Gold Induces a Metallothionein Gene and a CPx-ATPase Gene Through Regulation of a Repressor BxmR in *Oscillatoria brevis*

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Abstract

A cysteine-rich metal binding protein MT (metallothionein) gene (*bmtA*) and a CPx-ATPase metal transporter gene (*bxaI*) are induced upon exposure to multiple heavy metal ions in the cyanobacterium *Oscillatoria brevis*. The SmtB/ArsR family repressor BxmR from *O. brevis* represses the expression of an operon encoding *bmtA* and *bxmR*, and another operon encoding *bxaI*. In the present study, the expression of *bmtA* and *bxaI* was induced in vivo by 5–15 μ M gold (Au) treatment for 1–4 h with a different level in *O. brevis* cells. In vitro electrophoretic gel mobility shift analysis (EMSA) revealed that the Au treatment promotes a disassembly of BxmR protein from the BxmR-*bxmR/bmtA* operator (O)/promoter (P) or BxmR-*bxaI* O/P complex with a different affinity. Our results indicate that Au treatment can cause a release of BxmR protein in gene-expression level of these genes in *O. brevis*.

Keywords: CPx-ATPase, Cyanobacterium, Gold (Au) ion toxicity, Metallothionein, Repressor

INTRODUCTION

Gold (Au) is one of the rare metals on earth. Demand for gold in modern industry and nanotechnology has been increasing more and more. Exploration for new Au resource in the nature is very important and recovering Au from waste solutions (bioaccumulation of gold by the microorganisms) has been noticed as one of useful strategies for this purpose. Many studies have been performed to understand the interaction of Au with microorganisms (*Bacillus subtilis*, *Escherichia coli*, *Salmonella typhirium*, *Pseudomonas maltophilia* and so on) including cyanobacteria (*Plectonem terebrans* and *Plectonema boryanum*)^{1–10}. A filamentous cyanobacterium, *P. boryanum*, and Fe (III)-reducing bacteria reduce Au (III)-chloride prior to or simultaneously with adsorption of Au onto the cell surfaces by their reducing systems^{2,11}.

Therefore it has been suggested that the formation of an intermediate Au (I) species is important for Au-uptake.

Biological information for metal homeostasis, tolerance mechanisms and gene response mechanisms against Au toxicity are also very important to establish the effective Au bioaccumulation system in microorganisms. Two major strategies are involved in metal homeostasis and tolerance in many organisms. One involves the uptake or efflux of specific heavy metal ions across cell membranes, mediated by CPx-ATPase family^{12, 13}. Another strategy involves the specific chelation of metal ions, e.g. metallothioneins (MTs). MTs are involved in the detoxification of toxic heavy metals and in the homeostasis of essential metals^{14–16}. Stoyanov & Brown showed that a transcription of *E. coli* Cu-transporting ATPase gene, *copA*, was activated by Au as well as by Cu via the

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CueR activator⁹⁾. Sharma & McQueen reported that the injected Au ions were recovered in the MT fraction of rat kidney tissue and suggested a reaction between MT and Au¹⁷⁾. Moreover, Saito et al. reported that Au can bind to MT and induce MT gene in rat, but the exact mechanisms of gene-expression and regulation of MT by Au have not been elucidated^{18, 19)}.

The filamentous freshwater cyanobacterium, *Oscillatoria brevis*, is the first bacterium identified that harbors both CPx-ATPase (named Bxa1; accession number AB073990) and MT (named BmtA; accession number AB085749) in order to achieve multiple heavy metal co-tolerance for Ag (I), Cu (I), Zn (II) and Cd (II)²⁰⁾. Furthermore, Liu et al. have shown that a SmtB/ArsR metal-sensing family repressor, BxmR (accession number AB085750), binds to the operator (O)/promoter (P) regions of the *bmtA*, *bxa1* and *bxmR* genes and represses the expression of the *bmtA* and *bxa1* genes without heavy metals²¹⁾. This metal homeostasis system is regulated by both monovalent (Cu (I) and Ag (I)) and divalent (Zn (II) and Cd (II)) heavy metal ions by direct interactions between BxmR protein and these ions, which in turn reduces the affinity as a regulator for the O/P region^{21, 22)}. Release of BxmR repressor from O/P region induces their transcription. While all SmtB/ArsR repressors except for BxmR regulate either monovalent or divalent ions^{23, 24)}.

There is no report describing the regulation mechanism of SmtB/ArsR repressors under Au stress. It was therefore very interesting for us to clarify whether BxmR can response to Au as well as other metals and regulate the gene expression of the *bmtA* and *bxa1* under Au condition. Moreover, it is expected that the induced BmtA protein can trap and recover Au within *O. brevis* cells as a bioaccumulation system. The present study was undertaken to clarify the BxmR dependent gene-response mechanism of the *bmtA*, *bxa1* and *bxmR* genes under Au stress in *O. brevis*.

MATERIALS AND METHODS

Growth conditions *O. brevis* was grown at 25 °C in 200 ml of modified CT medium as

described²¹⁾. Cells were grown to an optical density of 0.5 (660 nm) and then used in this study.

Gene expression analysis of *bmtA*, *bxa1* and *bxmR* under Au stress To determine the Au concentration-dependent gene-expression of the *bmtA*, *bxa1* and *bxmR* genes, we suspended *O. brevis* cells (about 0.05 g fresh weight) in 30 ml of CT medium containing AuCl₃ (0, 5, 10 and 15 µM). Cells were mildly shaken for 5 h (40 rpm) and then harvested for total RNA extraction after Au treatment. To determine the effect of the duration of treatment, we treated *O. brevis* cells (about 0.05 g fresh weight) with 10 µM AuCl₃ at 25 °C for 1, 2, 4, 6, 10 and 24 h with a mild shaking (40 rpm). Total RNA was also extracted from these samples. Copy numbers of gene-specific mRNA were measured by real-time quantification RT-PCR (qRT-PCR) using Light Cycler 1.5 (Roche). Total RNA was extracted with an RNasy Plant Mini kit (QIAGEN). Then we adjusted the RNA concentration to 100 ng/µl after measurement with a spectrophotometer. Reverse transcription was done to synthesize cDNA with a Primer Script RT reagent Kit (Perfect Real time) (TaKaRa). qRT-PCR was performed using SYBR Premix Ex Taq (Roche Diagnostics). As primers, we used (5'-AAT GCGCCTGTCCTTCTTGCTT-3') and (5'-TTA TTTGTGGCACTCGCAACCG-3') for *bmtA*, (5'-CACAGATACCAGTCGCAGCCTG-3') and (5'-CAGTCAGCCTCACCAAGAGAACG-3') for *bxmR* and (5'-TGCTGACATTGTGATTA TGGCA-3') and 5'-TGCTAAAGCCACCCCA CAT-3') for *bxa1*. Experimental run protocol is shown below: denaturation program (95 °C for 10 min), amplification and quantification program repeated 45 cycles (95 °C for 5 s, 55 °C for 10 s, 60 °C for 15 s with a single fluorescence measurement). In this PCR, 146 bp product for the *bmtA*, 196 bp product for the *bxa1* and 172 bp product for the *bxmR* were amplified. The external standard curve for each gene was generated according to the method described previously and used for a calculation of the copy numbers of each mRNA^{20, 25)}. The validity of qRT-PCR products was confirmed by melting-curve analysis.

Over-expression and purification of BxmR Log-phase *E. coli* transformant expressing

the *bxmR* gene²¹), was incubated at 37 °C with a vigorous shaking (120 rpm) for 4 h with 0.02% L-arabinose. The cells were harvested by centrifugation and washed with 10 mM Tris-HCl buffer (pH 7.3) containing 1 mM phenylmethylsulfonyl fluoride. The resuspended cells were disrupted by ultrasonication (Ultrasonic Generator MODEL US-50, Nissei, strength 7, 12 times of sonication for 15s at 0 °C). After centrifugation at a low speed (5000 × g, 10 min at 4 °C), the supernatant was collected. Recombinant BxmR fusion protein containing an N-terminal polyhistidine tag (N-His₆-BxmR) was purified from the supernatant by Ni (II)-chelate chromatography using a MagExtractor fusion protein purification kit (Toyobo). Purified N-His₆-BxmR fusion protein was digested with an enterokinase (Invitrogen) at 25 °C for 16 h and the N-His₆ was removed according to the manufacture's protocol.

Electrophoretic gel mobility shift analysis (EMSA) The *bmtA/bxmR* DNA probe for EMSA (231 bp of *bmtA/bxmR* O/P) and the *bxa1* DNA probe (200 bp of *bxa1* O/P) were prepared by PCR using the primers (5'-CAGACTTCGGTGACATCTTTTCC-3') and (5'-AGTGAAGACTTAGGCTGGTCAGTACA-3') for *bmtA/bxmR* O/P and (5'-TGCCCGCAATTGAGAATAGTT-3') and (5'-GCTCGATTGGCTTTTGGCA-3') for *bxa1* O/P.

These amplified DNA include 12-2-12 inverted repeat sequences, respectively²¹). The 3'-end of each DNA fragment was labeled with digoxigenin (DIG) using a terminal transferase and DIG-ddUTP. Purified BxmR (45 nM) was incubated for 30 min with 0.3 nM of DIG-*bmtA/bxmR* O/P or DIG-*bxa1* O/P in a buffer containing 25 mM Tris-HCl (pH 7.3), 10% glycerol, 1 µg of poly (dI-dC) and 1 mM dithiothreitol in a total volume of 25 µl at 25 °C. The effect of exogenous Au metal on protein-DNA complex was examined by addition of AuCl₃ to the reaction mixture. DNA-protein complexes were separated at 4 °C on an 8% nondenaturing PAGE and then transferred to a positively charged nylon membrane (Pall Corporation). The DIG-derived chemiluminescent signal was detected on X-ray films.

RESULTS

BxmR repressor mediates gene-regulation of *bmtA*, *bxa1* and *bxmR* under Au stress To investigate whether BxmR regulates transcription of *bmtA*, *bxa1* and *bxmR* genes under Au stress, we measured the Au concentration dependency by qRT-PCR. Compared with the expression level of each gene at Au 0 µM condition, expression of both *bmtA* and *bxa1* was induced at a high Au concentration (Fig. 1), with a maximum induction at 15 µM Au (approximately 60-fold *bmtA* and 8-fold *bxa1* mRNA expression). A slight induction of *bxmR* was also observed.

Kinetics for the expression of *bmtA*, *bxa1* and *bxmR* in response to Au was examined at a fixed concentration of 10 µM (Fig. 2).

Fig. 1 Quantification of the expression of *bmtA*, *bxa1* and *bxmR* under Au stress. *O. brevis* cells were exposed to various concentration of Au (0–15 µM) for 5 h. Data were obtained from at least three independent qRT-PCR and expressed as the mean ± S.E.

Fig. 2 Time-course study of *bmtA*, *bxa1* or *bxmR* expression under Au stress. *O. brevis* was treated with 10 µM of Au for 0–24 h. Data were obtained from at least three independent qRT-PCR and expressed as the mean ± S.E.

Expression of *bmtA* gene was induced approximately 50-fold by Au treatment for 1–4 h and then reduced. Expression of *bxal* was also approximately 10-fold induced by Au treatment for 1–4 h compared with the 0 h condition. Maximum expression of these two genes was observed within the first 2 h treatment. A slight induction of *bxmR* gene was also detected by Au treatment for 1–4 h. The expression of all three genes was reduced to the original level (at 0h) after 24 h of treatment.

BxmR is dissociated from both *bmtA/bxmR* O/P and *bxal* O/P by Au The gene-expression of *bmtA*, *bxal* and *bxmR* was induced by several heavy metal stressors due to a release of BxmR from the O/P region of *bmtA/bxmR* or from the O/P region of *bxal*²¹⁾. EMSA experiments were carried out to determine whether a release of BxmR repressor from each O/P region is occurred under Au stress in vitro. As shown in Fig. 3, a slower migrating band (C in Fig. 3) decreased with an increase of Au concentra-

tion. The BxmR/DNA complex band almost disappeared and only the free DNA probe (FP in Fig. 3) was detected by 300 μ M Au treatment in both BxmR-O/P region of *bmtA/bxmR* (Fig. 3A) and BxmR-O/P region of *bxal* (Fig. 3B). This means that the Au stress promotes a release of BxmR repressor from these two BxmR-DNA complexes.

DISCUSSION

BxmR is an unique repressor which can regulate a high expression of *bmtA* in response to both monovalent heavy metals (Ag (I) and Cu (I)) and divalent heavy metals (Cd (II) and Zn (II)), and a moderate expression of *bmtA* to a specific thiol oxidant, diamide, in *O. brevis*^{21,26)}. In this study, we determined the expression of *bmtA*, *bxal* and *bxmR* genes under Au stress in *O. brevis* and this treatment induced 50-fold and 10-fold expression levels of *bmtA* and *bxal* in early stage of Au treatment (0–4 h), respectively. Therefore BxmR can be regarded as an unique repressor with a wide range adaptation.

It has already been reported that Au ion can bind to MT fraction and induce MT in rat kidney tissue^{17–19)}. Saito et al. showed an order of the binding stability of heavy metals to MT (Hg > Au > Cu > Cd > Zn) and also showed that Au can bind to MT stronger than Cu or Zn in vitro. Stability (=affinity) constant of each metal to MT was also determined (Cu, 10^{19} – 10^{17} ; Cd, 10^{17} – 10^{15} ; Zn, 10^{14} – 10^{11})¹⁸⁾. These results indicated that Au shows a very high affinity to MT. Unfortunately there was no clear evidence which ion form (Au (I) or Au (III)) can preferentially bind to MT, but it has been suggested that Au (I), like as Cu (I), binds to MT. We suppose that Au can bind to induced MT and be detoxified in *O. brevis*. BxmR must be an interesting regulator candidate in the Au-mediated homeostasis/detoxification system in *O. brevis*.

EMSA results showed that Au ion induces a release of BxmR protein from the BxmR-DNA complexes, such as BxmR-*bxmR*/ *bmtA* O/P and BxmR-*bxal* O/P. The disassembly is Au concentration-dependent and occurs in the early stage of Au treatment (0–4 h). Thus, it is suggested that BxmR

Fig. 3 Release of BxmR from the DNA fragment including putative promoter region of *bmtA/bxmR* (A) O/P or *bxal* O/P (B) by Au treatment. DNA fragment (0.3 nM) was incubated with 45 nM or without the BxmR monomer. These mixtures were treated with various concentrations of Au (0–300 μ M). FP and C correspond to free DNA probe and BxmR-DNA complex, respectively. DNA-protein samples were separated at 4 °C on an 8% nondenaturing PAGE and then transferred to a nylon membrane. Lane 1, FP alone. In lane 7, the condition for the binding reaction was the same as in lane 2 except for an addition of 100-fold excess unlabeled DNA fragment. Three independent experiments were performed and almost similar results were obtained.

harbors stressor-binding site(s) for Au as well as other heavy metals such as Ag (I), Cu (I), Cd (II) and Zn (II). These metals allosterically regulate the binding capacity of BxmR repressor to the O/P regions of the three genes. Au dependent release of repressor from the complex has not been reported in any other previously characterized SmtB/ArsR regulator as far as we know. Release of BxmR from the O/P region due to an exposure to Au leads the induction of the three genes (*bmtA*, *bxal* and *bxmR*). Approximately half of the BxmR proteins were released from the *bmtA/bxmR* O/P by 30 μ M Au treatment, and from the *bxal* O/P by 3 μ M Au, respectively in our EMSA condition (Fig. 3). There may be a difference in the binding strength of BxmR protein to these two O/P regions under the condition. EMSA was also performed using BxmR-*bmtA/bxmR* O/P and BxmR-*bxal* O/P complexes under La (III) treatment. But La did not affect the release of BxmR protein from these two complexes (data not shown). We suppose that Au (III) ion is reduced to Au (I) ion in its uptake process like as Cu (I). Absorbed Au (I) ion probably shows a biological toxicity to *O. brevis* and causes a release BxmR from the O/P regions. This speculation should be addressed by further studies.

Expression level of the *bmtA* was much higher than that of the *bxal* in the Au treated *O. brevis* cells. Furthermore, MT has a high affinity to Au, as described above. We have not yet done influx and efflux experiment of Au in *O. brevis*, but it is supposed that Au ions in cytoplasmic region may be trapped by MT rather than excluded to out side by BxaI transporter. Moreover, we suppose that filamentous cyanobacteria, *O. brevis*, can be easily recovered from hydrosphere and the Au ions trapped by MT also can be easily recovered from cell pellet at the final step of bioaccumulation process. We believe that this study is a fundamental research in recovery of Au from hydrosphere by *O. brevis*.

CONCLUSIONS

O. brevis bmtA (MT gene) and *bxal* (metal ion transporter gene) were induced by 5–15 μ M Au treatment. Maximum induction of these two genes was observed within 2 h by

an exposure to 10 μ M Au. Especially, approximately 50-fold of gene expression was seen in the case of the *bmtA* gene. Our EMSA moreover indicated that BxmR repressor was released from O/P region of the three genes by an addition of Au ion. These results indicated that the *O. brevis bmtA* and *bxal* genes can be controlled through BxmR repression in the presence of Au ion in hydrosphere.

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