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Molecular characterization of Alr1105 a novel arsenate reductase of the diazotrophic cyanobacterium *Anabaena* sp. PCC7120 and decoding its role in abiotic stress management in *Escherichia coli*

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Abstract This paper constitutes the first report on the Alr1105 of *Anabaena* sp. PCC7120 which functions as arsenate reductase and phosphatase and offers tolerance against oxidative and other abiotic stresses in the *alr1105* transformed *Escherichia coli*. The bonafide of 40.8 kDa recombinant GST+Alr1105 fusion protein was confirmed by immunoblotting. The purified Alr1105 protein (mw 14.8 kDa) possessed strong arsenate reductase (Km 16.0 ± 1.2 mM and Vmax 5.6 ± 0.31 μmol min^{-1}mg protein^{-1}) and phosphatase activity (Km 27.38 ± 3.1 mM and Vmax 0.077 ± 0.005 μmol min^{-1} mg protein^{-1}) at an optimum temperature 37 °C and 6.5 pH. Native Alr1105 was found as a monomeric protein in contrast to its homologous *Synechocystis* ArsC protein. Expression of Alr1105 enhanced the arsenic tolerance in the arsenate reductase mutant *E. coli* WC3110 (Δ*arsC*) and rendered better growth than the wild type W3110 up to 40 mM As (V). Notwithstanding above, the recombinant *E. coli* strain when exposed to $CdCl_2$, $ZnSO_4$, $NiCl_2$, $CoCl_2$, $CuCl_2$, heat, UV-B and carbofuron showed increase in growth over the wild type and mutant *E. coli* transformed with the empty vector. Furthermore, an enhanced growth of the recombinant *E. coli* in the presence of oxidative stress producing chemicals (MV, PMS and H_2O_2), suggested its protective role against these stresses. Appreciable expression of *alr1105* gene as measured by qRT-PCR at different time points under selected stresses reconfirmed its role in stress tolerance. Thus the Alr1105 of *Anabaena* sp. PCC7120 functions as an arsenate reductase and possess novel properties different from the arsenate reductases known so far.

Keywords *Anabaena* sp. PCC7120 · Arsenic · *alr1105* · Overexpression · Complementation · Abiotic stress

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Introduction

Arsenic, a naturally occurring potentially toxic metalloid, exists in two predominant forms e.g. oxidized As (V) and reduced As (III) (Oremland and Stolz 2005). The use of As contaminated water for irrigation hampers not only the agricultural productivity directly but also through negatively affecting the survival of microbial communities including diazotrophic cyanobacteria which are the major contributors to the nitrogen economy of rice paddy soils.

To combat arsenic toxicity different organisms are blessed with arsenic resistance systems in the form of operon having an arsenite ion-inducible repressor (*arsR*), a cytosolic arsenate reductase (*arsC*) and a membrane associated arsenite export system (*arsB*) (Ji and Silver 1992; Carlin et al. 1995). Following transport into the cell, the As (V) using arsenate reductase (ArsC) undergoes reduction forming As (III) which is effluxed out of the cell through an arsenite efflux system. While this fundamental strategy of conferring arsenic resistance is fairly conserved, the arsenate reductases are quite varied in different organisms. The four types of arsenate reductases such as plasmid encoded R773 ArsC and pI258 ArsC from *Escherichia coli* (Chen et al. 1986; Oden et al. 1994; Gladysheva et al. 1994) and *Staphylococcus aureus* (Ji and Silver 1992) respectively, Acr2p from *Saccharomyces cerevisiae* (Bobrowicz et al.

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1997; Mukhopadhyay et al. 2000) and hybrid type arsenate reductase SynArsC from *Synechocystis* PCC6803 (Li et al. 2003; Lopez-Maury et al. 2009) characterized as yet possess dissimilar sequences and differ from one another in several of their physical and catalytic properties (Mukhopadhyay and Rosen 2002; Li et al. 2003). While R773 ArsC and pI258 ArsC are monomeric, the Acr2p and SynArsC are homodimer. The R773 ArsC, Acr2p and SynArsC enzymes obtain their reducing equivalents from glutathione and glutaredoxin (Gladysheva et al. 1994; Mukhopadhyay et al. 2000; Lopez-Maury et al. 2009) whereas pI258 ArsC uses thioredoxin (Ji et al. 1994). The enzyme pI258 ArsC and SynArsC contain three catalytically essential cysteine residues (Messens et al. 1999; Li et al. 2003) while R773 ArsC and Acr2p each possess only one (Liu et al. 1995; Mukhopadhyay and Rosen 2001).

The disruption of *arsC* gene has been shown to develop arsenate hypersensitivity in both *E. coli* and *S. cerevisiae* (Mukhopadhyay and Rosen 1998, 2002). Apart from its role in As stress the ArsC protein in *Synechocystis*, *E. coli,* and transgenic *Arabidopsis* and *Nicotiana* overexpressing bacterial ArsC conferred Cd tolerance also (Houot et al. 2007; Dhankher et al. 2003). Besides, a 3.5-fold induction of *arsC* gene under peroxide stress has also been observed in *Synechocystis* PCC6803 (Li et al. 2004).

While diazotrophic *Anabaena* sp. PCC7120, a photosynthetic prokaryote having plant like oxygenic photosynthesis, contains a gene *alr1105* annotated as arsenate reductase (http://genome.kazusa.or.jp/cyanobase/Anabaena/genes/alr1105), its functional characterization has remained unexplored except for our own report of an 8.5-fold induction of its transcript under As stress (Pandey et al. 2012). Alr1105 protein sequence shows 65 and 43 % identity respectively with ArsC of *Synechocystis* PCC 6803 (PDB id 2l17) and *S. aureus* (PDB id 1LJL) (http://www.ebi.ac.uk/Tools/webservices/). The conserved domain analysis of Alr1105 encoding 131 residue suggested it to be a member of the low-molecular weight protein phosphotyrosine phosphatases (LMWPTP) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) similar to *Synechocystis* PCC6803 and *S. aureus*. However, unlike *arsC* of *Synechocystis* and *Staphylococcus*, *alr1105* lacks an operonic structure. Furthermore, the Alr1105 protein is dissimilar to *E. coli* ArsC but similar to eukaryotic ArsC in terms of conserved PTP signature motif $C(X)_5R$ required for the activities of reductase and phosphatase (Ramponi and Stefani 1997a, b). The LMW PTP plays a key role in signal transduction, regulation of cellular processes, differentiation, and resistance to stresses like heat and ethanol (Musumeci et al. 2005; Rusnak and Reiter 2000). In view of the dual function of switching between reductase and phosphatase under different circumstances (Zegers et al. 2001) ArsC may be regarded as moonlighting protein (Jeffery 1999).

Taking recourse to the above Alr1105 appears to be a novel arsenate reductase envisioned to offer tolerance against a variety of abiotic stresses and hence could be a potential gene for developing transgenic cyanobacteria capable of proliferating under metals (Cd, Cu, Co, Ni and Zn), UV-B radiation, heat, pesticide and others besides As stress. To address these issues, the *alr1105* gene of *Anabaena* sp. PCC7120 was cloned, over expressed, purified and biochemically characterized to understand its properties and role in oxidative and abiotic stress management.

Materials and methods

Cyanobacterial and bacterial strains and plasmid

The diazotrophic cyanobacterium *Anabaena* sp. PCC7120 was grown in BG-11 medium (Rippka et al. 1979) buffered with HEPES pH 7.5, at 24 ± 2 °C under day light fluorescent tubes emitting 72 µmol photon m^{-2} s^{-1} PAR (photosynthetically active radiation) light intensity with a photoperiod of 14:10 h. *E. coli* strain DH5α and *E. coli* BL21 (DE3) (Novagen) were used as host for cloning and expression, respectively. *E. coli* W3110 and mutant *E. coli* [WC3110 K12F2IN(*rrnD-rrnE*) Δ*arsC*] (hereafter mutant *E. coli* WC3110) were used for complementation and arsenate sensitivity assay. Cells harboring recombinant plasmids were grown and maintained on Luria–Bertani (LB) medium supplemented with 100 µg ml^{-1} ampicillin (Sambrook and Russell 2001). Plasmid pGEX-5X-2 (GE Healthcare, USA) was used as a vector for cloning.

Cloning of the ORF *alr1105* from *Anabaena* sp. PCC7120

Genomic DNA from *Anabaena* sp. PCC 7120 was isolated by the method of Srivastava et al. (2007). Using genomic DNA as the template *alr1105* was amplified by polymerase chain reaction (PCR) with a pair of primers, Pf (5′CGGGATCCCG ATGAAAAAAGTAATGTTTGTATG3′) and Pr (5′GGAATTCCTTAGTTGAGTAATGCGATTAACT3′). The underlined bases are BamH1 and EcoR1 recognition sites, respectively. The PCR was done in a reaction mixture of 25 µl for 30 cycles at 94 °C for 1.30 min, 61 °C for 1 min, and 72 °C for 2 min using standard PCR conditions (100 ng of DNA, 2.5 µl of 10× Taq buffer with 15 mM $MgCl_2$, 200 µM dNTPs, 10 pmol of each primer and 0.2 U Taq DNA polymerase in an Icycler (Bio-Rad, USA). Amplified PCR product was confirmed by DNA sequencing (Macrogen, Korea).

Construction of the over expressing recombinant vector

The purified PCR product was digested with BamHI and EcoRI (NEB) and the resultant DNA fragment was cloned

into the pGEX-5X-2 expression vector, digested with the same restriction enzymes. After ligation the recombinant plasmid pGEX-5X-2-*alr1105* was introduced into *E. coli* BL21 grown in LB medium. The recombinant plasmid was isolated and the DNA sequence of *alr1105* was further confirmed by sequencing (Macrogen, Korea).

qRT-PCR of *alr1105*

RNA was isolated using TRIzol reagent from *E. coli* cells grown in LB medium with and without IPTG. First-strand cDNA synthesis was performed using 1 µg of DNaseI-treated RNA with the Bio-Rad cDNA kit in a 20-µl reaction volume according to the manufacturer's protocol. The Icycler profile for *alr1105* gene was as follows: initial denaturation for 5 min at 94 °C followed by 40 incubation cycles each consisting of 1 min denaturation at 94 °C, 1 min annealing at 61 °C, 45 s extension at 72 °C and a final 10 min extension at 72 °C. To ascertain the equal loading of RNA in different samples RT-PCR of 16S rDNA was also performed and used as an internal control. Further to reconfirm the transcript results quantitative real time PCR was performed. Transcript levels were quantified by qPCR using Bio-Rad CFX-96 system. Reactions were performed in a total volume of 20 µl using 15 ng cDNA, 10 pmol of forward and reverse primer and 1× SsoFast evagreen qPCR supermix (Bio-Rad). The thermal amplification protocol was as follows: 1 cycle at 95 °C for 5 min, 40 cycles of 95 °C for 15 s, 52 °C for 30 s and 72 °C for 30 s. Transcript levels were normalized to 16S transcription and calculated relative to 0 h using the $2^{-\Delta\Delta Ct}$ method. The comparative $\Delta\Delta C_t$ method was used to evaluate the relative quantities of each amplified product in the samples. The threshold cycle (C_t) was automatically determined for each reaction by the system set with default parameters. The specificity of the PCR was determined by melt curve analysis of the amplified products.

SDS-PAGE, native PAGE and immunoblotting

In order to confirm the expression of *alr1105* gene in *E. coli* cells, SDS-PAGE was carried out as per the method of Sambrook and Russell (2001). For isolation of protein, harvested bacteria were washed twice with extraction buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, 20 mM KCl, pH 7.5). The pellet was resuspended in 2 ml extraction buffer and subjected to grinding under liquid nitrogen for protein extraction. The extract was centrifuged at 10,000 rpm for 60 min. Supernatant was mixed with 1× SDS loading dye and SDS-PAGE was carried out. For native PAGE denaturing detergent SDS and reducing agent was not used.

The gel was transferred to a PVDF membrane (Millipore Immobilon-P) using a dual mini-electroblot system (Precision Instruments, Varanasi, India). The gel cassette was kept in transfer buffer (3.03 gl^{-1} tris base, 14.4 gl^{-1} glycine, and 200 ml methanol (99 % v/v pure) for 1 h at 100 V at 4 °C. Membrane was blocked for 4 h in TTBS (Tris buffer saline containing 0.1 % Tween-20) and 5 % (w/v) non-fat dried milk. The membrane incubated overnight at 4 °C in the diluted solution of the primary antibody anti-GST, was washed five times for 5 min each in TTBS. This was then incubated in a Goat anti Rabbit IgG HRP (horseradish peroxidases) conjugated secondary antibody (Genei, India) for 4 h. Following four consecutive 5 min wash in TTBS the membrane was developed with TMB/H_2O_2 (Tetra methyl benzidine) visualization solution. The reaction was terminated by washing the PVDF membrane with deionized distilled water and the blots were dried between filter paper and scanned.

Multiple sequence alignment

Basic local alignment search tool (BLAST) was used to find out homologs of Alr1105 in PDB database. Proteins so obtained from different species were used for multiple sequence alignment (MSA) using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Complementation of arsenate sensitive *E. coli* WC3110 by *alr1105*

The arsenate resistance phenotype of cells expressing *alr1105* gene was determined in *E. coli*. *E. coli* strains W3110 (wild-type) and WC3110 (DE3) (Δ*arsC*) were used for arsenate sensitivity assays (Shi et al. 1999). *E. coli* WC3110 was transformed with the recombinant plasmid pGEX-5X-2-*alr1105* and pGEX-5X-2 empty vector. Both wild type and transformed strains were grown overnight in low phosphate medium (Oden et al. 1994). The cells were diluted 100-fold in the same medium containing 0.3 mM IPTG and different amounts of sodium arsenate (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 5.0, 10.0, 20.0 and 40.0 mM) and allowed to grow at 30 °C for an additional 48 h. Growth was estimated from the absorbance at 600 nm. Further these *E. coli* strains were also spotted on solid medium having different concentrations of As (2, 4, 8 and 10 mM) to check the As sensitivity.

Enzyme assays

Arsenate reductase assay

Arsenate reductase assay of cell extract was done by the method of Anderson and Cook (2004). Cells were lysed by sonication in reaction buffer (10 mM Tris, pH 7.5, with 1 mM Na_2EDTA and 1 mM $MgCl_2$) and centrifuged at

14,000 (6,500×*g*) for 5 min and supernatant was used for enzyme assay. The NADPH oxidation was initiated at 37 °C by mixing 50 µl of crude extract in 820 µl of reaction buffer, 30 µl of 10 mm DTT, 50 µl of 2 mM arsenate, and 50 µl of 3 mM NADPH. Activity was monitored by measuring the decrease in NADPH absorbance at 340 nm and the NADPH oxidation was calculated using a molar extinction coefficient of 6,200 M^{-1} cm^{-1}. Absorbance decreases as NADPH is oxidized coupled to arsenate reduction to arsenite.

Phosphatase assay

Phosphatase activity was assayed as per the method of Zhou et al. (2006). Assay was performed at 37 °C with 10 mM p-nitrophenyl phosphate (pNPP) in 0.1 M MOPS/MES buffer, pH 6.5. The assay was initiated by the addition of pNPP, and the rate of hydrolysis was estimated by measuring increase in absorption at 410 nm. Each value was corrected for non-enzymatic pNPP hydrolysis.

Purification of recombinant protein

The recombinant protein was extracted in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) from the cell pellet of 500 ml IPTG induced *E. coli* culture. Extract was centrifuged as mentioned earlier and the supernatant was loaded at 0.2 ml/min flow rate on to a 1 ml GSTrap HP column (GE Healthcare) preequilibrated with binding buffer (PBS, pH 7.3, 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4). The column was then washed with 25 ml binding buffer followed by elution with 5 ml elution buffer (50 mM Tris–HCl, 10 mM reduced glutathione, pH 8.0). 1 mg purified GST+Alr1105 protein was incubated with 10 U Xa factor (GE Healthcare) at 22 °C for 16 h. After digestion GST was removed by GSTrap HP column followed by HighTrap Benzamidine FF column (GE Healthcare) to remove Xa factor. Purified protein was electrophoretically resolved by 15 % SDS-PAGE and visualised by Coomassie Brilliant Blue (R250) staining. MALDI/TOF MS analysis of the purified protein identified a single protein corresponding to Alr1105 with a mawse score of 270, and mw 14.9 kDa confirming the purity and identity of the purified native protein.

Assay of arsenate reductase activity of purified Alr1105

Arsenate reductase activity was measured using a coupled assay system that measures the arsenate-dependent oxidation of NADPH (Li et al. 2003). Briefly, the purified protein (25 µg) was incubated at 37 °C in 1.0 ml of 100 mM Tris–HCl (pH 7.5) containing 0.1 mg of bovine serum albumin/ml, 0.25 mM NADPH, 0.2 µM yeast glutathione reductase, 8 mM reduced glutathione, 40 mM sodium arsenate, and 4 µM *E. coli* glutaredoxin (grxB) (Fitzgerald). Reductase activity was monitored at 340 nm and expressed as nmol of NADPH oxidized per mg of Alr1105 using a molar extinction coefficient of 6,200 M^{-1} cm^{-1} for NADPH. Enzyme kinetics was determined at different As (V) concentrations. Kinetic constants were obtained by non linear regression (curve fit) of Michaelis–Menten equation using GraphPad Prism software version 5.04.

Effect of pH and temperature on enzyme activity

The pH dependence of enzyme activity was checked by incubating it at different pH (4.5–9.5) at 37 °C for 30 min. Acetate buffer (100 mM) was used for the pH range 4.5–5.5, MES (100 mM) for pH 6.0–6.5, Tris–HCl buffer (100 mM) for pH 7.0–8.5 and Gly-NaOH (100 mM) for pH 9.0–9.5. For optimum temperature determination the standard reaction mixture (MES, pH-6.5) was incubated for 30 min at different temperatures (27–97 °C) and the enzyme activity was measured.

Phosphatase assay of purified Alr1105

The phosphatase assay was performed as per the method of Li et al. (2003). The phosphatase activity was initially assayed using *p*NPP as substrate. Purified protein was incubated for 30 min at 37 °C in 200 µl of 100 mM Tris–HCl (pH 7.5) containing 5 mM DTT. Assay was performed with different concentrations of pNPP (10, 20, 40, 80, 160 and 320 mM). The reaction was terminated by the addition of 400 µl of 0.5 M NaOH and the absorbance of the resulting solution was determined at a wavelength of 410 nm. The quantity of *p*-nitrophenol produced was calculated using an extinction coefficient of 17,800 M^{-1} cm^{-1}. Kinetic constants were obtained by non linear regression of Michaelis–Menten equation using GraphPad Prism software version 5.04.

Evaluation of abiotic stress tolerance and response to oxidative stress generating chemicals

In order to examine the role of *alr1105* in abiotic stress tolerance the effects of sodium arsenate ($Na_2AsO_4{\cdot}7H_2O$), $ZnSO_4$, $NiCl_2$, $CoCl_2$, $CdCl_2$, $CuCl_2$, carbofuron, UV-B, and temperature on the growth behavior of wild type *E. coli* W3110, mutant *E. coli* WC3110 and mutant *E. coli* WC3110 transformed with pGEX-5X-2-*alr1105* was compared. Survival of *E. coli* W3110 was measured by treating with different concentrations of sodium arsenate (1.0, 2.0, 3.0, 4.0 and 5.0 mM), Cd (0.1, 0.2, 0.3, 0.4 and 0.5 mM), $CuCl_2$ (1.0, 2.0, 3.0, 4.0 and 5.0 mM), $ZnSO_4$

(0.1, 0.5, 1.0, 1.5, and 2.0 mM), $NiCl_2$ (0.5, 1.5, 2.5, 3.0 and 3.5 mM), $CoCl_2$ (0.1, 0.5, 1.0, 1.5, and 2.0 mM) and carbofuron (0.010, 0.015, 0.02, 0.025 and 0.03 mg ml^{-1}). Similarly growth at different temperature (40, 45, 50 and 55 °C) and UV-B (5.0, 10, 15 and 20 min exposure) was measured. Approximately 50 % survival of *E. coli* was observed at sodium arsenate (4 mM), Cd (0.35 mM), ZnSO4 (1.5 mM), $NiCl_2$ (3.0 mM), $CuCl_2$ (4.0 mM), $CoCl_2$ (1.5 mM) and carbofuron (0.03 mg ml^{-1}).

To assess the effect of oxidative stress, *E. coli* strains were grown in oxy radical generating chemicals e.g. 100 µM methyl viologen (MV), 20 µM phenazine methosulphonate (PMS) and 4 mM hydrogen peroxide (H_2O_2).

Specific growth rate and doubling time were calculated by using the equations: $l = [\ln(n2/n1)/(t2 - t1)]$ and $G = 0.693/l$ where l stands for specific growth rate; n1, n2 are absorbance of culture suspension at the beginning (t1) and end (t2) of the selected time interval and G for doubling time. All growth experiments were conducted on a rotary shaker (200 rpm) at 37 °C. Three independent measurements were taken and the average value was used for making the final data.

Transcript analysis of *alr1105* in response to different abiotic stress

Escherichia coli cells subjected to different stresses were withdrawn at 0, 3, 6 and 12 h, their RNA was isolated using TRIzol reagent and expressional characterization of *alr1105* gene was done using qRT-PCR as described earlier (Shrivastava et al. 2012).

Metal accumulation analysis

After growth for 24 h in the presence of IPTG and metal ($CuCl_2$, $CdCl_2$, $NiCl_2$, $ZnSO_4$ and $CoCl_2$) and 5 mM buthionine sulfoximine (BSO) cells were harvested, rinsed three times using LB medium and dried at 55 °C for 24 h. Following addition of 5 ml HNO_3 (70 %), mineralization was carried out on hot plate. After cooling this was again treated with 70 % HNO_3 and digested on a hot plate at 70 °C. To this 30 % H_2O_2 was added dropwise till the solution became colourless. The resulting solution was analysed in a Perkin–Elmer-2380 atomic absorption spectrophotometer.

Results

Molecular cloning of *alr1105*

The PCR amplified product of *alr1105* was about 396 bp (Fig. 1a), which matched with the theoretical length of the *alr1105* gene (396 bp). It was digested with BamHI and EcoRI, ligated to the pGEX-5X-2 and transformed into *E. coli* strain BL21 (DE3). The recombinant construct pGEX-5X-2-*alr1105* was verified by DNA sequencing (Macrogen, Korea).

Expression analysis of *alr1105* using qRT-PCR and SDS-PAGE

A 4 h exposure of cells transformed with recombinant plasmid to 0.3 mM IPTG produced a 4.0-fold increase in the transcript compared to non-induced cells (Fig. 1b). The control *E. coli* harbouring the empty vector (pGEX-5X-2) failed to show detectable *alr1105* transcript. This data suggested successful transcription of the recombinant plasmid (pGEX-5X-2-*alr1105*). The induction of fusion protein was also observed on the SDS-PAGE after IPTG treatment (Fig. 1c). The molecular mass of fusion protein GST+Alr1105 as estimated by comparison with protein molecular weight marker was 40.8 kDa.

Immunoblotting

Immunoblot analysis with anti-GST produced a single 40.0 kDa band of recombinant protein that was in good agreement with the molecular weight (40.8 kDa) deduced from the nucleotide sequence of *alr1105* gene (Fig. 1d). This confirmed that *E. coli* cells containing recombinant construct pGEX-5X-2-*alr1105* were successfully translated and resulted in the overexpression of the GST+Alr1105.

SDS-PAGE and native PAGE of purified Alr1105

Purified GST+Alr1105 after digestion with Xa factor showed GST band at 26.0 kDa and Alr1105 band at 14.8 kDa. Further, after removal of GST a single band of Alr1105 was observed confirming its theoretical mw 14.8 kDa (Fig. 1e). Native-PAGE analysis of purified recombinant protein GST+Alr1105 (mw 67.0 kDa) as well as Alr1105 (mw 14.8 kDa) clearly showed that Alr1105 is a monomeric protein while purified GST a dimeric protein (mw 52.0 kDa) (Fig. 1f).

Multiple sequence alignment

Multiple sequence alignment of alr1105 protein sequence with the known arsenate reductase of *Synechocystis* PCC6803, *S. aureus* and *Bacillus subtilis* suggested the presence of conserved PTP signature motif and catalytic cysteine residues (Fig. 2a).

alr1105 complements arsenate-sensitive phenotype in *E. coli*

The ability of the *alr1105* gene to complement the arsenate-sensitive phenotype of an *E. coli* Δ*arsC* strain

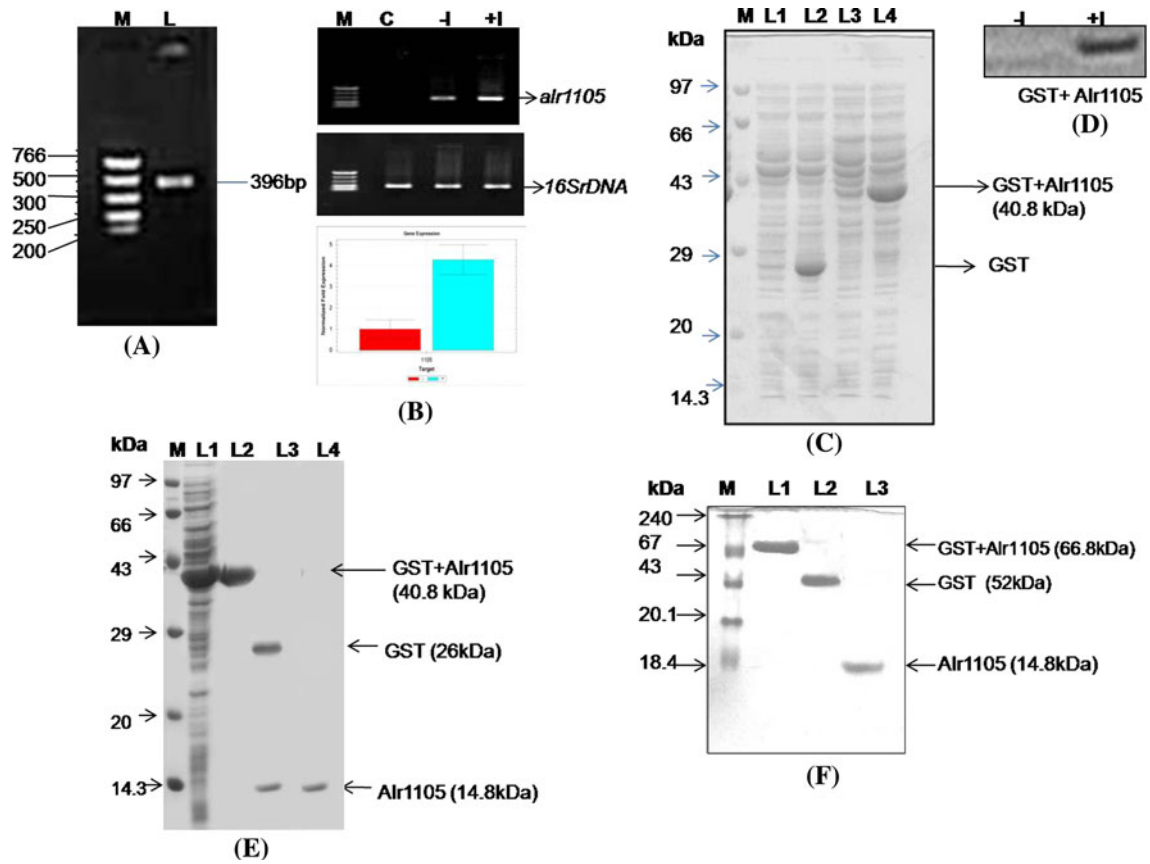

Fig. 1 **a** PCR amplification of *Anabaena* sp. PCC 7120 ORF *alr1105* Lane (*M*) DNA ladder, (*L*) PCR product of *alr1105* (396 bp), **b** RT-PCR of *alr1105* gene and 16S rDNA as an internal control, Lane (*M*) DNA ladder, (*C*) control, (−*I*) without IPTG, (+*I*) with IPTG; graph showing qRT-PCR result of *alr1105*, **c** SDS-PAGE (12 %) analysis of recombinant protein of *E. coli*. Lane (*M*) protein marker, (*L1*) whole cell lysate of *E. coli* cells containing empty vector pGEX-5X-2 without IPTG induction, (*L2*) cell lysate containing the empty vector pGEX-5X-2 after 4 h induction with 0.3 mM IPTG, (*L3*) cell lysate of *E. coli* transformed with plasmid pGEX-5X-2-*alr1105* without IPTG, (*L4*) plasmid pGEX-5X-2-*alr1105* obtained after 4 h induction with 0.3 mM IPTG. The number on the right side is the apparent molecular weight of the recombinant Alr1105 protein (40.8 kDa), **d** Immunoblot detection of GST+Alr1105 protein in transformed *E. coli* with empty and recombinant vectors, **e** SDS-PAGE profile, (*M*) protein marker, (*L1*) whole cell lysate of *E. coli* cells containing the pGEX-5X-2-*alr1105* after IPTG induction, (*L2*) purified recombinant protein GST+Alr1105, (*L3*) Xa digested recombinant protein showing GST and Alr1105 and, (*L4*) purified Alr1105 protein, **f** Native-PAGE profile, (*M*) protein marker, purified (*L1*) recombinant protein GST+Alr1105, (*L2*) GST, and (*L3*) Alr1105

WC3110 was examined. Cyanobacterial *alr1105* not only restored arsenate resistance up to 2 mM sodium arsenate in the wild-type strain W3110 but demonstrated a 7.0-fold better growth than the wild type even at 40 mM As (V) (Fig. 2b). Further when these strains were spotted on solid media containing different As concentrations (2, 4, 8 and 10 mM) no growth in case of *E. coli* Δ*arsC* strain WC3110 was noticed. Nevertheless, the *E. coli* Δ*arsC* WC3110 transformed with *alr1105* was not only able to grow at different concentrations of As but showed better growth than the wild type *E. coli* W3110 (Fig. 2c).

Arsenate reductase and phosphatase activity

The cells containing pGEX-5X-2-*alr1105* plasmid showed 3.1-fold increased activity over those containing empty vector. Further a marked arsenate reduction activity in the complemented *E. coli* strain and absence in the mutant strain attested functionality of *alr1105* (Fig. 3a). Besides, the phosphatase activity in the form of pNPP hydrolysis was also higher in recombinant cells over the control cell (Fig. 3b).

Arsenate reductase activity of purified protein

Spectrophotometric assay was performed with and without recombinant enzyme and oxidation of NADPH coupled with reduction of arsenate was measured. Decrease in absorbance at 340 nm in the presence of purified protein indicated arsenate reductase activity. However, in the absence of glutathione, glutaredoxine, glutathione reductase and As (V), arsenate reductase failed to show any

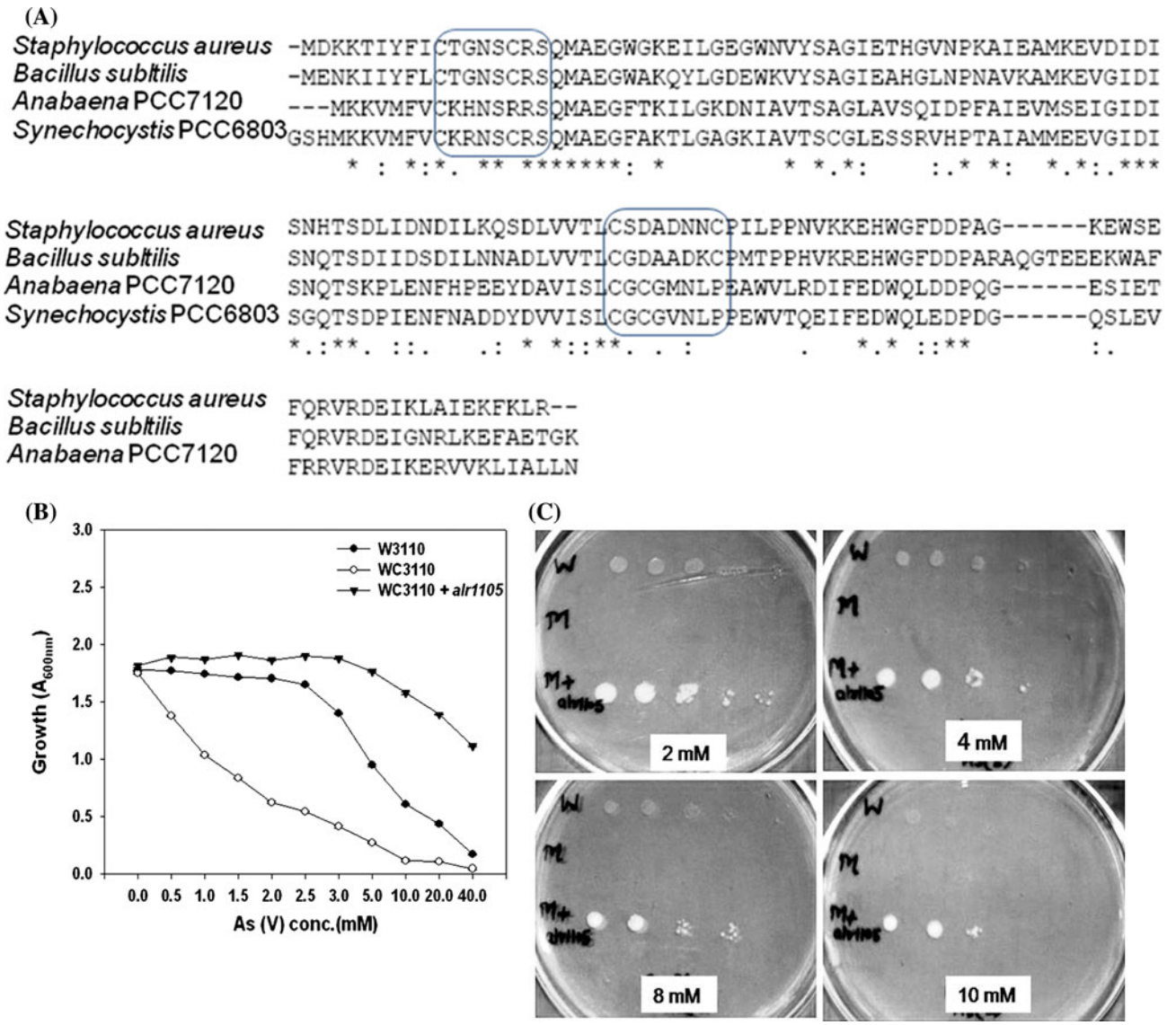

Fig. 2 **a** Multiple sequence alignment of Alr1105 with known arsenate reductase of *Synechocystis* PCC6803, *S. aureus*, and *B. subtilis*. The first encircled amino acid sequence represents conserved PTP-signature sequence and the second represents presence of conserved cystein residues, **b** Arsenate sensitivity assay of *E. coli* W3110, WC3110 and WC3110 containing *alr1105* gene at different concentrations of sodium arsenate in liquid medium, **c** Arsenate sensitivity assay at 2, 4, 8 and 10 mM sodium arsenate concentration in agar plate. The agar plates were spotted with wild type (*E. coli* W3110), mutant (*E. coli* WC3110) and mutant containing *alr1105*

activity (Fig. 4a). Kinetic parameters of Alr1105 at different As (V) concentrations were determined. The enzyme exhibited a Km value of 16 ± 1.2 mM and Vmax of 5.6 ± 0.31 µmol min^{-1} mg $protein^{-1}$ (Fig. 4b, Table 1).

Effect of temperature and pH

The purified arsenate reductase showed activity at a temperature range between 27 °C and 87 °C, but the highest activity was observed at 37 °C (Fig. 5a). Likewise the activity was maximum at pH 6.5 (Fig. 5b).

Phosphatase assay of purified Alr1105

The purified Alr1105 protein demonstrated phosphatase activity in the presence of substrate pNPP (Fig. 6a). The dephosphorylation of para-nitrophenyl phosphate (pNPP) in the presence of enzyme proceeds significantly faster than in the control without enzyme, proving that catalysis occurs. Kinetic parameters of Alr1105 at different pNPP concentrations showed a Km value of 27.38 ± 3.1 mM and Vmax of 0.077 ± 0.005 µmol min^{-1} mg $protein^{-1}$ (Fig. 6b).

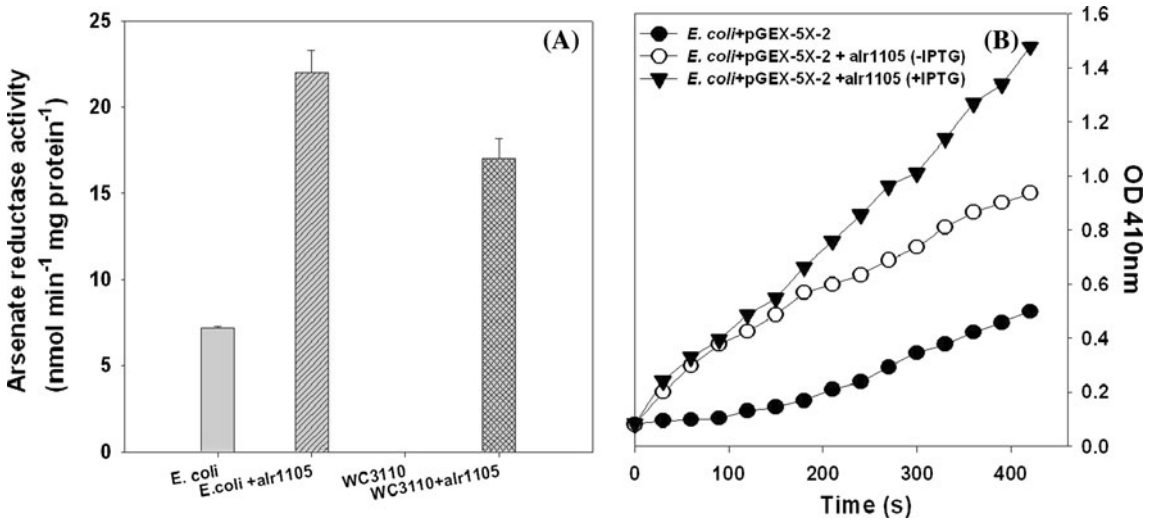

Fig. 3 **a** Arsenate reductase activity of *E. coli* (BL21) and WC3110 transformed with empty and recombinant vectors. **b** Phosphatase assay of *E. coli* transformed with empty and recombinant vectors grown in LB medium supplemented with 100 μg ml^{-1} ampicillin, 0.3 mM IPTG

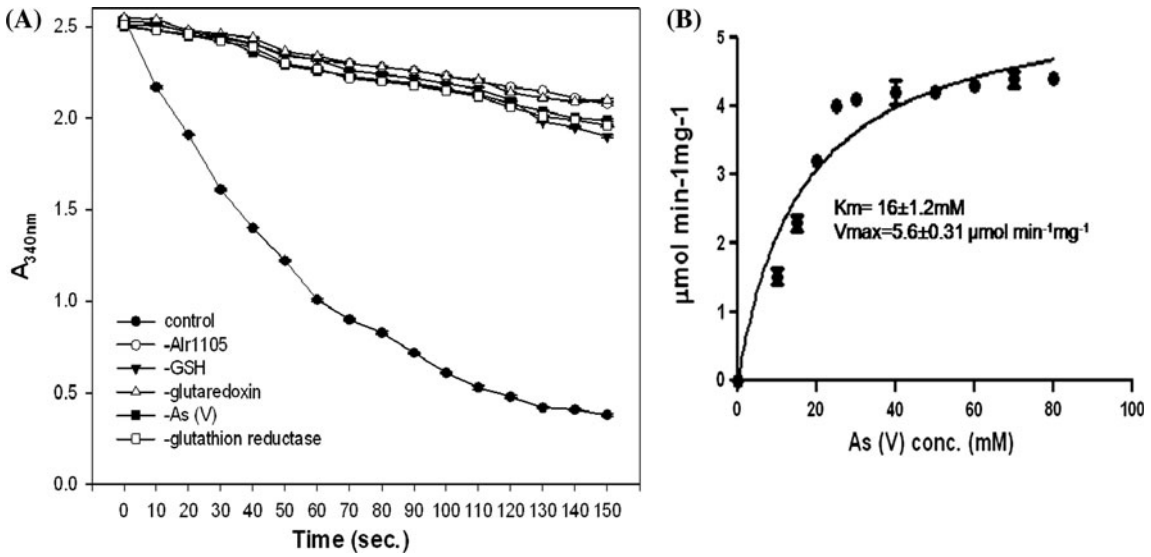

Fig. 4 **a** Purified Alr1105 showing arsenate reductase activity (*closed circle*). Dependence of arsenate reductase activity is also shown by changes in absorbance observed in control assays either Alr1105 (*open circles*), glutathione (*closed triangles*), glutaredoxin (*open triangles*), arsenate (*closed squares*), or glutathione reductase (*open squares*) was omitted, **b** arsenate reductase activity at different concentrations of As (V)

Effect of abiotic and oxidative stresses on the growth of wild typeW3110, mutant WC3110 and transformed (WC3110+*alr1105*) *E. coli* strains

The mutant *E. coli* cells transformed with recombinant plasmid (pGEX-5X-2-*alr1105*) depicted approximately 18 and 15.3 % better growth than the wild type and the mutant strains respectively under control condition (Fig. 7). Impact of $Na_2AsO_4.7H_2O$, $CdCl_2$, $NiCl_2$, $ZnSO_4$, $CoCl_2$, $CuCl_2$, 47 °C temperature, carbofuron, and UV-B on the wild type, mutant and mutant *E. coli* cells transformed with pGEX-5X-2-*alr1105* was demonstrated (Figs. 7, 8). The specific growth rate of these *E. coli* strains was decreased and doubling time was increased in presence of above stresses in comparison to their respective genotypes under control condition (Table 2). Mutant *E. coli* transformed with pGEX-5X-2-*alr1105* showed 39.7, 16.5, 22.7, 20.3, 16.3, 61.0, 15.1, 17.0, and 17.0 % better specific growth rate and 28.0,16.6, 18.0, 17.5, 13.7, 38.3, 13.5, 14.0 and 13.3 % decrease in doubling time under $Na_2AsO_4.7H_2O$, $CdCl_2$, $NiCl_2$, $ZnSO_4$, $CoCl_2$, $CuCl_2$, 47 °C temperature, carbofuron, and UV-B stresses respectively than wild type under stress. Likewise, with respect to mutant, the mutant *E. coli* transformed with pGEX-5X-2-*alr1105* showed 112,

Table 1 Comparison of Alr1105 with the arsenate reductase of other organisms

Organism	Group	Class	$HAsO_4^{2-}$ Km (mM)	Vmax (μmol/min/mg)	Optimal pH	Optimal temperature (°C)	Reference
Anabaena sp. PCC7120	Cyanobacteria	LMW PTP-like	16	5.6	6.5	37	This paper
Synechocystis sp. strain PCC 6803	Cyanobacteria	LMW PTP-like	1.25	3.1	7.5	37	Li et al. (2003)
E. coli plasmid R773	Gram-negative bacteria	Unique	8	0.8–1.5	6.3–6.8	37	Gladysheva et al. (1994)
Staphylococcus aureus plasmid pI258	Gram-positive bacteria	LMW PTP-like	0.068	14.5	7.5	37	Messens et al. (2002)
Saccharomyces cerevisiae	Yeast	Cdc25-like	35	0.3-0.4	6.5–8.5	37	Mukhopadhyay et al. (2000)
Oryza sativa	Plant	Cdc25-like	12.2	0.117	6.5	37	Duan et al. (2007)
Pteris vittata	Plant	Cdc25-like	28	0.19	6.5	37	Ellis et al. (2006)

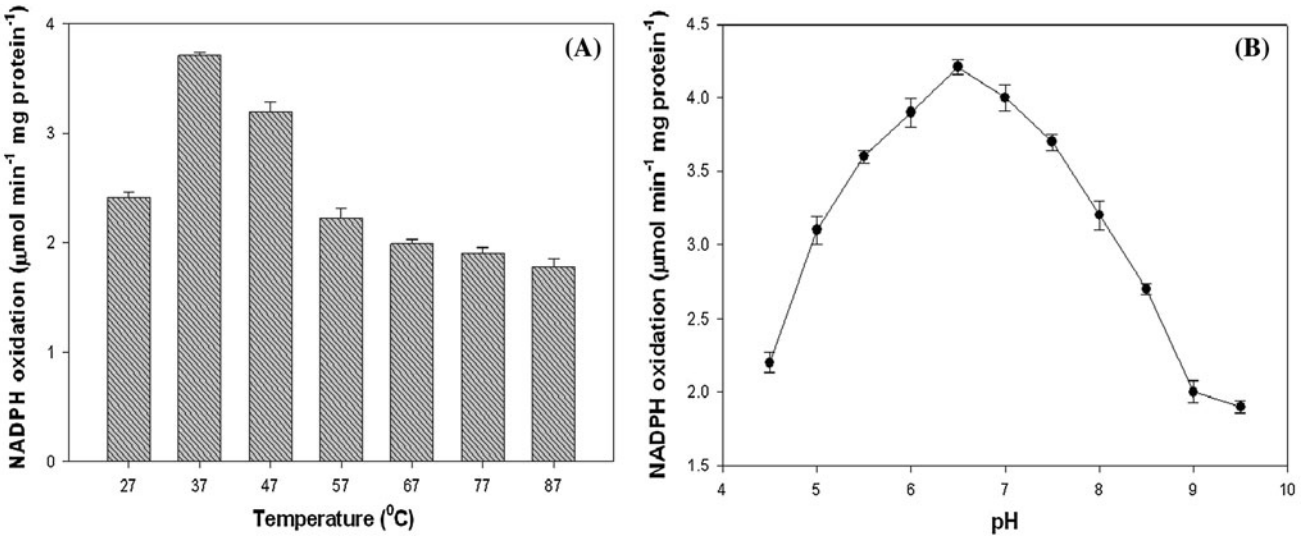

Fig. 5 **a** Effect of temperature, and **b** pH on arsenate reductase activity

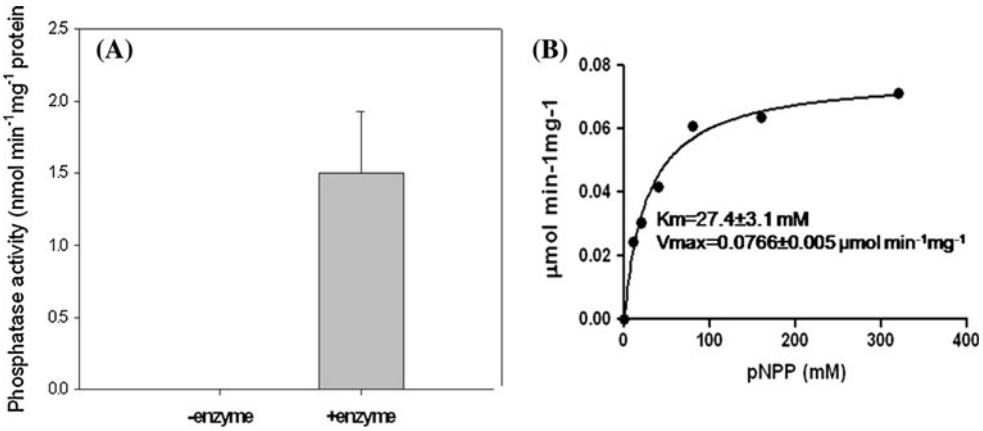

Fig. 6 **a** Phosphatase activity with and without purified Alr1105, **b** Phosphatase activity at different pNPP concentrations

101, 44.7, 22.7, 21.1, 62, 16.4, 12.1 and 15.4 % better specific growth rate as well as 52.8, 50.0, 30.5, 18.3, 18.4, 38.6, 14.2, 10.4 and 13.3 % decrease in doubling time under $Na_2AsO_4.7H_2O$, $CdCl_2$, $NiCl_2$, $ZnSO_4$, $CoCl_2$, $CuCl_2$, 47 °C temperature, carbofuron, and UV-B stresses respectively.

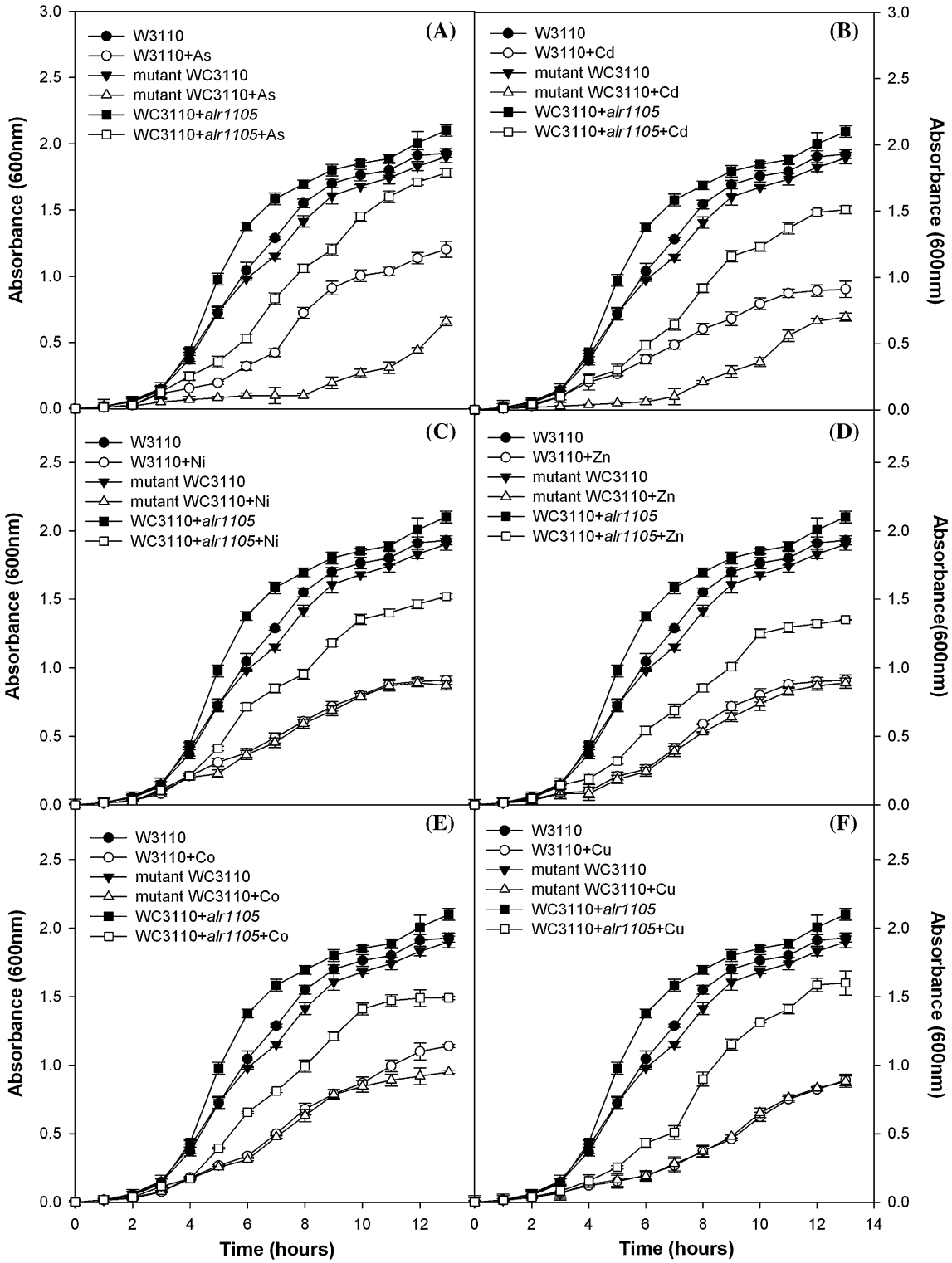

Fig. 7 Effect of **a** sodium arsenate, **b** $CdCl_2$, **c** $NiCl_2$, **d** $ZnSO_4$, **e** $CoCl_2$, and **f** $CuCl_2$ on the growth of *E. coli* W3110, mutant WC3110 and mutant having *alr1105*. The mean of three independent replicates are plotted with error bars indicating standard deviations. The cells were cultivated in LB medium (supplemented with 100 μg ml^{-1} ampicillin and 0.3 mM IPTG)

Effects of oxy radical generating chemicals MV, PMS and H_2O_2 on the growth of *E. coli* strains was demonstrated (Fig. 8d–f). While the wild type and the mutant *E. coli* cells did not depict significant difference in growth, the *alr1105* complemented *E. coli* registered much better growth. Mutant *E. coli* transformed with pGEX-5X-2-

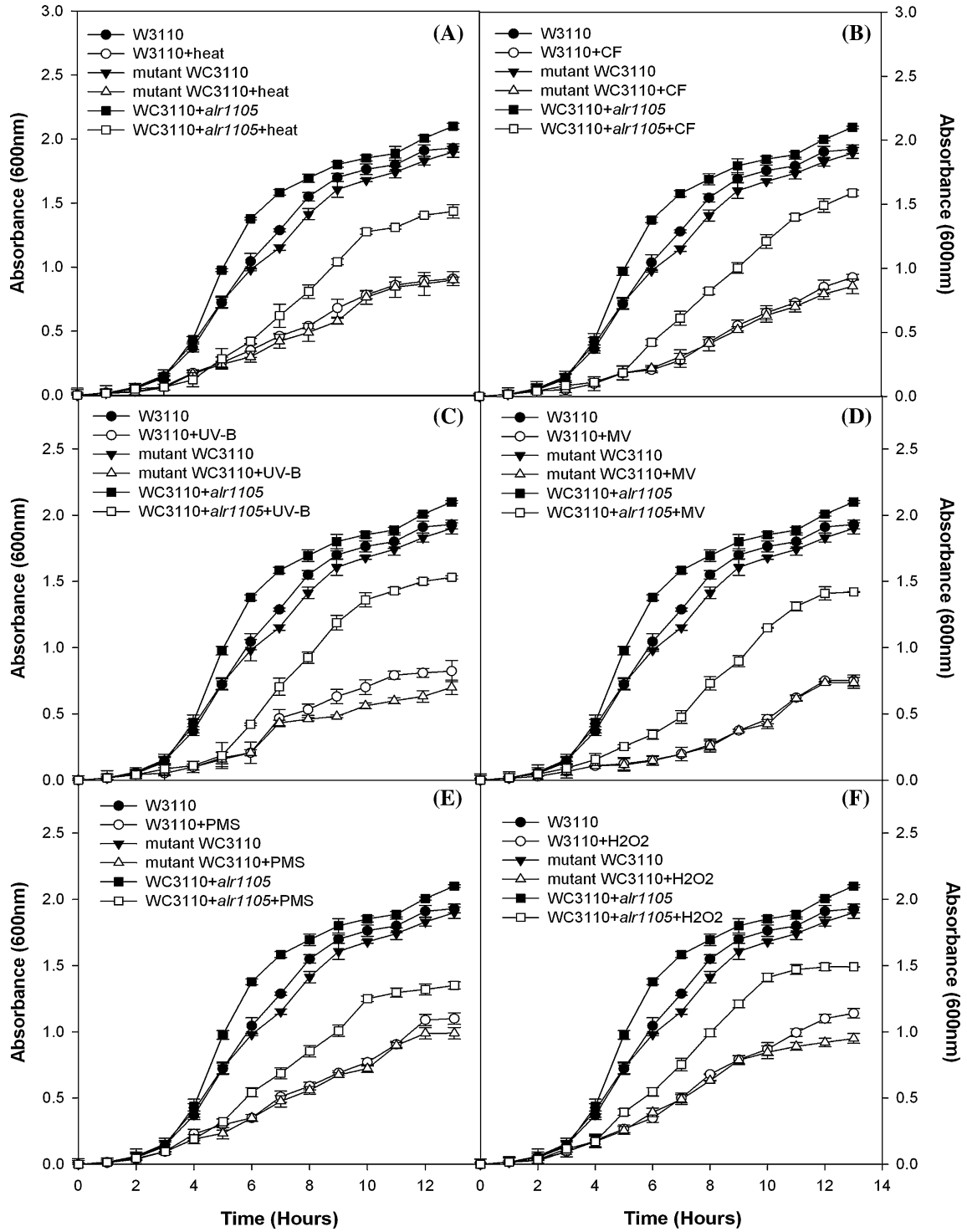

Fig. 8 Effect of **a** 47 °C temperature, **b** carbofuron, **c** UV-B, **d** MV, **e** PMS, and **f** H_2O_2 on the growth of *E. coli* W3110, mutant WC3110 and mutant having *alr1105*. The mean of three independent replicates is plotted with *error bars* indicating standard deviations. **a**–**f** represents the growth curves of *E. coli* in LB medium (supplemented with 100 μg ml^{-1} ampicillin and 0.3 mM IPTG)

alr1105 registered 59.6, 33.7 and 20.0 % better specific growth rate as well as 37.3, 25.6 and 18.8 % decrease in doubling time in the presence of MV, PMS and H_2O_2 respectively. Likewise, with respect to mutant, mutant *E. coli* transformed with pGEX-5X-2-*alr1105* demonstrated 66.0, 34.4 and 20 % better specific growth rate and 39.7, 25.6 and 18.8 % decrease in doubling time in the presence of MV, PMS and H_2O_2 respectively.

Table 2 Percentage change in doubling time of *E. coli* strains under different stresses with their respective genotype in control condition

Abiotic stresses	W3110 (% change)	Mutant WC3110 (% change)	Mutant WC3110+ *alr1105* (% change)
As	88.6	177.0	60.0
Cd	47.1	136.0	44.8
Ni	26.4	43.6	22.2
Zn	78.3	73.6	73.7
Co	31.1	33.6	33.6
Cu	89.6	83.6	38.1
Heat	18.9	15.5	21.3
CF	41.5	27.3	43.6
UV-B	41.5	36.6	44.7
MV	135.0	135.0	73.7
PMS	31.3	31.4	32.5
H_2O_2	50.9	50.9	49.0

Expression of *alr1105* in response to various abiotic stresses

Significant increase in the transcript of *alr1105* gene at various time points under given stresses was observed (Fig. 9a–i). This increase being 1.4, 1.6, 2.5-fold in $Na_2AsO_4.7H_2O$; 1.8, 2.1, 2.3-fold in $CdCl_2$; 1.1, 1.6, 2.1-fold in $NiCl_2$, 1.4, 1.7, 2.4-fold in $ZnSO_4$; 1.4, 1.6, 1.7-fold in $CoCl_2$, 3.8, 4.0, 4.4-fold in Cu; 1.3, 1.4, 1.7-fold in heat; 1.4, 2.0, 2.7-fold in carbofuron and 1.4, 1.9, 2.8-fold in UV-B as compared to the control after 3, 6 and 12 h respectively.

Metal accumulation

Metal accumulation analysis demonstrated that the mutant WC3110 transformed with *alr1105* accumulated 2.8, 2.3, 2.2, 1.8 and 1.6-fold Cu, Cd, Zn, Ni and Co respectively in comparison to the wild type and 2.8, 2.4, 2.1, 1.9 and 1.6-fold over the mutant WC3110 (Table 3). Mutant strain, however, in presence of BSO demonstrated 95, 90, 93, 96 and 93 % decreased accumulation of Cd, Ni, Zn, Co and Cu respectively. On the other hand WC3110+*alr1105* showed an appreciable decrease of 90, and 81 % for Cd and Co; the decrease in accumulation was extremely low e.g. 7.9, 8.9, and 3.4 % for Ni, Zn, and Cu respectively (Table 3).

Discussion

The data compiled in the present paper is the first attempt to functionally and biochemically characterize the Alr1105 of *Anabaena* sp. PCC7120 and to decode its role in oxidative and abiotic stress management. For characterization, the *alr1105* gene was cloned into pGEX-5X-2 and expressed in *E. coli* BL21. The expression of the gene was confirmed by its transcript and accumulation of 40.8 kDa GST+Alr1105 fusion protein (Fig. 1c, d). Furthermore, the fusion protein was purified by affinity column and digested with Xa factor to obtain the purified Alr1105 protein. While SDS-PAGE analysis and protein sequencing attested the theoretical mw (14.8 kDa) of Alr1105 (Fig. 1e), the native PAGE clearly revealed it as a monomeric protein similar to *S. aureus* but different from *Synechocystis* (Fig. 1f). Alr1105 possessed conserved motif $C(X)_5R$ as known for *Synechocystis, S. aureus* and *B. subtilis* (Fig. 2a). The Cys residue of the conserved motif is known for both arsenate and phosphatase activity. Like *Synechocystis* ArsC, the Alr1105 contains Cys^{80} and Cys^{82} residues which play essential role in arsenate reduction (Li et al. 2003).

While Alr1105 of *Anabaena* sp.PCC7120 is totally unrelated to *E. coli* with respect to sequence and conserved catalytic active site, its heterologous expression in *E. coli*, complemented the arsenate sensitive phenotype of an *E. coli* Δ*arsC* strain (WC3110) (Fig. 2b, c). These findings are supported by Duan et al. (2007) and Mukhopadhyay and Rosen (2002) who demonstrated complementation of rice and *Saccharomyces* arsenate reductase in *E. coli* Δ*arsC* strain respectively. The Alr1105 not only bestowed 2 mM sodium arsenate tolerance over the wild type strain W3110 but depicted better growth up to 40 mM As (V) (Fig. 2b, c). Further, the crude enzyme extract as well as the purified Alr1105 protein from *alr1105* overexpressing *E. coli* demonstrated strong arsenate reductase activity (Figs. 3a, 4a). This activity was dependent on the presence of glutathione, glutaredoxine, As (V) and glutathione reductase (Fig. 4a).

The arsenate reductase activity of purified Alr1105 followed the Michaelis–Menten saturation kinetics with respect to different As (V) concentrations (Fig. 4b, c). Different levels of arsenate reductase activity, i.e. >30 % of maximum, were detectable over a wide range of pH i.e., 4.5–9.5 and temperature i.e. 27 °C–87 °C, with highest activity obtained at pH 6.5 and at 37 °C (Fig. 5a, b). Its Vmax, a measure of enzyme activity under substrate saturation, was 1.8-fold higher than that of SynArsC and 3.7, 29.4, 14.0 and 47.8-fold higher than other arsenate reductases like R773, PvACR2, Acr2p, and OsACR2 respectively, but it was 2.6-fold less than *S. aureus* (Table 1). The Km of Alr1105 for arsenate (16.0 mM) was 12.8 and 63.2-fold higher than that reported for SynArsC and pI258 ArsC respectively, although it was significantly lower than Acr2p and PvACR2 (Table 1). It merits mention that the sequence analysis, complementation assay and biochemical characterization of Alr1105 strongly advocated it to be a functional arsenate reductase.

Apart from its arsenate reductase activity, this protein was found to catalyze hydrolysis of *p*-nitrophenyl phosphate thus acting as a phosphatase (Duan et al. 2007) (Fig. 6). This role

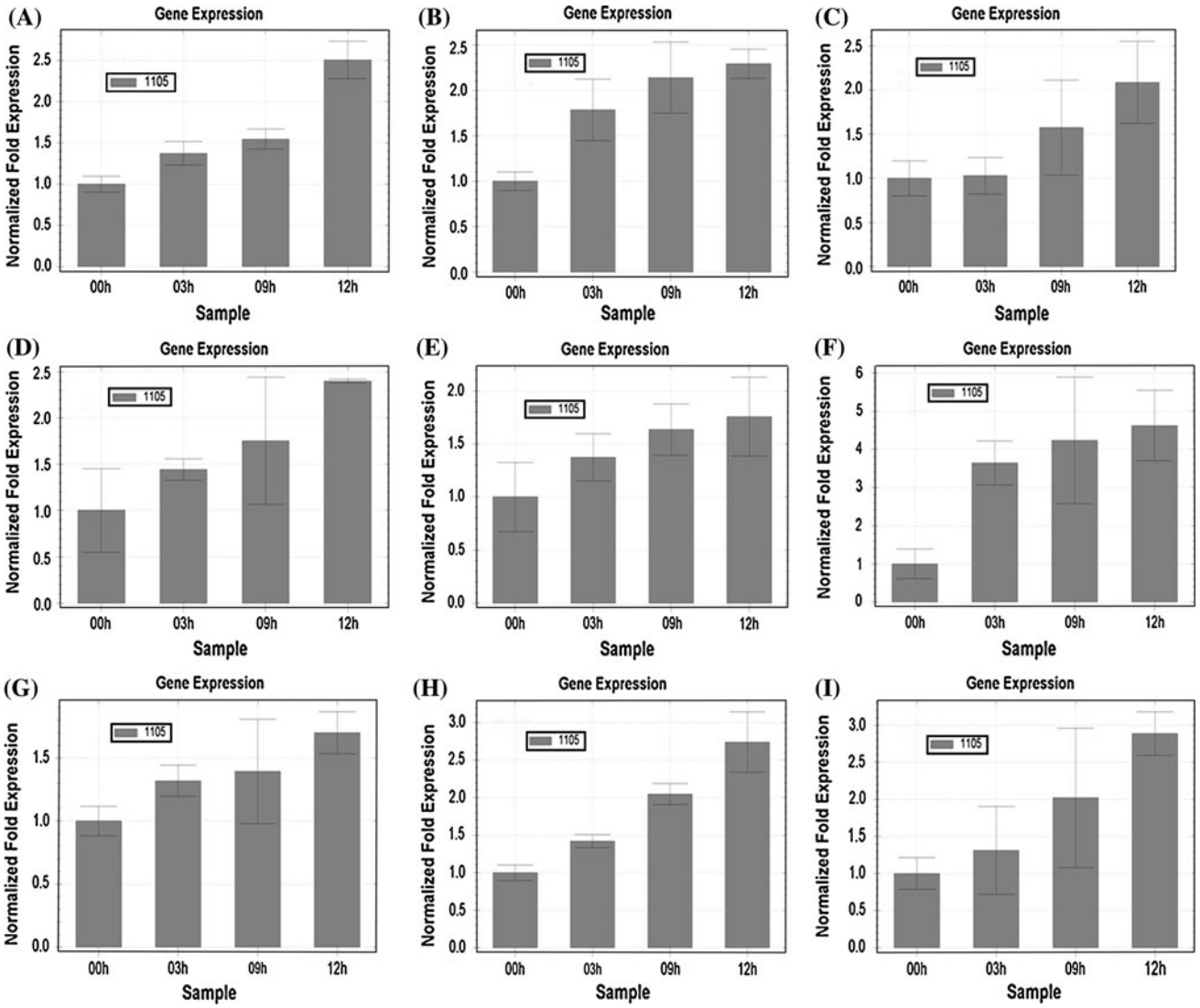

Fig. 9 qRT-PCR analysis of *alr1105* gene showing effect of **a** sodium arsenate, **b** $CdCl_2$, **c** $NiCl_2$, **d** $ZnSO_4$, **e** $CoCl_2$, **f** $CuCl_2$, **g** 47 °C, **h** carbofuron, and **i** UV-B at different time points

has also been demonstrated in *S. aureus* and *Synechocystis* with which our protein shares 43 and 65 % sequence identity respectively suggesting that it belongs to LMW PTP family proteins (Zegers et al. 2001; Li et al. 2003).

Furthermore, to score the role of *alr1105* in abiotic stress tolerance, growth response of *E. coli* was monitored under different stresses. Specific growth rate of complemented *E. coli* cells were much better than wild type and mutant under different stresses (Figs. 7, 8). In view of the fact that abiotic stresses like heavy metals, UV-B, heat and carbofuron generate reactive oxygen species (ROS) which upset the redox homeostasis of the cell and are highly toxic to nucleic acids, proteins, and cell membrane fatty acids hence impair the cellular metabolism leading to cell death (Imlay 2002). Better growth of complemented *E. coli* under above mentioned stresses suggests its role in combating oxidative stress which was further attested by its better growth in oxidative stress invoking chemicals such as MV, PMS and H_2O_2 (Fig. 8d–f). The above observations are supported by an induction of transcript of *arsC* gene of *Synechocystis* under H_2O_2 stress (Li et al. 2004) and by the work of Houot et al. (2007) where deletion mutant of the *arsC* gene of *Synechocystis* PCC6803 became more sensitive to Cd than the wild type strain. Results of our study are supported by Dhankher et al. (2003) where overexpression of bacterial arsenate reductase gene (*arsC*) provided Cd tolerance in *E. coli*, *Nicotiana tabacum* and *Arabidopsis thaliana*. The role of ArsC in offering protection against different stresses may be due to its protein tyrosin phosphatase activity which is known to tolerate heat shock and ethanol toxicity (Musumeci et al. 2005).

Table 3 Intracellular metal content in *E. coli* grown in medium supplemented with different metals

Metal	Wild type W3110 metal accumulation (ppm ± SD)	Wild type W3110+BSO metal accumulation (ppm ± SD)	Mutant WC3110 metal accumulation (ppm ± SD)	Mutant WC3110+BSO metal accumulation (ppm ± SD)	Mutant WC3110+*alr1105* metal accumulation (ppm ± SD)	WC3110+*alr1105*+BSO metal accumulation (ppm ± SD)
Cd	0.240 ± 0.019	0.014 ± 0.0025 (−94.1 %)	0.231 ± 0.019	0.012 ± 0.0020 (−95 %)	0.555 ± 0.061	0.055 ± 0.0089 (−90 %)
Ni	0.172 ± 0.042	0.017 ± 0.0017 (−90 %)	0.164 ± 0.042	0.016 ± 0.0017 (−90 %)	0.315 ± 0.066	0.290 ± 0.0920 (−7.9 %)
Zn	0.254 ± 0.034	0.015 ± 0.0010 (−94 %)	0.266 ± 0.034	0.019 ± 0.0010 (−93 %)	0.560 ± 0.72	0.510 ± 0.0078 (−8.9 %)
Co	0.471 ± 0.051	0.023 ± 0.0010 (−95.1 %)	0.478 ± 0.051	0.020 ± 0.0010 (−96 %)	0.765 ± 0.034	0.145 ± 0.0080 (−81 %)
Cu	0.330 ± 0.043	0.021 ± 0.0021 (−93.6 %)	0.328 ± 0.043	0.023 ± 0.0021 (−93 %)	0.921 ± 0.99	0.890 ± 0.0960 (−3.4 %)

In case of As (V) stress, an enhanced growth of recombinant *E. coli* may be due to reduction of As (V) into As (III) and its efflux from the cell (Lopez-Maury et al. 2003). Likewise a better growth of recombinant cells under Cd (II), Zn (II), Co (II), Cu (II) and Ni (II) may be due to their binding with amino acid residues of Alr1105 or sequestration of the metals by GSH-metal complex (Houot et al. 2007) leading eventually to metal accumulation inside the cell (Table 3). Increased intracellular metal content in recombinant *E. coli* in comparison to the control (Table 3) supports our proposition and finds support from the work of Dhankher et al. (2003) where transgenic tobacco over expressing bacterial arsenate reductase accumulated 30–50 % more Cd than the wild type. However, the difference in accumulation of metals may due to their binding affinity and stoichiometry (Dixit and Dhankher 2011). Furthermore, the differential metal accumulation may be explained in the light of the report of Sauge-Merle et al. (2003) in *E. coli* overexpressed with *A. thaliana phytochelatin synthase* gene. The increased metal content in recombinant *E. coli* correlated to an increased expression of *alr1105* as measured by qRT-PCR (Fig. 9) and an increase in the growth rate of the recombinant cells (Figs. 7, 8).

Further in order to find out whether *alr1105* or GSH is responsible for metal tolerance, metal accumulation was measured in the presence of BSO a known inhibitor of GSH biosynthesis (Table 3). Significant reduction in metal accumulation of the mutant in presence than in absence of BSO was observed. In contrast to this, *E. coli* WC3110+*alr1105* depicted some interesting findings. While Ni, Zn and Cu accumulation was low in the presence of BSO than in its absence, Cd and Co registered approximately 90 and 81 % decrease in accumulation thereby vividly demonstrating a role of GSH in Cd and Co accumulation and *alr1105* in Cu, Zn and Ni accumulation.

In view of the multiple abiotic stress tolerance, as also known for other cyanobacterial genes like *pcs, AhpC, dps* and *usp* (Chaurasia et al. 2008; Mishra et al. 2009; Narayan et al. 2010; Shrivastava et al. 2012), and metal accumulation the *Anabaena* sp. PCC7120 Alr1105 appeared more robust than the *E. coli* arsenate reductase. Thus, it may be used for developing transgenic cyanobacteria tolerant to oxidative as well as other abiotic stresses.

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