereas the maximum for *bmtA* mRNA took 8 h, suggesting that Bxa1 is a rapid response factor, and BmtA is a relatively slow emergent in the handling of Zn^{2+} and/or Cd^{2+}.

2. Materials and methods

2.1. Materials and DNA manipulation

The cultures of the filamentous cyanobacterium *O. brevis*, DNA/RNA manipulations and Southern blotting were described previously [16].

2.2. MT isolation and sequence analysis

For the isolation of MT, *O. brevis* cells (about 0.7 at OD_{660}) were collected after incubation in medium containing 20 μM of Cd^{2+}. The cells were sonicated ($140\,000 \times g$) and the supernatants were chromatographed on a Sephadex G-50 column after treatment at 80°C for 2 min. The fractions containing a high concentration of Cd^{2+} were collected and further purified on a DEAE-Sephadex-25 column and by reverse-phase high-performance liquid chromatography (Hitachi). The resulting product was separated by tricine-sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subsequently sequenced with a protein sequencer (PPSQ-10, Shimadzu).

For the isolation of *bmtA*, degenerate polymerase chain reaction (PCR) was performed using two degenerate primers as shown in Fig. 1 designed from the N-terminus of MT, and thermal asymmetric interlaced PCR (tail PCR) was performed to obtain a complete open reading frame (ORF) using the method described before [16].

2.3. Electrospray ionization mass spectrometric (ESI-MS) analysis

The apo-MT purified from *O. brevis* was diluted with 0.1% trifluoroacetic acid for analysis on a M-1200H ESI-mass spectrometer (Hitachi). The molecular mass of apo-MT was calculated using the software supplied.

*Corresponding author. Fax: (81)-86-434 1223.
E-mail addresses:* liutong@server.rib.okayama-u.ac.jp (T. Liu), snakashi@rib.okayama-u.ac.jp (S. Nakashima), k-hirose@rib.okayama-u.ac.jp (K. Hirose), smine@rib.okayama-u.ac.jp (M. Shibasaka), kmaki@rib.okayama-u.ac.jp (M. Katsuhara), kasamo@rib.okayama-u.ac.jp (K. Kasamo).

2.4. Construction of E. coli *strains with recombinant* bmtA *and* bxa1, *and minimal inhibitory concentration (MIC) analysis for* bmtA *and* bxa1 *transformants*

The cDNA of *bmtA* was generated by PCR using *O. brevis* genomic DNA as a template. The resulting products were subcloned into the vector pBAD/HisA (Invitrogen) to construct the plasmid pBAD/bmtA. The *bxa1* recombinant plasmid was constructed as described previously [16]. *E. coli* TOPO 10 cells were then transformed with recombinant plasmids or empty vectors. The expression of BmtA and Bxa1 was induced by addition of L-arabinose using the araBAD promoter. For determining the MIC, 20 μl of the pre-incubated *bmtA* or *bxa1* transformant was inoculated into 2 ml of LB medium containing 0.0002–0.02% arabinose (0.0002% for the *bxa1* transformant), 50 μg of ampicillin/ml, and increasing concentrations of Zn^{2+} or Cd^{2+}. After incubation at 37°C for 12 h, the amplification of *E. coli* strains was detected by measuring the absorbance at 600 nm. The MIC was defined as the lowest concentration at which complete inhibition of colony amplification was observed. Successful transformation and expression was confirmed by DNA sequencing and Western blotting (data not shown).

2.5. Transcriptional induction of bmtA *and* bxa1 *by heavy metal ions*

O. brevis cells grown in CT medium for 40 days (about 0.5 at OD_{660}, 0.1 g wet weight) were collected and suspended in 200 ml of medium containing Zn^{2+} or Cd^{2+} at the concentration described in Figs. 4 and 5. For concentration-dependent experiments, samples were harvested after 6 h. For time-course experiments, the cultures were incubated with the indicated metal concentration at 25°C for 0.5–168 h before harvest. Total RNA was isolated from samples as described [16].

2.6. Quantification of the absolute amounts of bmtA *and* bxa1 *mRNA using the rQRT-PCR method*

The real-time quantitative reverse-phase (rQRT) PCR analysis was carried out with a Smart Cycle system (Cepheid) using a Real-time One Step RNA PCR kit (Takara). Two primers encoding a 145 bp fragment were used to amplify the *bmtA* transcript. Another two primers as described [16] were employed for *bxa1*.

The external standards were created using PCR products corresponding to the entire *bmtA* and *bxa1* genes, using an introduced promoter T7 transcription site with the MEGAscript™ in vitro Transcription kit (Ambion) [16]. Total RNA (100 ng for *bmtA* or 150 ng for *bxa1*) was first reverse-transcribed at 50°C in a 25 μl reaction solution for 10 min. The PCR profile was as follows: 94°C for 30 s, 64°C (*bmtA*) or 62°C (*bxa1*) for 30 s and 72°C for 30 s with optics on. The validity of the amplification was assessed by melting curve analysis and reconfirmed by DNA sequencing.

3. Results

3.1. Isolation and identification of a MT and a CPx-ATPase from O. brevis

A Cys-rich metal-binding protein was purified from *O. brevis* by chromatography-based methods, and the N-terminus (40 amino acids) of this protein was sequenced. The partially sequenced amino acid sequence of the purified protein shows high identity with SmtA (Fig. 1). In order to confirm that this

Table 1
Analysis of the MIC for Zn^{2+} and Cd^{2+} in Bxa1 and BmtA transformant strains

	Zn^{2+}	Cd^{2+}
Vector control for BmtA	0.99 ± 0.03	0.80 ± 0.05
Vector control for Bxa1	0.99 ± 0.04	0.80 ± 0.04
Bxa1 transformant	1.77 ± 0.11	1.48 ± 0.09
BmtA transformant	1.16 ± 0.13	0.98 ± 0.12

Data were obtained from at least three independent analyses and the S.D. is indicated.

protein is a bacterial MT, a PCR-based method was employed to isolate the gene encoding this protein using *O. brevis* genomic DNA as template. A fragment of about 1718 bp was isolated and sequenced. This fragment contained one ORF of 168 bp, encoding a 55 amino acid sequence (Fig. 1). This gene has been termed *bmtA*. The proportion of Cys (16.4%) in BmtA is similar to that in bacterial MTs. The conserved and semi-conserved amino acid residues also showed remarkable similarity with MTs in bacteria (Fig. 1). Unlike eukaryotic MTs [5,17–19], BmtA contains three His residues (His[40], His[48] and His[54]), making it similar to SmtA (Fig. 1).

To confirm whether or not BmtA and the purified Cys-rich metal-binding protein are in fact the same gene product, an ESI-MS analysis was performed to estimate the molecular mass of the apoprotein purified from *O. brevis*. The results gave a value of 5483 Da, which is consistent with the molecular mass predicted for BmtA lacking an N-terminal Met residue (5478 Da).

The isolation and identification of Bxa1 from *O. brevis* were described in a previous study [16]. Southern hybridization showed that both *bmtA* and *bxa1* were present in the *O. brevis* genome (data not shown).

Amino acid analysis shows that BmtA is a homologue of *Synechococcus* PCC 7942 SmtA; Bxa1 also shares high homology with the zinc transport CPx-ATPase (ZiaA) in *Synechocystis* PCC 6803 except for a unique His-rich N-terminus (Fig. 2). The presence of both *bmtA* and *bxa1* genes in *O. brevis* raised questions of whether, and if so why, *O. brevis* utilizes both CPx-ATPase and MT for primary metal resistance.

3.2. bmtA and bxa1 expression can be induced in vivo by Zn^{2+} or Cd^{2+} at low to high concentrations

The basal mRNA expression (without excess metal ions) of *bmtA* and *bxa1* remained relatively constant at low levels during the incubation period from 40 to 90 days (Fig. 3). However, the expression of both genes could be markedly


```
                          10        20        30        40        50
                          |         |         |         |         |
O. brevis(BmtA)           MTTVTQIKCACPSCLCVVSLTEAIEKSGKSYCSSACADG-HPNGT.GCGHTGCECHK
O. brevis(purified apo-protein)  .TTVTQIKCACPSCLCVVSLTEAIEKSGKSYCSSACADG-H...............
S. PCC7942 (SmtA)         MTSTTLVKCACEPCLCNVDPSKAIDRNGLYYCSEACADG-HTGGSKGCGHTGCNCHG
S.vulcanus (MtnA)         MTTVTQMKCACPHCLCIVSLNDAIMVDGKPYCSEVCANGTCKENS-GCGHAGCGCGSA
A. PCC7120                MTTVTQMKCACPSCLCIISVEDAINKEGKYYCSEGCAEG-HKTIK-GCNHNGCGC---
P. aeruginosa             ---MNSETCACPKCTCQPG-ADAVERDGQHYCCAACASG-HPQGEP-CRDADCPCGGT
P. putida KT2440          ---MNDQRCACTHCSCTVD-ANALQRDGKAYCCEACASG-HRKGEP-CRMQDCHCG--
```

Fig. 1. Sequence alignment of BmtA and purified apo-MT homologues with other microbial MTs or predicted microbial MTs from *Synechococcus* PCC 7942 (SmtA, P30331), *Synechococcus vulcanus* (MtnA, P30565), *Anabaena* PCC 7120 (deduced products from genomes), *Pseudomonas aeruginosa* (deduced products from genomes) and *Pseudomonas putida* (deduced products from genomes). The conserved or semi-conserved residues are indicated in gray, and the weak peaks present in the apo-MT amino acid analysis are indicated by a dot. The primers used for degenerate PCR are underlined. The homologue analysis was performed with the Genetyx software (Software Development Co. Ltd.).

```
Bxa1       1  ------------------------------------------------------------------------------------------    1
ZiaA       1  ------------------------------------------------------------------------------------------    1
alr7622    1  MITIVQLNSCSIVVLEYWQLTFCLNSKAAMTQTPSLKTQTLQVGGMDCGSCAKTIEVALQQLHGVTEATVNFTTGKARVSYDAEVLSEKSIY   92

Bxa1       1  --MQKAKSSDSGCCSHDAHHEHSHENHNHDHDH-GHDH----------------------------------------------------   35
ZiaA       1  ------------------------------------MTQSSPLKTQQVGGMDCTSCKLK-EGSLERLKGVAEASVTVATGRLTVTY        52
alr7622   93  NQIKGLGLYTIEQSHQAQSHQQSHSCGGEHDHDHQNHDHNVDIV-SLQTLQLQISGMDCGSCA-KTIEAGVQKIIGVQEASVSFASERLHISY  182

Bxa1      36  --------------------------------------------------------QNGDFNL-KQELIPVISVVLLF-IGG-LIFE       63
ZiaA      53  DPKQ-VSEITIQERIAALGYTLAEPKSSVTL-NG-HK-IP---ISHREEGHS----HS--HGAGEFNL-KQELLPVLTAIALFTTI-AIL-FE  128
alr7622  183  DP-QLVNEKTIYDRIQDLGYTVEEGVETSSNHHTDACGHD-HEFHEDHDYS-HN----HEFSQP-INKSKQKQKSDLTSWR-FWIENRR-GQ  264

Bxa1      64  EK-LHNTPY-S-IAFYL-VFIPAYLLSGWNV--ILTSAGRNILRGRVFDENFL--------MTVATLGAVAIH-KLPE-AVGVMLFFKIGELF  139
ZiaA     129  QP-LHNT-PGQ-IAEFA-VIIPAYLLSGWTV--LKTAGRNILRCQIFDENFL--------MTIATLGALATH-QLPE-AVAVMLFFRVGELF   204
alr7622  265  SVILAGIGLVLGLLTQYLV-LPIWIARAFYGIGIVIACYPIARAGLFELR-LRRADMNLLMTISVIGAVILGDWF-EGGLVVFLI-FSLGTTL  352

Bxa1     140  QEFAVGRSRQSIKSLLEIRPDSANLKENGDIKKVSPETVAVGDIIL-VKPGEKIPLDGEIIDGNSQIDTSALTGESVPRTVKV-GETVLAGT   229
ZiaA     205  QEYSVGRSRSRIKALLLEARPDTANLKRNGTVQQVSPETVQVDDLIL-VKPGEKVPLDGEILGTSQVDTSALTGESVPGIVKP-GDIILAGM   294
alr7622  353  QVFTFGRTRNAIRSLMDLTPPTATVKRGNQEFTVPVESIQLGEII-LTIRPCGRVALDGVVVSGNSAIDQSPIITGESIPED-KDVGDTVFAGT  442

Bxa1     230  INQTGVLTVKVTKIFGESSISRILELVENARSKKAETEKFISKFASYYTPFVVFASLGVALIPPL-FISGATNADRLLWVYRALVLLVISCP   320
ZiaA     295  INQSGVLTIRVTKLFSESSIAKVLDLVENASSKKASTEKFIITQFARYYTPVIVFLSLAVALLPPL-FIPGADRAD---WVYRALVLLVISCP  382
alr7622  443  LNQTGFLEVKVTHTSSDTTVAKIINLVEQAQESRAPSQQWVDKFAQVYTPIVILAATAIALIPPLAFAQ-PFN-V---WLYRALVMLVIACP   529

Bxa1     321  CGLVISIPLGYFGGIGGAAKRGILVKGS-TFLDTLTAVKTVVLDKTGTLTKGVFKVAQIVPONGFSQSDLMAMA-AKVESQSNHPVAKSILE   410
ZiaA     383  CGLVISIPLGYFGGIGGAAKHIGILIKGS-TFLDSLTAVKTVVFDKTGTLTKGIFKVTQVVTKNGFSESELLTILA-AKAESHSTHPIALSIRE  472
alr7622  530  CALVISTPVSIVSAICAATRRCVLFKCGNA-LEIIAGHLTTLAFDKTGTIIIQG-LPIVQQVYDLGVVSGDMVLILAASLEQKSEHPLAKAIVV  619

Bxa1     411  AYGG-KIDSSEVTDYEEIAGHGILAKVNNQIVLAGNDRLLHRENIPH-DVCNAEGTVV-HLAVDNRYA-----GYIL--IAD-EL-KDDAVQ   490
ZiaA     473  AYAQ-SHADSEVADYEEIAGHGIRAVVQNQVVIAGNDRLLHREKIDF-DTGDVAGTVV-HLAVDGRYG-----GYIL--IAD-EI-KEDAVQ   552
alr7622  620  KAQELGLELETPLNFTALPGKGIQANFGEQLYLVGNRRLFSDQGICLSDEAESLLTEIEQFG-QIPVLVGTNGG-LLGAIALSDGIRLEATE   709

Bxa1     491  AIRDLKKLGVERIVMLTGDNQAVADSVARKLGLDSYIIAELLPEGKVEAIEKLISQSKKGDKIVFVGDGINDAPVLARADVGIAMGGLGSDAA  582
ZiaA     553  AIRDLKRMGVEKTIVMLTGDSEIVAQSVAQQIGLDAFVAELLPEEKVDEIEQLLDPSGKA-KLAFVGDGINDAPVIIARADVGIAMGGLGSDAA  643
alr7622  710  ALRQLQRVGLKRLVMLTGDRASVAKQIAQQVGITEYQAELLPEDKLQATIQQLRRHGVVGMV---GDGINDAPALATADISFAVGGIDI--A   795

Bxa1     583  IETADIVIMADAPSKVAEAIQIARKTHNIVVQNIIFAMSVKALIFIALGAFGLATMWE-AVFADVGVALAAIFNATRVLK------        660
ZiaA     644  IETADVVLMTDAPSKVAEAIHVARKTRQIVVQNIVLALGIKALFIALGTIGLATLWE-AVFADVGVALLAILNATRIAK------        721
alr7622  796  LETADVVLVGSDLRQLAYAVDLSRRIVSVIQQNVVFSLVTKALFLLLGTFGFVG-LAIAVLADTGTSLLLVTANGMRLFKTKTLKN        879
```

Fig. 2. Homology analysis of Bxa1 with ZiaA and the ZiaA homologue from the genome of *Anabaena* PCC 7120 (deduced products from alr7622). The conserved residues are inverted.

enhanced by addition of Zn^{2+} or Cd^{2+} (Fig. 4). The corresponding induction of *bmtA* expression by Zn^{2+} or Cd^{2+} reached a maximum at the same concentration (0.5 mg/l), with almost the same magnitude (Fig. 4A). On the other hand, the maximum level of transcription for *bxa1* was obtained at 0.5 mg/l for Cd^{2+} and almost the same level of expression was found at 4 mg/l for Zn^{2+} (Fig. 4B). A further increase in the concentration of both metals led to a gradual reduction in *bmtA* and *bxa1* expression. However, the expression levels of the two genes were greater than basal levels even at lethal concentrations.

3.3. Over-expression of bmtA and bxa1 in E. coli shows both genes mediate Zn^{2+} and Cd^{2+} tolerance

Over-expression of Bxa1 in *E. coli* strain TOPO 10 markedly enhanced both Zn^{2+} and Cd^{2+} tolerance in transformant strains (Table 1). Over-expression of BmtA in *E. coli* also increased tolerance to both Zn^{2+} and Cd^{2+} in transformants, although not as extensively (Table 1). We have no way of efficiently deleting *bmtA* or *bxa1* in filamentous cyanobacteria at present. Thus, it is difficult to state with confidence which gene participates in Zn^{2+} or Cd^{2+} tolerance in *O. brevis*. However, as shown in Table 1, the higher resistance of the CPx-
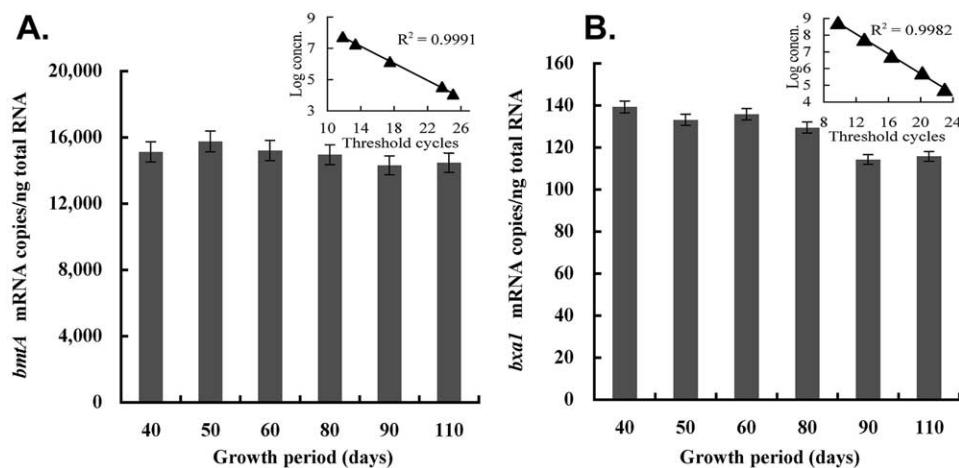

Fig. 3. Basal transcription level of *bmtA* (A) and *bxa1* (B). *O. brevis* was grown in modified CT at 25°C. Cells were collected after 40, 50, 60, 80, 90 and 110 days. Total RNA was isolated and real-time quantification RT-PCR was performed as described in Section 2. The external standards for BmtA and Bxa1 were generated as described in Section 2. The standard curve generated with the same batch of samples for absolute quantification is shown in the inset.
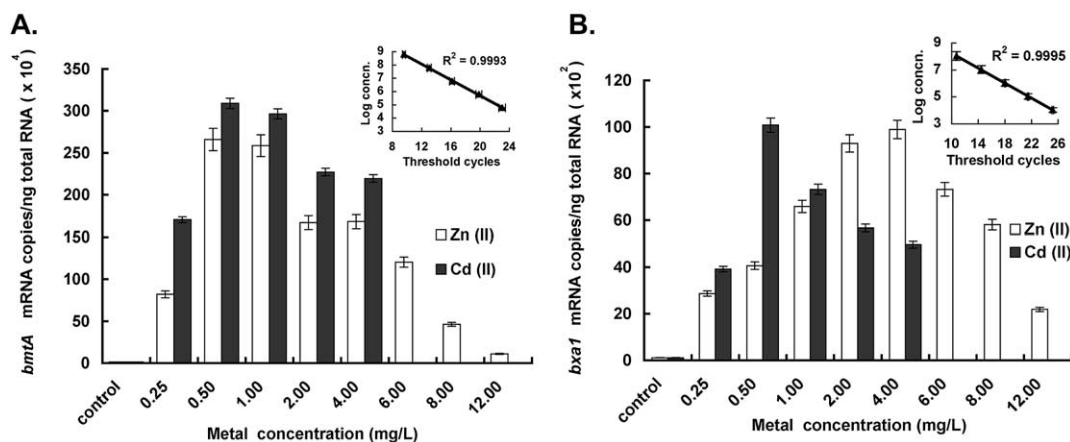
Fig. 4. Quantification of transcriptional induction of *bmtA* (A) or *bxa1* (B) in response to an elevated concentration of heavy metal ions. Samples were treated with the indicated concentrations and varieties of heavy metal ions for 6 h. Sample treatment, RNA isolation, the generation of external standards and rQRT-PCR were conducted as described in Section 2. Data were obtained from at least three analyses and the S.D. is indicated.

ATPase transformant and relatively low resistance of the MT transformant to Zn^{2+} and Cd^{2+} might reflect differences in the basal functions and efficiencies of the two genes in metal homeostasis.

3.4. The expression of bxa1 induced by metal ions is more rapid than that of bmtA

The dynamic expression of *bxa1* and *bmtA* induced by Zn^{2+} and Cd^{2+} in vivo was performed to facilitate understanding of the function and interrelation in vivo between the two genes in *O. brevis*. As shown in Fig. 5A, the amount of *bmtA* mRNA expressed in response to the metal ions increased gradually with the incubation time. After about 8 h, the expression of *bmtA* induced by Cd^{2+} peaked. Zn^{2+} had a maximum effect at 24 h incubation, 16 h later than Cd^{2+}.

The inducible expression of *bxa1* peaked more rapidly (Fig. 5B), the maximum being reached at 1 h for both Zn^{2+} and Cd^{2+}. Prolonged incubation beyond the optimal time resulted in a decrease in the transcription level of both *bxa1* and *bmtA*. The high-level induction of *bmtA* and *bxa1* mRNA expression was still observed for at least 168 h (data not shown). The rapid induction of *bxa1* expression to a maximum suggests

that CPx-ATPase in *O. brevis* is a quick response factor and MT is a relatively slow emergent in Zn^{2+} and/or Cd^{2+} handling.

4. Discussion

BmtA is a homologue of *Synechococcus* PCC 7942 SmtA, and Bxa1 shares high homology with *Synechocystis* PCC 6803 ZiaA except for a unique His-rich N-terminus [12,16]. High levels of both *bmtA* and *bxa1* mRNA expression were induced by Zn^{2+} and Cd^{2+} over a wide range of concentrations. Experiments on heavy metal tolerance in *BmtA*-recombinant *E. coli* demonstrated that BmtA also slightly enhanced the resistance to Zn^{2+} and Cd^{2+}. Although Cd^{2+} could also induce the expression of both genes, amino acid homology analysis and induction expression properties suggest that Zn^{2+} might be the basal substrate for both Bxa1 and BmtA. As we described above, the zinc-related SmtA (MT) and ZiaA (CPx-ATPase) have been found in *Synechococcus* PCC 7942 [5,15] and *Synechocystis* PCC 6803 [12,13], respectively. ZiaA and SmtA appear to be 'interchangeable' in handling zinc tolerance [12]. The presence of *bmtA* and *bxa1* in *O. brevis*
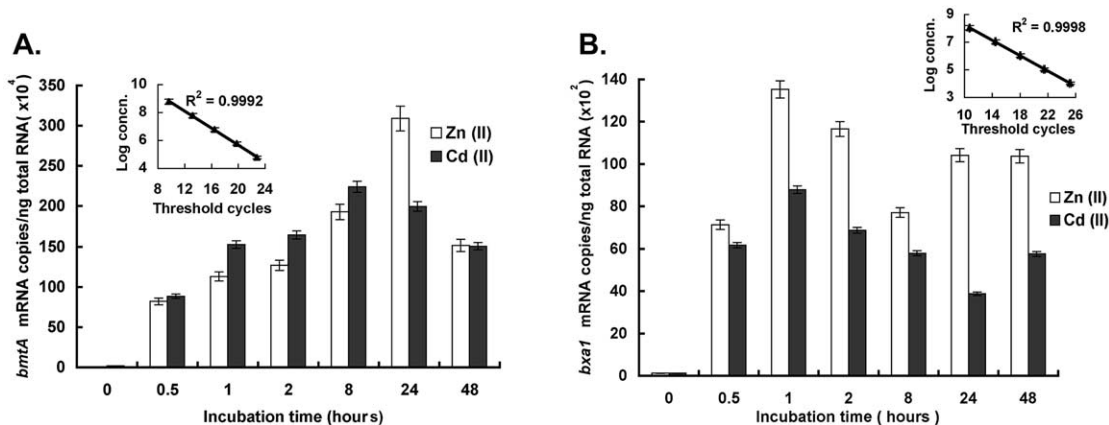

Fig. 5. Time-course study of *bmtA* (A) and *bxa1* (B) expression induced by heavy metal ions. A 200 ml volume of medium containing about 0.1 g of *O. brevis* was treated with 4 mg/l of Zn^{2+} or 2 mg/l of Cd^{2+} for 0.5–48 h. Sample treatment, RNA isolation, the generation of external standards and rQRT-PCR were conducted as described in Section 2. Data were obtained from at least three analyses and the S.D. is indicated.

raised the questions of why and how *O. brevis* utilizes both CPx-ATPase and MT for its primary heavy metal resistance, when others utilize only one of them.

In MIC analysis, the *bmtA* transformant showed less heavy metal tolerance than the *bxa1* transformant, implying a difference in the efficiency of the two genes in handling Zn or Cd. However, we have no way of efficiently deleting *bmtA* or *bxa1* in filamentous cyanobacteria, so it is difficult to state clearly which gene confers higher Zn^{2+} or Cd^{2+} tolerance in *O. brevis*. In the in vivo time-course induction experiment, the expression of *bxa1* reached a maximum within 1 h for both Zn^{2+} and Cd^{2+} (Fig. 5). On the other hand, the maximum of *bmtA* was reached at 8 h for Cd^{2+} and 24 h for Zn^{2+} (Fig. 4). The same induction pattern was observed when *O. brevis* was exposed to a lower or higher metal concentration (data not shown). These results suggest that *bxa1* is a rapid response determinant in the handling of Zn^{2+} and/or Cd^{2+}, which might function as a first line of defense against intracellular heavy metal ions; *bmtA* might be a relatively slow emergent factor and a second line of defense in heavy metal tolerance.

In higher organisms, a similar relation between BmtA and Bxa1 has been found recently; however, instead of CPx-ATPase, a cation diffusion facilitator (ZIP4 or Zhf) acts as a first line of defense against the uptake of excess Zn^{2+} through exclusion, and MTs provide a second line of defense through the chelation and detoxification of excess metal ions [19–22]. Most prokaryotes have been reported to utilize only either CPx-ATPase or MTs as a primary mechanism for heavy metal tolerance [8–10]. Our results suggest that the increased heavy metal tolerance in *O. brevis* is achieved by more complex mechanisms; utilizing a CPx-ATPase (Bxa1) to rapidly efflux excess metal ions out of cells and a MT (BmtA) to buffer and store excess heavy metal ions.

What might be the mechanism regulating *bxa1* and *bmtA*? Sequence analysis of the promoter region of *bxa1* and *bmtA* showed that both regions contain a 12-2-12 inverted repeat element (no published data). Similar elements have been found in the promoter region of SmtA and ZiaA, which functions as a DNA–protein binding site in response to two SmtB homologous metalloregulators (SmtB and ZiaR). The identification and characterization of the regulators for Bxa1 and BmtA are now in progress.

References

[1] Clemens, S., Kim, E.J., Neumann, D. and Schroeder, J.I. (1999) EMBO J. 18, 3325–3333.
[2] Hall, J.L. (2002) J. Exp. Bot. 53, 1–11.
[3] Murphy, A., Zhou, J.M., Goldsbrough, P.B. and Taiz, L. (1997) Plant Physiol. 113, 1293–1301.
[4] Rensing, C., Ghosh, M. and Rosen, B.P. (1999) J. Bacteriol. 181, 5891–5897.
[5] Robinson, N.J., Tommey, A.M., Kuske, C. and Jackson, P.J. (1993) Biochem. J. 295, 1–10.
[6] Williams, L.E., Pittman, J.K. and Hall, J.L. (2000) Biochim. Biophys. Acta 1465, 104–126.
[7] Pena, M.M.O., Koch, K.A. and Thiele, D.J. (1998) Mol. Cell. Biol. 18, 2514–2523.
[8] Riggle, P.J. and Kumamoto, C.A. (2000) J. Bacteriol. 182, 4899–4905.
[9] Binet, M.R. and Poole, R.K. (2000) FEBS Lett. 473, 67–70.
[10] Mitra, B. and Sharma, R. (2001) Biochemistry 40, 7694–7699.
[11] Weissman, Z., Berdicevsky, I., Cavari, B.Z. and Kornitzer, D. (2000) Proc. Natl. Acad. Sci. USA 97, 3520–3525.
[12] Thelwell, C., Robinson, N.J. and Turner-Cavet, J.S. (1998) Proc. Natl. Acad. Sci. USA 95, 10728–10733.
[13] Robinson, N.J., Whitehall, S.K. and Cavet, J.S. (2001) Adv. Microb. Physiol. 44, 183–213.
[14] Kanamaru, K., Kashiwagi, S. and Mizuno, T. (1994) Mol. Microbiol. 13, 369–377.
[15] Turner, J.S. and Robinson, N.J. (1995) J. Ind. Microbiol. 14, 119–125.
[16] Liu, T., Nakashima, S., Shibasaka, M., Katsuhara, M. and Kasamo, K. (2002) J. Bacteriol. 184, 5027–5035.
[17] Hamer, D.H. (1986) Annu. Rev. Biochem. 55, 913–951.
[18] Nies, D.H. (1999) Appl. Microbiol. Biotechnol. 51, 730–750.
[19] Palmiter, R.D. (1998) Proc. Natl. Acad. Sci. USA 95, 8428–8430.
[20] Borrelly, G.P.M., Harrison, M.D., Robinson, A.K., Cox, S.G., Robinson, N.J. and Whitehall, S.K. (2002) J. Biol. Chem. 277, 30394–30400.
[21] Kelly, E.J. and Palmiter, R.D. (1996) Nat. Genet. 13, 219–222.
[22] Vasak, M. and Hasler, D.W. (2000) Curr. Opin. Chem. Biol. 4, 177–183.