AzuR From the SmtB/ArsR Family of Transcriptional Repressors Regulates Metallothionein in *Anabaena* sp. Strain PCC 7120

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Metallothioneins (MTs) are cysteine-rich, metal-sequestering cytosolic proteins that play a key role in maintaining metal homeostasis and detoxification. We had previously characterized NmtA, a MT from the heterocystous, nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120 and demonstrated its role in providing protection against cadmium toxicity. In this study, we illustrate the regulation of *Anabaena* NmtA by AzuR (Alr0831) belonging to the SmtB/ArsR family of transcriptional repressors. There is currently no experimental evidence for any functional role of AzuR. It is observed that azuR is located within the *znuABC* operon but in the opposite orientation and remotely away from the *nmtA* locus. Sequence analysis of AzuR revealed a high degree of sequence identity with *Synechococcus* SmtB and a distinct α5 metal binding site similar to that of SmtB. In order to characterize AzuR, we overexpressed it in *Escherichia coli* and purified it by chitin affinity chromatography. Far-UV circular dichroism spectroscopy indicated that the recombinant AzuR protein possessed a properly folded structure. Glutaraldehyde cross-linking and size-exclusion chromatography revealed that AzuR exists as a dimer of ~28 kDa in solution. Analysis of its putative promoter region [100 bp upstream of *nmtA* open reading frame (ORF)] identified the presence of a 12–2–12 imperfect inverted repeat as the cis-acting element important for repressor binding. Electrophoretic mobility shift assays (EMSAs) showed concentration-dependent binding of recombinant dimeric AzuR with the promoter indicating that NmtA is indeed a regulatory target of AzuR. Binding of AzuR to DNA was disrupted in the presence of metal ions like Zn$^{2+}$, Cd$^{2+}$, Cu$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Pb$^{2+}$, and Mn$^{2+}$. The metal-dependent dissociation of protein–DNA complexes suggested the negative regulation of metal-inducible *nmtA* expression by AzuR. Overexpression of *azuR* in its native strain *Anabaena* 7120 enhanced the susceptibility to cadmium stress significantly. Overall, we propose a negative regulation of *Anabaena* MT by an α5 SmtB/ArsR metalloregulator AzuR.

**Keywords:** *Anabaena* 7120, AzuR, regulation, metallothionein, cadmium stress
INTRODUCTION

Trace metal ions are crucial for nearly all aspects of metabolism in the prokaryotic cells. These are involved in various biological processes like enzymatic reactions that require metal ions as cofactors, for folding and structural stabilization of the proteins or for the maintenance of the metal-sensing regulatory factors (Rees, 2002; Bertini et al., 2007; Chandrangsu et al., 2017). Although the essential metal ions are indispensable, these are toxic in excess amounts (Chandrangsu et al., 2017). As a result, the microorganisms have developed mechanisms to regulate the homeostasis of the essential metal ions. Metal homeostasis is mediated by balancing the uptake, storage, transfer, and efflux of the metals so that the cellular requirements are fulfilled and the right metal is introduced into the right macromolecule in the cells for various biological processes (Tottey et al., 2005; Waldron and Robinson, 2009).

Metallothioneins (MTs) are cysteine-rich, low-molecular-weight, metal-sequestering proteins that are known to bind metal ions via metal–thiolate clusters and are involved in maintaining homeostasis of physiologically important metals like zinc (Zn\(^{2+}\)) and copper (Cu\(^{2+}\)) (Klaassen et al., 1999; Blindauer, 2011). Apart from binding to the essential metals, MTs are implicated in the detoxification of toxic metals including cadmium (Cd\(^{2+}\)) and mercury (Hg\(^{2+}\)) from the cells (Klaassen et al., 1999). MTs are induced in the presence of ionic species of various metals like Cd, Zn, Cu, Hg, Au, Ag, Bi, Pb, Ni, and Cr (Palmiter, 1987; Huckle et al., 1993) as well as oxidative stress (Andrews, 2000). MT expression is strictly regulated owing to its role in maintaining metal homeostasis. While eukaryotic MT gene expression has been shown to be under positive regulation (Klaassen et al., 1999), prokaryotic MT expression is proposed to be negatively regulated (Turner and Robinson, 1995). The first characterized prokaryotic MT is Synechococcus sp. SmtA (Blindauer and Leszczyszyn, 2010). The smtA gene expression is negatively regulated by a zinc responsive transcriptional repressor SmtB (Erbe et al., 1995; Turner et al., 1996) of the SmtB/ArsR family of transcriptional regulatory proteins. The SmtB/ArsR family of proteins bind to specific regulatory sequences present upstream of the gene. Derepression of transcription by such regulators results from direct binding of the metal to the repressor, which inhibits its binding to the operator/promoter (O/P) region of the gene under regulation (Busenlehner et al., 2003; Osman and Cavet, 2010).

Analysis of the genome sequence of Anabaena PCC 7120 (hereby referred as Anabaena 7120) revealed two SmtB-like repressors of the SmtB/ArsR family, namely, (a) AztR (Alr7621) and (b) AzuR (Alr0831) (Liu et al., 2005). AztR has been identified as a Zn\(^{2+}\)/Pb\(^{2+}\)/Cd\(^{2+}\)-responsive metalloregulator constituting a Zn\(^{2+}\)/Pb\(^{2+}\)/Cd\(^{2+}\) efflux operon (aztAR operon) regulating AztA, a Zn\(^{2+}\)/translocating CpxA-ATPase (Liu et al., 2004, 2005). However, presently, there is no experimental evidence toward the functionality and regulation of the other repressor, AzuR in Anabaena 7120, that shares 60% identity with SmtB (Figure 1A).

Previously, we had identified and characterized a MT from the heterocystous, filamentous cyanobacterium Anabaena 7120 (also belonging to the BmtA family) referred to as NmtA. Overexpression of NmtA in its native strain conferred tolerance to cadmium stress (Divya et al., 2018). We had observed increased abundance of the nmtA transcripts in the presence of elevated concentrations of metal ions like Zn\(^{2+}\), Cu\(^{2+}\), and Cd\(^{2+}\) (Divya et al., 2018), indicating transcriptional regulation of nmtA expression. It is proposed that the expression of the proteins associated with metal homeostasis is largely regulated at the transcriptional level in bacteria (Finney and O’Halloran, 2003). It is, therefore, worthwhile to explore whether AzuR, which is an SmtB-like repressor, has any role in the regulation of NmtA expression in Anabaena 7120.

The present study provides a comprehensive characterization of Anabaena AzuR (Alr0831). We show here that AzuR indeed binds to the upstream region of the mntA open reading frame (ORF). DNA binding was repressed in the presence of various divalent metal ions, indicating a negative regulation of mntA expression by AzuR. Our results showed that overexpression of azuR in Anabaena enhanced the susceptibility of the recombinant strain to cadmium stress significantly. The present investigation advances our understanding of the mechanisms of metal-regulated gene expression in the nitrogen-fixing cyanobacterium Anabaena 7120.

MATERIALS AND METHODS

Organism and Growth Conditions

Anabaena 7120 cultures were grown in BG-11 liquid medium, pH 7.2, with combined nitrogen (17 mM NaNO\(_3\)) under continuous illumination (30 \(\mu\)E m\(^{-2}\) s\(^{-1}\) without or with shaking (100 rpm) at 27°C ± 2°C (Allen, 1968). Escherichia coli cultures were grown in Luria–Bertani (LB) medium at 37°C (DH5\(\alpha\), HB101) or 30°C (SHuffle) with shaking at 120 rpm. The neomycin antibiotic was used for recombinant Anabaena cultures in BG-11 liquid medium (15 \(\mu\)g ml\(^{-1}\)) or BG-11 agar plates (25 \(\mu\)g ml\(^{-1}\)), whereas chloramphenicol (34 \(\mu\)g ml\(^{-1}\)) or carbenicillin (100 \(\mu\)g ml\(^{-1}\)) was used for E. coli cultures. Primers, plasmids, E. coli, and Anabaena strains used in this study are listed in Table 1.

Bioinformatic Analysis

Alignment of DNA and protein sequences was determined using ClustalW (Thompson et al., 1994) and Clustal Omega (Madeira et al., 2019), respectively. Jalview was used to visualize and edit aligned protein sequences (Waterhouse et al., 2009). A phylogenetic tree was constructed using MEGA version X (Kumar et al., 2018) by the maximum likelihood method. The I-TASSER software was used to predict the tertiary structure of AzuR and metal-binding residues (Zhang, 2008; Roy et al., 2010). Pattern search analysis of conserved sequences was carried out using the online tool Pattern Locator (Mrázek and Xie, 2006). The –10 and –35 boxes of the upstream region of nmtA were predicted from BPROM (Salamov and Solovyevand, 2011).

Cloning, Expression, and Purification of AzuR

The azuR ORF (363 bp) was PCR amplified from Anabaena 7120 genomic DNA and cloned into pTwin1 vector at NdeI–SapI sites.
The resulting construct pTwinazuR was confirmed by sequencing and transformed into an E. coli SHuffle strain. Overexpression of chitin-binding domain (CBD)-tagged AzuR was induced by the addition of 0.5 mM IPTG. The protein purification was carried out by chitin affinity chromatography as per the manufacturer’s protocol (New England Biolabs). The protein was cleaved from its tag and eluted following incubation with 40 mM DTT at 4°C for 3 days. CBD was also eluted as the contaminating protein. This eluate was loaded onto the fresh chitin resin after DTT removal. The flow-through was collected, which contained purified Anabaena AzuR without CBD. The purified protein band following electrophoresis on 15% SDS-PAGE was excised and processed for LC-MS/MS analysis (Q Exactive Plus BioPharma High-Resolution Orbitrap MS system, Thermo Fischer Scientific) at the Sophisticated Analytical Instrument Facility (SAIF), IIT Bombay, India. Spectrum was acquired in positive ion mode in a mass range from 350 to 2,000 m/z. The resultant spectrum was used for peptide identification using the Anabaena 7120 protein database available at UniProt.

Structural Characterization of AzuR

Determination of the oligomeric status of AzuR was done by glutaraldehyde cross-linking of protein in the native state. Purified AzuR was incubated with 10 mM glutaraldehyde at room temperature (RT) for 10–15 min in 10 mM Tris, pH 7.5. The resulting cross-linked protein was analyzed by 15% SDS-PAGE. The native molecular mass of AzuR was determined by size-exclusion chromatography (AKTA FPLC system, GE Healthcare) using the GE Superdex 75 column equilibrated with 20 mM Tris, 100 mM NaCl, pH 7.5 at 25°C at a flow rate of 0.5 ml min⁻¹. The column was previously calibrated using a set of gel filtration markers [bovine serum albumin (66 kDa), ovalbumin (44 kDa), carbonic anhydrase (44.3 kDa), and cytochrome c (29 kDa)] (GE Healthcare).

Analysis of the secondary structure of AzuR was performed by circular dichroism (CD) spectroscopy (MOS-500 Biologic CD spectrometer equipped with a Peltier-type thermostatic cell holder) at 25°C. The CD spectrum was recorded in the wavelength range of 200–260 nm using a cuvette with a path length cell of 0.1 mm. The alpha helical content was calculated using the online tool K2D2 (Perez-Iratxeta and Andrade-Navarro, 2008). CD spectra were also recorded for titrations of AzuR with increasing concentrations of zinc (molar equivalents ranging from 1 to 10).

Rapid Amplification of cDNA Ends

Total RNA was isolated from Anabaena 7120 treated with 10 µM cadmium for 1 h as described earlier (Divya et al., 2018). cDNA was synthesized with 0.5 µg of total RNA using ReadyScript cDNA Synthesis Mix (Sigma-Aldrich). Following dA tailing of
cDNA by terminal transferase (Roche), PCR was performed with the oligo(dT)-anchor primer and nmtA primer as listed in Table 1. The PCR product was then sequenced.

Electrophoretic Mobility Shift Assay

The putative promoter region (100 bp DNA sequence upstream of nmtA ORF) was PCR amplified (primers listed in Table 1) and end-labeled with DIG-ddUTP as per manufacturer’s instructions (Roche). Two nanograms of a DIG-labeled probe (P

Was performed with 100 ng of AzuR (360 nM) with either 20 ng of P

For protein specificity, 20 ng of P

EMSAs were also carried out in the presence of 1 mM DTT (Erbe et al., 1995) for reactions containing all the aforesaid metals.

Overexpression of AzuR in Anabaena 7120

Overexpression of azuR gene in its native strain was achieved by triparental conjugation (Divya et al., 2018). The azuR gene was cloned downstream to the light-inducible psbA promoter in the pFPN vector at Ndel and BamH1 sites. A SalI–Xmal fragment from pFPNazuR was excised and cloned into the

| Primer | Description | References |
|--------|-------------|------------|
| nmtA Rev | CCGGGATCCCTTAAACGCGCAAGCCCCAGTATG | Divya et al., 2018 |
| azuR_pTwinC Fwd | GGTGGTCATAGTATTAATAAATCACAATTTGTTAC | This study |
| azuR_pTwinC Rev | GGTGGTCATAGTATTAATAAATCACAATTTGTTAC | This study |
| azuR_Ndel Fwd | GGAATCCATATAGTATTAATAAATCACAATTTGTTAC | This study |
| azuR_BamHI Rev | CCGGATCCCTTAAACGCGCAAGCCCCAGTATG | This study |
| Prom_Fwd | ATATTTCCTCGGGTTCCTTGT | This study |
| Prom_Rev | GAAAGTTTAAATAACGCTTGGAT | This study |
| Oligo(dT) anchor primer | GACCGGCGTATGATGCTGACTTTTTTTTTTTTTTT | 5’3’ RACE kit, Roche |
| 16S Fwd | CACACTGCGACTGAGACAC | Pinto et al., 2012 |
| 16S Rev | CTGCTGGCAGGAGTTAG | |

## Plasmid

| Plasmid | Description | References |
|---------|-------------|------------|
| pTwin | Expression vector resulting in protein fusion with CBD and cleavable intern tag, CbR | NEB |
| pTwinazuR | 360 bp azuR fragment cloned in pTwin vector | This study |
| pFPN | CbR, KanR, integrative expression vector | Chaurasia et al., 2008 |
| pAM1956 | KanR, promoterless gfpmut1 reporter gene | Yoon and Golden, 1998 |
| pFPNazuR | 360 bp azuR fragment cloned in pFPN | This study |
| pAMpsbA | Xmal-SalI fragment from pFPN cloned in pAM1956 vector | Divya et al., 2018 |
| pAMazuR | Xmal-SalI fragment from pFPNazuR cloned in pAM1956 | |
| pAMnmtA | Xmal-SalI fragment from pFPNnmtA cloned in pAM1956 vector | |

## E. coli strain

| E. coli strain | Description | References |
|----------------|-------------|------------|
| DH10A | F− recA141 endA1 gyrA96 thr-1 hsdR17 (d− r− m−) supE44 relA lacU169 | Lab collection |
| BL21(DE3)pLysS | F−ompT gal dcm lon hsdS30 (rB− mB−) λDE3 | Lab collection |
| HB101 | F− mcrB mrr hsdS20 (rB− mB−) recA138lexB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 psL20 (SmR) λlacU44 λ− | Lab collection |
| HB101R2 | Donor strain carrying pRL623 (encoding methylase) and pRL443 (conjugal plasmid) | Elhai et al., 1997 |
| Shuffle T7 Express lysY | MinF lysY (CamR) hsdR142 lacZ77 gene1 [lom ompT ahpC gal lac-thrNEB3-r1-cDsbC (SpecR, lacIq) ΔtnxB suA11 R(mcr-73::minTn10–TesS)] dcm Δlgr Δn114::IS10 | NEB |

## Anabaena strain

| Anabaena PCC 7120 | Wild-type strain | Lab collection |
| AnpsbA+ | Anabaena 7120 harboring light inducible promoter psbA from pFPN, NmR | This study |
| AnazuR+ | Anabaena 7120 harboring pAMazuR, NmR | This study |
| AnnmtA+ | Anabaena 7120 harboring pAMnmtA, NmR | This study |
E. coli/Anabaena shuttle vector pAM1956 upstream of the promoterless gfpmut2 gene. pAMazuR was then transferred into Anabaena 7120. The recombinant Anabaena strain was designated as AnazuR⁺. In a similar way, AnmmtA⁺ (Anabaena strain overexpressing NmtA) was also generated. ApnpsbA⁺ (Anabaena harboring Pam1956 with constitutive expression of GFP) was generated by excising the Pₜₚ₃₈₁ fragment from the pFPN vector and cloning it into the vector pAM1956 upstream of the promoterless gfpmut2 gene and transferred conjugally into Anabaena 7120. The recombinant Anabaena strains were repeatedly subcultured and maintained under the selective pressure of neomycin (Nm¹⁵). Visualization of GFP fluorescence in the recombinant cells confirmed the expression of the azuR gene placed upstream of the gfpmut2 gene.

Transcript Analysis by RT-PCR
For RT-PCR, 1 µg RNA was used for cDNA synthesis (ReadyScript cDNA Synthesis Mix, Sigma-Aldrich). RT-PCR was carried out with azaR-specific primers (Table 1) with 16S rRNA serving as the internal control. RT-PCR products were resolved by electrophoresis on 1% agarose gel and detected by staining with ethidium bromide. For quantification of mmtA transcripts, real-time PCR was performed with mmtA-specific primers in QIagen rotor-Gene Q real-time PCR cyclers. 16S rRNA was used as the internal control.

Cadmium Exposure Studies
Exponential phase cultures (3-day-old cultures) of AnpsbA⁺, AnmmtA⁺, and AnazuR⁺ were inoculated in BG-11 N⁺ (Nm¹⁵) liquid medium at a chlorophyll a (Chla) density of ~4 µg ml⁻¹ and incubated for 10 days under illumination without or with cadmium at 10 and 20 µM concentrations. Growth was assessed by measuring Chla content at regular intervals. For spot assays, exponentially growing cultures of AnpsbA⁺, AnmmtA⁺, and AnazuR⁺ were spotted onto BG-11 N⁺ (Nm²⁵) agar plates without or with cadmium (10, 20, and 40 µM) at the chlorophyll density mentioned in the figure and incubated under continuous illumination for 7 days.

Microscopy of Anabaena Strains
Bright-light and fluorescence microscopy (FM) images were taken at ×600/×1,500 magnification on a Carl Zeiss Axioscope 40 microscope with a charge coupled device (CCD) AxioCam MRc camera (Zeiss). Green fluorescence of GFP was visualized using a Hg-arc lamp (excitation BP: 450–490 nm, emission LP: 515 nm). Chla fluorescence of Anabaena was visualized with green light excitation (excitation BP: 546/12, emission LP: 590 nm). It should be noted here that the microscopic settings for GFP fluorescence used the emission filter (λ_emission: 515 nm) that could detect both GFP and Chla fluorescence. For scanning electron microscopy (SEM), exponential-phase cells of WT, AnpsbA⁺, AnmmtA⁺, and AnazuR⁺ were harvested by centrifugation, and the resulting cell pellets were washed with 0.9% NaCl and fixed with 2.5% glutaraldehyde at 4°C for 1–2 h. Post fixation, the cells were serially dehydrated in 20, 30, 50, 70, 90, and 100% ethanol. The dehydrated sample was then gold coated with a sputtering device (Q 150R ES, Quorum) and visualized using SEM (EVO 18 Research, Carl Zeiss, United Kingdom).

Statistical Analysis
Growth experiments were repeated three times. Average values with standard deviations are shown for a representative experiment. For determination of cell size, data are represented as average values ± standard deviation. One-way ANOVA was employed for calculating the significance of the difference in cell size between WT, AnpsbA⁺, AnmmtA⁺, and AnazuR⁺ cultures.

RESULTS AND DISCUSSION
Sequence Analysis and Genomic Context of AzuR (Alr0831)
The genome of Anabaena PCC 7120 harbors two proteins belonging to the ArsR-SmtB family of proteins, Alr7621 and Alr0831. The ArsR-SmtB family of transcriptional metalloregulators represses the expression of genes/operons involved in maintaining metal homeostasis or toxic metal detoxification (Osman and Cavet, 2010). Among the 15 characterized metal binding motifs (Saha et al., 2017), the metal-sensing members of the regulators include two structurally diverse metal-binding sites, namely, α3N, and α5 (Busenlehner et al., 2003). Alr7621 in Anabaena 7120 encodes for AztR, a regulator of AztA [Zn(II)/Pb(II) CPx-ATPase efflux pump] (Liu et al., 2005), and belongs to the α3N group of proteins. The α3N site consists of cysteine thiolate ligands—two from the α3 helix with signature motifs Cx₁₋₂C or Cx₂GD and one or two cysteine ligands derived from the amino-terminus (Saha et al., 2017). The sequence analysis of the yet-uncharacterized Alr0831 (AzuR) revealed the absence of a functional α3N site in AzuR as it contained only one cysteine residue each in the α3 helix and at the amino-terminus (Figure 1A). Protein sequence alignment of AzuR with the Synechococcus transcriptional repressor SmtB showed 60% sequence identity, and the key amino acids in the α5 site important for metal sensing, i.e., His, Gln, and Asp in SmtB (VanZile et al., 2000, 2002), were found to be conserved in AzuR (Figure 1A). It is likely that the function of AzuR is similar to that of SmtB owing to the high degree of sequence identity. Tertiary structure prediction of AzuR using the software I-TASSER showed the presence of all the secondary structural folds (α₁–α₂, β₁, and β₂) similar to that of SmtB (Figure 1B, i). Structural modeling predicted zinc-binding residues Asp102 and His104 (Figure 1B, ii) of AzuR comparable to that of Staphylococcus aureus CadC as well as His115 and Gln118 (Figure 1B, iii) similar to that of Synechococcus SmtB. Hence, AzuR could possibly be grouped into α5 SmtB/ArsR metalloregulators with the signature motif Dhx₁₀Hx₂E present in the α5 helix (Figure 1A). Phylogenetic analysis of representative sequences from SmtB/ArsR family members showed that AzuR shared maximum identity to BxmR (67%), which contain both α3N and α5 sites (Figure 1C). It also showed that SmtB (α5) and proteins belonging to different groups—ZiaR (α3N, α5), AztR (α3N), BxmR (α3N, α5), and AzuR (α5)—evolved independently but were linked to a common ancestor (Figure 1C).
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Metallothionein Regulation in Anabaena

Several metal-responsive proteins and their repressors of SmtB/ArsR family members have been shown to exist as operons. For example, BmtA (MT of Oscillatoria brevis) and its repressor BxmR (Liu et al., 2004), ZiaA (Zn efflux protein of Synechocystis PCC 6803) and its repressor ZiaR (Thelwell et al., 1998), and AztA (Zn\(^{2+}\)-translocating CPx-ATPase) and its repressor AztR (Liu et al., 2005) are organized in operons. In Synechococcus PCC 7942, the smtB gene and smtA gene are separated by 100 bp, forming a divergon (Huckle et al., 1993). However, there is a deviation in the genetic organization of Anabaena MT, which is not organized in an operon. The nmtA ORF (located between positions 3938083 and 3937925) is present within a larger ORF of an unknown protein, asr3266, but in the opposite orientation (Bose et al., 2006). Similarly, the putative regulator azuR is not placed adjacent to the nmtA locus but is present within the ZnuABC operon (Figure 1D). Alr0831 is positioned at 956795→957157 between alr0830 (ZnuC, ABC transporter permease protein) and alr0832 (ZnuA, ABC transporter ATP binding protein) in the opposite orientation. Similar to AzuR, the SmtB ortholog has been identified within an operon with an ABC-type transporter system in other cyanobacteria like Nodularia and Anabaena variabilis (Blindauer, 2008). Analysis of the genomic organization of other prokaryotic MTs like Pseudomonas MT also revealed an absence of regulatory protein adjacent to the Pseudomonas fluorescens Q2-87 MT locus. Also, the genes adjacent to the Pseudomonas MT gene code for proteins of unknown function (Habjanić et al., 2020). Genomic arrangement of MT and its regulator as operons apparently is not mandatory as such regulators function as trans-acting factors on cis-regulatory elements.

Overexpression, Purification, and Structural Characterization of AzuR

To characterize the regulatory role of AzuR, the corresponding gene (alr0831) was cloned in the pTwin1 vector. The resulting construct pTwinazuR was expressed in the E. coli SHuffle strain. Induction with IPTG expressed a ∼42 kDa protein corresponding to CBD-tagged AzuR (Figure 2A). The cloning at NdeI–SapI sites ensured that no extra amino acids were incorporated in the purified protein following removal of the tag. AzuR was purified by chitin affinity chromatography, and the removal of the CBD tag was achieved by thiol-induced cleavage with 40 mM DTT at 4°C. The purified AzuR was visualized on SDS-PAGE as a monomer under reducing conditions with a molecular weight of ∼13.9 kDa (Figure 2B), which was further confirmed with LC-MS/MS analysis. The MS analysis identified

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six unique peptides, showing 77% coverage of the *Anabaena* AzuR protein sequence.

The SmtB/ArsR family of proteins binds to the regulatory DNA sequences as homodimers (Osman and Cavet, 2010). To ascertain the native form of AzuR, the oligomeric status of AzuR was evaluated by glutaraldehyde cross-linking. The protein was predominantly found to be present in the dimeric state as observed by glutaraldehyde cross-linking (Figure 2C). The dimeric state was also confirmed with size-exclusion chromatography (Figure 2D). This is in agreement with the previously characterized SmtB/ArsR family of prokaryotic metalloregulatory transcriptional repressors that existed as stable dimers in solution (Busenlehner et al., 2001; Liu et al., 2005, 2008). It was observed that AzuR existed as a monomer under reducing conditions and dimer under non-reducing conditions (Figure 2C). These observations suggested the involvement of cysteine residues in AzuR dimerization. Secondary structure analysis by CD showed that AzuR is composed of 67.5% α helical content (Figure 2E), suggesting that the purified recombinant AzuR protein was properly folded. This is in agreement with the theoretical secondary structure prediction of AzuR using the SOPMA software, which projected 67% α helical content followed by 17% random coil and 11% extended strand.

**Mapping and Characterization of AzuR-DNA Binding Sequence**

The SmtB/ArsR family of transcriptional regulators binds to 12–2–12 inverted repeats present upstream or within the genes that they regulate (Erbe et al., 1995; Turner et al., 1996). RACE analysis with total RNA isolated from the cadmium-treated (IC₅₀ 10 µM) *Anabaena* 7120 showed an ∼200 bp cDNA product (Figure 3A). Sequence analysis of the product identified the transcriptional start site (TSS) to be at 23 nt upstream of the translational start of the nmtA ORF (Figure 3B). The palindromic sequence (12–2–12 imperfect inverted repeat), corresponding to the consensus of the α3N and α5 groups of SmtB/ArsR-binding sites (Saha et al., 2017), was found to be located 36 nt upstream of the nmtA translation start site (Figure 3B). Its position overlaps with the theoretical prediction of the −35 element of the promoter. It is shown that the cis-regulatory element of metal-inducible operons is composed of one or two inverted 12–2–12 repeats present in the vicinity or overlapping the transcriptional start site of the gene under regulation. For example, one of the two such inverted 12–2–12 repeats found in *Synechococcus* 7942 was essential for the regulation of smtA expression by its repressor, SmtB (Turner et al., 1996). Similarly, the *Synechocystis zia* O/P region has a single 12–2–12 inverted repeat between the −10 box and the translational start site of ziaA, which is regulated by a divergently transcribed repressor, ziaR (Thelwell et al., 1998). Pattern search analysis was performed with the conserved bases in the 12–2–12 imperfect repeat along the entire *Anabaena* 7120 genome. Similar repeats were found at sites upstream and within other genes that include all1178, which codes for a two-component hybrid sensor and regulator, alr7622 (also designated as aztA), encoding for cation-transporting ATPase and other hypothetical proteins (Table 2). The conserved 12–2–12 inverted repeat of SmtB/ArsR-regulated O/Ps are shown in Figure 3C. Although nmtA and its putative regulator azuR do not constitute an operon in *Anabaena* 7120,
the inverted 12–2–12 imperfect repeat could be located at the appropriate upstream distance from the nmtA translation start site. Although the azuR ORF is present within the znuABC operon, a detailed search for conserved bases in the 12–2–12 imperfect repeat following global search analysis by PATLOC in the close vicinity of the znuABC operon (corresponding to the 500 bp upstream region to 500 bp downstream of the operon) and within the operon did not show any such repeat.

**TABLE 2 |** Anabaena genes possessing conserved sequences in the 12–2–12 inverted repeat identified by PATLOC.

| S. No. | Inverted repeat | Position | Gene and distance |
|--------|-----------------|----------|------------------|
| Chromosome | | | |
| 1. * | AATACTTGAGTA-AT-TTATCAAGTTCT | 1386159–1386184 | all1178 (two-component hybrid sensor and regulator) (−); 31–2429 |
| 2. | AATAACCTGAACA-GA-TGTTCAAGTATT | 3938119–3938144 | asr3266 (hypothetical protein) (−); 10 |
| 3. * | CACAATTGATGA-TA-TCTTCTAGTGGG | 4556777–4566802 | alr3769 (hypothetical protein) (−); 314–383 |
| 4. | TAAATGATGA- TA-TACCATCATTTA | 5585215–5585240 | alr4684 (hypothetical protein) (−); 291 |
| Alpha plasmid | | | |
| 5. | GAAAACTGAGTA-AT-TTATCAATTGCT | 40552–40577 | asr7047 (hypothetical protein) (−); −12 |
| Beta plasmid | | | |
| 6. * | TACAATTGAATA-GT-TGTTCAATTGTT | 114477–114502 | alr7622 (cation-transporting ATPase) (−); 13–2601 |
| 7. * | GAAATTTGAAAA-CT-TCCTCACCTCAA | 153412–153437 | alr7649 (hypothetical protein) (−); 5492–2228 |

*Denotes repeat sequence present within the gene. Arrows represent transcription direction.

**FIGURE 4 |** Binding of AzuR to the upstream region of nmtA. (A) Electrophoretic mobility shift assay (EMSA) of DIG-labeled 100 bp DNA sequence upstream of nmtA (2 ng) with purified AzuR. Different concentrations of AzuR protein were incubated with DIG-labeled DNA, and the assay mixtures were resolved on 10% native PAGE in 0.5× TBE. Detection with the DIG-labeled probe was carried out as per manufacturer’s protocol (Roche) using NBT-BCIP. Lane 1 contains 2 ng of P<sub>nmtA</sub>. Lanes 2–8 contain increasing concentrations of AzuR as indicated. Representative data from three independent experiments are shown. (B) Representative plot showing the percentage of bound complex against the concentration of AzuR protein fitted to the Hill equation. (C) EMSA for evaluation of non-specific interaction of DNA–protein binding. Lane 1: 100 bp DNA ladder. Lane 2: 20 ng of 100 bp P<sub>nmtA</sub> only or with 360 nM (100 ng) AzuR (lane 3) or 360 nM (478 ng) BSA (lane 4) or 360 nM (41 ng) NmtA (lane 5) or 360 nM (158 ng) AnLexA (lane 6). Lane 7 contains 20 ng of the nmtA gene only or with 360 nM (100 ng) AzuR (lane 8). The DNA–protein complexes were resolved on 10% native PAGE and visualized by ethidium bromide staining. (D) The CD spectrum of AzuR was recorded with increasing molar equivalents of zinc, which was an average of three scans. (E) EMSA of P<sub>nmtA</sub> with AzuR in the presence of Zn<sup>2+</sup>. Lane 1 contains 2 ng of P<sub>nmtA</sub>. Lanes 2–7 contain DNA with 100 ng of AzuR in the presence of increasing concentrations of Zn<sup>2+</sup> as indicated. EMSA of P<sub>nmtA</sub> with AzuR protein with Zn<sup>2+</sup> and Cd<sup>2+</sup> in the absence of DTT (F) or in the presence of 1 mM DTT (G). Lane 1 contains 2 ng of probe only. Lanes 2–8 contain P<sub>nmtA</sub> with 100 ng of AzuR, lanes 3–5 with increasing concentrations of Zn<sup>2+</sup>, and lanes 6–8 with increasing concentrations of Cd<sup>2+</sup> as indicated in (F,G). (H) EMSA of DNA probe with 70 ng of AzuR with 100 µM of divalent cations as indicated in the figure. All the reactions were performed in the absence of DTT.
sequence in the entire analyzed region. The regulation of the znuABC operon by the zur (all2473)/furB regulator has been demonstrated previously in *Anabaena* 7120 (Napolitano et al., 2012). Zur (zinc uptake regulator), known to be the master regulator for zinc homeostasis in *Anabaena* 7120, regulated the expression of genes involved in zinc homeostasis like *alr0830* (ZnuC), *alr0833* (ZnuA), and *all7621* (AztR). On analysis, we did not find zur-binding sequences upstream of the azuR ORF, indicating that the global regulator of zinc homeostasis, Zur, did not regulate azuR expression.

*Anabaena* 7120 AztA is transcriptionally regulated by AztR (belonging to the SmtB/ArsR family) by recognizing and binding to the inverted 12–2–12 imperfect repeat region. EMSA studies done with AztR and the nmtA/bmtA upstream region showed its binding *in vitro* (Tottey et al., 2007). Similar inverted repeat sequences identified by AztR and AzuR indicate that AztR and AzuR might be sharing the function of regulating AztA and NmtA. As described above, AztR belongs to the α3N group and AzuR to the α5 group of the SmtB/ArsR family. The α5 group members sense physiologically important metals like Zn$^{2+}$, Cu$^{2+}$, Co$^{2+}$, and Ni$^{2+}$, while the α3N group prefers larger, more thiophilic metal ions like Cd$^{2+}$ or Pb$^{2+}$ (Busenlehner et al., 2003). It is possible that AzuR and AztR preferred different groups of metal ions but could regulate both MT and efflux proteins, thus enabling the cell to respond to a wide range of metal ions.

### AzuR Binds to the Upstream Sequence of nmtA Open Reading Frame

Electrophoretic mobility shift assays (EMSA) were done in order to identify the AzuR-DNA binding site using a 100 bp fragment (P$nmtA$) upstream of the nmtA gene (probe) containing the 12–2–12 inverted repeat sequence. The results showed that AzuR could bind and form complexes with P$nmtA$ in a concentration-dependent manner (**Figure 4A**). The Hill coefficient of AzuR binding to DNA was calculated to be 2.48 ± 1.14 (>1) (**Figure 4B**), which indicated positive cooperative binding (Hill, 1910). SmtB has been shown to bind to the smt O/P in a multimeric state (Erbe et al., 1995). The positive cooperative binding suggested that AzuR bound to the target DNA as an oligomer similar to that of SmtB. The specificity of DNA–protein binding was confirmed by using the nmtA gene or DNA-binding protein LexA from *Anabaena* 7120 (AnLexA) or other proteins like BSA and NmtA. No retardation in the mobility of P$nmtA$ was observed in the presence of AnLexA. Also, AzuR could not bind to the nmtA gene sequence, confirming that AzuR regulated nmtA expression by binding to the upstream sequence and not to its internal region (**Figure 4C**). Our results established the specific binding of P$nmtA$ with the AzuR protein.

SmtB senses metal ions through the α5 site. Zinc binding to residues present in this site allosterically regulates the DNA

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**FIGURE 5** | Overexpression of AzuR in *Anabaena* 7120. (A) Effect of AzuR overexpression on the morphology of *Anabaena* 7120. Bright-field (BF) and FM photomicrographs under blue light excitation (BLE) (excitation 470 nm, emission 508 nm) and green light excitation (GLE) (excitation 520 nm, emission 680 nm) at ×1,500 magnification and scanning electron micrographs (SEMs) at ×100,000 magnification of WT, AnpsbA$^+$, AnmntA$^+$, and AnazuR$^+$.

Non-uniformity of cell stacking in AnazuR$^+$ filament is indicated by red arrows in BF micrographs. (B) Confirmation of overexpression of azuR transcripts by RT-PCR. Total RNA of 1 µg was used for cDNA synthesis, which served as a template for PCR performed with azuR-specific primers. The amplified products were resolved on 1% agarose gel and visualized by ethidium bromide staining. The lower panel represents the products of 16S rRNA used as control. (C) Plot of average cell size of WT, AnpsbA$^+$, AnmntA$^+$, and AnazuR$^+$ as analyzed by SEM is presented. One-way ANOVA was employed for calculating significance of the difference. Data shown here represent mean ± standard deviation (n = 13), ns, non-significant.
binding activity of SmtB to the smtA O/P region (VanZile et al., 2002) similar to other reported SmtB/ArsR repressors (Busenlehner et al., 2003). The bound Zn\(^{2+}\) changes the conformation of the protein, which inhibits the DNA binding. Since AzuR contains a similar \(\alpha_5\) site, the conformational changes in AzuR as a result of metal binding was assessed by CD spectra of the protein in the presence of various concentrations of zinc (Figure 4D). The degree of the alpha helical region progressively decreased with increasing concentrations of zinc, indicating the changes in the secondary structure of AzuR in the presence of zinc. To further confirm whether zinc or other metal ions interfered with the AzuR DNA binding ability, EMSA was carried out in the presence of various metal ions. Dissociation of the DNA–AzuR complex was clearly evident with increasing concentrations of Zn\(^{2+}\) (Figures 4E,F). The interaction of Cd\(^{2+}\) with AzuR also disrupted the binding with \(P_{nmtA}\) (Figure 4F); however, the disruption was more prominent in the presence of DTT (Figure 4G), emphasizing the requirement of free sulphydryls for Cd\(^{2+}\) binding to AzuR \textit{in vitro}. It was interesting to see the reversal of AzuR binding to \(P_{nmtA}\) in the presence of other divalent metal ions like Cu\(^{2+}\), Co\(^{2+}\), Ni\(^{2+}\), Pb\(^{2+}\), and Mn\(^{2+}\) (Figure 4H), suggesting that AzuR not only senses toxic metal ions like Cd\(^{2+}\) and Pb\(^{2+}\) but also is capable of sensing essential metal ions like Zn\(^{2+}\), Cu\(^{2+}\), Co\(^{2+}\), Ni\(^{2+}\), and Mn\(^{2+}\). EMSAs attempted with metals other than Zn\(^{2+}\) and Cd\(^{2+}\) in the presence of DTT showed visible precipitates in the binding reaction and hence were not included here.

We have previously observed the induction of \(nmtA\) in the presence of Cd\(^{2+}\), Zn\(^{2+}\), and Cu\(^{2+}\) (Divya et al., 2018). AzuR, therefore, can be proposed as a negative regulator of \(nmtA\) as it binds to regulatory DNA sequence in the absence of the metals and the repression is relieved in the presence of metal ions. In view of our results, it can be suggested that AzuR might have a larger role in the metal resistance system of \textit{Anabaena} 7120.

![FIGURE 6](image_url)

**FIGURE 6** | Effect of AzuR overexpression on cadmium exposure. (A) Spot assays of AnpsbA\(^{-}\), AnnmtA\(^{-}\), and AnazuR\(^{-}\) following exposure to cadmium stress for 7 days. The cell densities are indicated in terms of Chl a content (\(\mu g\)). (B) Growth kinetics of AnpsbA\(^{-}\), AnnmtA\(^{-}\), and AnazuR\(^{-}\) as assessed by contents of Chl a. (C) The recombinant cultures were exposed to 10 or 20 \(\mu M\) cadmium for 10 days, and subsequently, the cultures were transferred to 12-well microtiter plate and photographed. (D) BF and FM microphotographs under BLE (excitation 470 nm, emission 508 nm) and GLE (excitation 520 nm, emission 680 nm) at \(x600\) magnification of AnpsbA\(^{-}\), AnnmtA\(^{-}\), and AnazuR\(^{-}\) cells after 10 days of cadmium exposure.
Overexpression of Anabaena AzuR (Alr0831) and the Alterations in the Cell Morphology

Overexpression of transcriptional regulators has been previously studied in Anabaena sp. (Wu et al., 2007). To gain insights into the effect of AzuR on various characteristics or phenotype of Anabaena 7120, we constructed a recombinant strain of Anabaena 7120 that overexpressed AzuR. The azuR gene was cloned and overexpressed constitutively in Anabaena 7120 from a strong light-inducible promoter, PpsbA. GFP fluorescence of the downstream reporter gene was the first indication of successful azuR gene expression (Figure 5A). GFP fluorescence was visualized in Anabaena harboring an empty vector with PpsbA upstream of the gfpmut2 gene, AnpsbA+, and Anabaena overexpressing nmtA, and Annmta+ (Figure 5A). WT cells did not show any such GFP fluorescence (Figure 5A). The observation of few cells appearing red in the filaments of recombinant cells under FM and blue-light excitation (BLE) conditions could be due to partial or reduced GFP expression (Figure 5A). The filament length in AnazuR+, AnpsbA+, and Annmta+ was comparable to that of WT Anabaena cells. The uniformity of the cell stacking in AnazuR+ filaments appeared to be compromised as compared to those in the filaments of WT, AnpsbA+, and Annmta+. However, the Chla fluorescence in AnazuR+ cells was intact and equivalent to that observed for WT, AnpsbA+, or Annmta+ cells (Figure 5A). A substantial increase in azuR transcript level was seen in RT-PCR performed with RNA isolated from AnazuR+ as compared to AnpsbA+ and Annmta+, thus confirming the overexpression of the regulator in vivo (Figure 5B).

Scanning electron microscopy (SEM) analysis of exponential-phase cells of AnazuR+ revealed a significant decrease in cell size with the cells showing spherical and globular morphology in contrast to AnpsbA+, Annmta+, and WT cells (Figure 5A). The average cell size of AnazuR+ cells was found to be 2.70 ± 0.26 µm as compared to 3.92 ± 0.50 µm for AnpsbA+ and 3.67 ± 0.34 µm for Annmta+. The cell size of AnazuR+ was lesser than the WT cells (3.214 ± 0.34 µm) (Figure 5C). Similar morphological changes regarding cell stacking and cell size were observed following overexpression of the global transcriptional regulator FurA in Anabaena 7120 (González et al., 2010). The elongated cell phenotype seen in AnpsbA+ and Annmta+ cells could be because of stress owing to neomycin and heterologous GFP overexpression. The gross morphological changes in AnazuR+ as compared to the empty vector AnpsbA+ indicate the possible involvement of AzuR in the regulation of genes involved in functions other than metal homeostasis. Chromatin immunoprecipitation (ChIP) studies need to be done in the future to identify direct binding of targets of AzuR in the Anabaena genome.

AzuR Overexpression Renders Anabaena 7120 Sensitive to Cadmium Stress

DNA binding studies by EMSA showed that AzuR bound to the upstream region of the nmtA ORF in vitro. Evaluation of nmtA expression levels in AnazuR+ by qRT-PCR with 16S rRNA as internal control showed the downregulation of nmtA expression in AnazuR+ by ~32-fold as compared to its empty vector AnpsbA+. These results are in agreement with the negative regulation of nmtA transcription by AzuR in vivo.

Previously, overexpression of NmtA in Anabaena 7120 had conferred tolerance to cadmium stress (Divya et al., 2018). Since the negative regulation of nmtA transcription by AzuR was observed here, we were interested to see the effect of the overexpression of AzuR on the cadmium tolerance ability of Anabaena 7120. We compared the response of cadmium stress in AnazuR+, AnpsbA+, and Annmta+ cultures. Spot assays showed increased sensitivity of AnazuR+ cells to cadmium stress following 7 days of exposure (Figure 6A). Growth of AnazuR+ assessed in terms of Chla content showed a substantial decrease even at concentrations of 10 µM cadmium as compared to AnpsbA+ (Figure 6B). Growth kinetics studies in the presence of 20 µM cadmium resulted in almost complete bleaching of cultures of both AnpsbA+ and AnazuR+ after 10 days of exposure to the stress (Figure 6C) including extensive cell lysis in AnazuR+ culture (Figure 6D). In contrast, filaments of Annmta+ appeared intact, long, and healthy on exposure to cadmium (Figure 6D). The spot assays and growth studies assessed in terms of Chla contents (Figures 6A–C) of Annmta+ also supported the microscopy observations, which are in agreement with our previous results showing superior tolerance of Annmta+ against cadmium stress (Divya et al., 2018). The GFP and Chla fluorescence were found to be unaffected in Annmta+ similar to AnazuR+ and AnpsbA+ in the presence of cadmium.
of cadmium (Figure 6D). The toxic effects of cadmium on the photosynthetic machinery have been studied extensively in *Synechocystis* PCC 6803 (Tóth et al., 2012). The major proteins involved in photosynthetic machinery include zinc-containing enzymes like carbonic anhydrase and sulphydryl groups in ribulose-5-phosphate kinase among others that lose their activity by replacement with cadmium (Tóth et al., 2012). MTs play a key role in metal detoxification by directly binding to the toxic metal, which results in lesser bioavailability (Klaassen et al., 1999). This protects the essential metalloproteins from the toxic metal. Since AzuR overexpression leads to a decrease in basal nmtA expression, the protective role of NmtA in imparting cadmium tolerance could be obliterated, resulting in the susceptibility of AnazuR⁺ to cadmium stress, which was evident from its decreased growth and increased cell lysis. The susceptibility of AnazuR⁺ to cadmium stress confirms the negative regulation of nmtA expression at the physiological level in *Anabaena*.

**CONCLUSION**

We have characterized the role of AzuR belonging to the SmtB/ArsR family of metalloregulators in the regulation of *Anabaena* MT NmtA. The sequence analysis of AzuR (Alr0831) identified a distinct α5 metal binding site similar to that of SmtB. Although the *azuR* gene locus was found to be situated remotely away from the *nmtA* locus, analysis of the region upstream of the *nmtA* ORF identified the presence of 12–2–12 imperfect inverted repeats, which are reportedly important for binding of metalloregulators belonging to the SmtB/ArsR family of proteins. EMSAs showed AzuR binding with putative PpstmA, indicating that NmtA is a regulatory target of AzuR. Dissociation of the protein–DNA complex was observed not only in the presence of toxic metal ions like Cd²⁺ and Pb²⁺ but also in the presence of essential metal ions like Zn²⁺, Cu²⁺, Co²⁺, Ni²⁺, and Mn²⁺, which suggested negative regulation of metal-inducible *nmtA* expression by AzuR. On the basis of our findings, we propose a model for *Anabaena* NmtA regulation by AzuR (Figure 7). In the absence of metals or basal conditions, the binding of AzuR to the upstream region of the *nmtA* ORF blocks the binding site for the RNA polymerase transcription initiation complex, resulting in the repression of *nmtA*. At elevated concentrations of the metals, the binding of AzuR to DNA is disrupted as a result of conformational changes in the protein resulting from metal binding. This leads to the induction of *nmtA* transcription in the presence of metals as seen earlier in our studies (Divya et al., 2018). The sensing of a large number of metal ions implies a greater role of AzuR in the modulation of metal ions in the intracellular environment in *Anabaena* 7120.

Although we have largely focused on the role of AzuR in MT regulation, the presence of cis-regulatory elements important for repressor binding at several locations in the *Anabaena* 7120 genome indicates that AzuR might act as a global transcriptional regulator. It will be interesting to study the role of AzuR beyond metal homeostasis. The similar inverted repeats recognized by AztR (repressor of CPx-ATPase) and AzuR (repressor of MT) suggest that these two repressors could share regulation of their respective effector genes *in vivo*. The direct interaction between the two regulators and possibly the cross-talk between the two processes of metal sequestration and efflux would help us to understand the regulation of the metal homeostasis system in *Anabaena*.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

**AUTHOR CONTRIBUTIONS**

CA conceived, designed, and supervised the research. TVD performed the experiments. CA and TVD analyzed the data, wrote the draft of the manuscript, and revised the manuscript. Both authors approved the submitted version.

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