Biochemical, molecular and *in silico* characterization of arsenate reductase from pH, salt and arsenic tolerant *Bacillus thuringiensis* KPWP1

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Abstract

The objective of the present study was to characterize arsenate reductase of pH, salt and arsenate tolerant *Bacillus thuringiensis* KPWP1, isolated from contaminated surface water. Interestingly, it was found that the *arsC*, *arsB* and *arsR* genes involved in arsenate tolerance are distributed in the genome of KPWP1. The inducible *arsC* gene was cloned, expressed and the purified ArsC protein showed profound enzyme activity with the K_M and K_{cat} values as 25 μM and 0.00119 s^{-1} , respectively. *In silico* studies of KPWP1 ArsC revealed that in spite of 19–26% differences in gene sequences, the ArsC proteins of *Bacillus thuringiensis*, *Bacillus subtilis* and *Bacillus cereus* are structurally conserved and KPWP1 ArsC structure is close to nature. Docking and analysis of binding site showed that arsenate ion interacts with three cysteine residues of ArsC of KPWP1 and predicts that the ArsC from *B. thuringiensis* reduces arsenate by using the triple Cys redox relay mechanism.

1. Introduction

Arsenic is poisonous metalloid which exists in nature as different allotropic forms. Arsenic toxicity related to drinking water or other household purposes is a major problem worldwide. Arsenic in different chemical states and forms can cause chronic and acute health problems including cancer (Hughes 2002). Some microorganisms have evolved mechanisms to tolerate high concentrations of arsenic, and some even use it as a source of energy for their own growth (Ehrlich 1978). They use arsenic compounds as electron acceptors, electron donors and some of them possess the mechanisms of detoxification of its adverse effects (Diorio et al. 1995; Oremland and Stolz 2003). Arsenic redox activity involved in transformation of arsenic between the two states as oxidized arsenate and reduced arsenite. During aerobic respiration, arsenate is used as terminal electron acceptor by arsenate reducers, whereas, arsenite is involved as electron donor for autotrophic growth by chemoautotrophic arsenite oxidizers (Anderson and Cook 2004; Glasser et al. 2018). Although arsenite is more toxic and more common in anaerobic conditions, arsenate is the predominant form of inorganic arsenic in aqueous aerobic environments and due to its structural similarity to inorganic phosphate, arsenate can uncouple glycolysis and block TCA cycle and thus exerts different levels of toxicity in the living world (Hughes 2002).

Bacterial resistance to arsenic is mediated mainly by the gene products of *ars* operon which help them to extrude arsenic out of the cell mediated by the arsenic resistance gene system. Presence of *ars* operon in bacteria has been found both in transmissible plasmids as well as in chromosomes. The organization of *ars* operon in bacteria varies from strain to strain, but some essential genes are always conserved. The *ars* operon is generally made up of three (*arsRBC*) or five (*arsRDABC*) genes that have been organized into a single transcriptional unit (Diorio et al. 1995; Sato and Kobayashi 1998; Kruger et al. 2013). In the chromosomes of *E. coli* (Carlin et al. 1995; Diorio et al. 1995), *Bacillus subtilis* (Sato and Kobayashi 1998), *Pseudomonas aeruginosa* (Cai et al. 1998), three gene system encoding operon was found which encodes the arsenite transcriptional repressor - *arsR*, dedicated arsenite specific transmembrane pump, arsenite permease - *arsB* and arsenate reductase - *arsC*. Apart from the three essential genes, five genes operon (*arsRDABC*) encodes a negative regulatory protein that helps in the fine tuning of operon

expression – *arsD* which in turns controls the over expression of *arsB* and an ATPase – *arsA* which couples ATP hydrolysis by extruding arsenicals out of the cell collectively working along with *arsB* (Wu and Rosen 1993).

Although, arsenate resistance genes, in most of the cases, are found to be clustered together in *ars* operons (Sato and Kobayashi 1998), but there are reports that the arsenate resistance genes can also be dispersedly present in genome. Cytoplasmic arsenate reductase encoded by *arsC* gene is the key enzyme for the arsenate reduction process and arsenate resistance. ArsC (*arsC* gene product) proteins vary in their molecular weight range between 13–16 kDa and the presence of ArsC in cell cytoplasm facilitates the reduction of arsenate to arsenite with the help of glutaredoxin and glutathione in Gram-negative bacteria (Chen et al. 1986) or with the help of thioredoxin and thioredoxin reductase in Gram-positive bacteria (Messens et al. 1999; Shi et al. 1999) as redox couplers. Thus, two distinct families of arsenate reductase proteins are present in bacterial world (Silver and Phung 2005; **Lloyd and Oremland 2006**).

Arsenate reductase from Gram-negative *E. coli* plasmid R773 and Gram-positive *S. aureus* plasmid pI258 shares a very low (10%) sequence homology, although both the proteins do catalyze the same chemical reaction (Liu et al. 1995). The difference in redox partners elucidates the difference in sequence homology in different arsenate reductases. It was also experimentally shown that in *S. aureus* arsenate reductase, a cysteine pair Cys82 and Cys89 is needed to perform its job where a disulfide bond is formed by oxidation of these two residues, but in *E. coli*, this cysteine pair is not present in its ArsC protein (Bennett et al. 2001).

Among the Gram-positive bacteria, *arsC* genes are found to vary in their locations (Sato and Kobayashi 1998; Chauhan et al. 2019) and sequences as well. 65% identity in sequence homology was found between arsenate reductase from *S. aureus* and *B. subtilis* where three cysteine residues are conserved in both viz. Cys10, Cys82 and Cys89. A triple cysteine redox relay system was proposed for arsenate reduction mechanism containing these cysteine residues (Bennett et al. 2001). Cys 10 was considered to be the arsenate binding site in CX5R motif compiled in P-loop. In 2011, Jain et al showed the similarity between *B. cereus* and *B. subtilis* strains' arsenate reductase proteins containing P-loop as well as the important cysteine residues and confers such motifs ability of arsenate reduction (Jain et al. 2011).

Bacillus thuringiensis are Gram-positive bacteria that are well known for agricultural, health and economic interests (Wang et al. 2016). *B. thuringiensis* produces Cry proteins which can act as insecticides for a variety of plant insects and mosquitos (Boniolo et al. 2012; Khoury et al. 2014). *B. thuringiensis* also cited for its biodegradation and detoxification potentials. The importance of *B. thuringiensis* has been further accentuated by the recent discovery of its therapeutic activities against hook- worm and cancer (Ohba et al. 2009) when it encodes Cry proteins.

In the present study, we have elucidated the ability of *Bacillus thuringiensis* KPWP1 (Roy et al. 2010; Banerjee et al. 2013) to tolerate different pH, and different salt and arsenate concentrations. Presence of genes responsible for arsenate resistance mechanism and arsenate reductase activity in KPWP1 have led us to clone and express *arsC* gene, and purification and characterization of ArsC protein of *Bacillus*

thuringiensis KPWP1. Results from homology modelling showed that the ArsC proteins are structurally conserved. Patchdoc predicted the binding site for arsenate ion which conferred that same residues are involved in arsenate binding for *B.subtilis* ArsC and the ArsC of *Bacillus thuringiensis*.

2. Materials And Methods

2.1. Bacterial strain

The bacterial strain KPWP1 used in this study was isolated from Kolkata port water and identified as *Bacillus thuringiensis* (Roy et al. 2010). The water sample, collected from Kolkata port was found to possess arsenic at a concentration of 0.03 mg/L that is equivalent to 30ppb which is three times more than the permissible limit set by WHO. The isolated strain (*Bacillus thuringiensis* KPWP1) was deposited at the National Chemical Laboratory, India and numbered as NCIM 5352. The NCBI deposition numbers of the 16S rDNA sequence of KPWP1 is FJ897722 (Roy et al. 2010).

2.2. Arsenate tolerance test

To evaluate the arsenate tolerance ability, *Bacillus thuringiensis* KPWP1 cells were grown in nutrient media supplemented with increasing concentrations of arsenate. Inoculated cells were grown at 37°C for overnight under shaking condition at 150 rpm. The turbidity of grown cultures was measured in Shimadzu Instruments UV 2600 Scanning Spectrophotometer at 600 nm.

2.3. Salinity tolerance test

The salinity tolerance limit of KPWP1 was determined by adding increased concentrations of NaCl ranging from 0–5% (W/V) in nutrient broth growth media. Bacterial cells were inoculated and then incubated for overnight at 37°C under shaking at 150 rpm. The turbidity of grown cultures was measured in Shimadzu Instruments UV 2600 Scanning Spectrophotometer at 600 nm.

2.4. pH tolerance test

The pH tolerance ability of KPWP1 was determined by varying pH range from 5 to 9 in nutrient broth growth media. pH was adjusted by using 1N HCl or 5N NaOH. Bacterial cells were inoculated and grown at 37°C for overnight under shaking condition at 150 rpm. The turbidity of grown cultures was measured in Shimadzu Instruments UV 2600 Scanning Spectrophotometer at 600 nm.

2.5. Assay of Phosphate mediated inhibition of arsenic toxicity

Arsenate is a structural analogue of phosphate and thereby it enters the bacterial system via the phosphate transport pathway. To check the competitive inhibition of toxic effect of arsenate towards KPWP1 cells by the presence of phosphate in the growth media, varying concentrations of phosphate viz. 2.5 mM and 5 mM were added to nutrient broth in presence of 3 mM arsenate and the bacteria were allowed to grow. Normal NB without any added arsenate or phosphate was used as a control growth

condition. Bacterial cells (10^7 cells) were inoculated and then incubated at 37°C. The growth of bacterial species was measured in terms of optical density (OD) in Shimadzu Instruments UV 2600 Scanning Spectrophotometer at 600 nm at various time points at a difference of 100 minutes till 600 minutes from the addition of the initial inoculum.

2.6. Atomic Absorption spectroscopy

To determine the possible accumulation of arsenic inside the *Bacillus thuringiensis* KPWP1 cells, atomic absorption spectroscopy was done. KPWP1 cells were grown at 37 °C overnight in presence and absence of 100 µM arsenate. After treatment, cells were harvested followed by nitric acid digestion. Then the samples were analyzed using atomic absorption spectroscopic analysis methods (Santo et al. 2011).

2.7. Arsenate reductase assay using crude bacterial cell extract

Arsenate reductase assay was done to check the presence of arsenate reductase activity in KPWP1. Cell free crude extracts were prepared from *Bacillus thuringiensis* KPWP1. For that, bacteria were grown in NB medium supplement with/without various concentrations of arsenate, harvested by centrifugation for 10 minutes at 7500g, washed twice in reaction buffer (10mM Tris, pH – 7.5, 1mM Na₂EDTA and 1mM MgCl₂) and then finally resuspended in reaction buffer. PMSF was added at a final concentration of 1mM. Cells were disrupted by sonication (Anderson and Cook 2004). Unbroken cells were removed by centrifugation at 8000g for 15 minutes at 4°C. Arsenate reductase activity was measured based on NADPH oxidation which is coupled to arsenate reductase activity. NADPH oxidation was initiated by mixing crude extract, reaction buffer, DTT (300 µM), arsenate (100 µM) and NADPH (0.15 mM). Measurements were recorded in Shimadzu Instruments UV 2600 Scanning Spectrophotometer at 340 nm. In order to calculate the specific activity, protein estimation was carried out by using standard Bradford method at 595 nm (Bradford 1976). Bovine serum albumin was used as the standard.

2.8. Primers for arsenic resistance genes and PCR amplification

All the primers used for determination of arsenic resistance genes are presented in Table 1. Three different primer sets were used to amplify genes of arsenate reductase (*arsC*), arsenite transporter (*arsB*), and transcriptional regulator (*arsR*). PCR reactions, using *Bacillus thuringiensis* KPWP1 genomic DNA as templates, were performed in a final volume of 25 µl containing 20 ng of genomic DNA, 0.2 mM of dNTPs, 1.5 mM of MgCl₂, 0.4 µM of each primer, 1 U of Taq polymerase and 1X PCR buffer.

Table 1
Primers used in the study

Primer name	Primer sequence	Gene	Reference
P52F	AGCCAAATGGCAGAAGC	<i>arsC</i>	Bachate et al., 2008
P323R	GCTGGRTCRTCAAATCCCCA		
arsB Bt F	GTGGTTTAAATGAGGCGTGG	<i>arsB</i>	This study
arsB Bt R	CACCAATTGGCGCACTAGA		
arsR AH F	GCAATTGCTGATCCAACC	<i>arsR</i>	This study
arsR AH R	CGTTCATAGTACGAGAGCCAC		
Btk F	GGGAATTCATATGGAGAACAAAAAGACAATCTAC	Full length <i>arsC</i>	This study
Btk R	CCGCCTGAGCTCCCCTGTTTCAGAAAAGC		

2.9. Determination of distribution of *arsR*, *arsB*, and *arsC* genes in *Bacillus thuringiensis* KPWP1

To check the distribution of *arsR*, *arsB* and *arsC* genes in *Bacillus thuringiensis* KPWP1, the genomic DNA, isolated from KPWP1, was used as template and PCR were performed by using gene specific primer sets and the combinations of primers as *arsR* Fwd and *arsB* Rev, *arsB* Fwd and *arsC* Rev, and *arsR* Fwd and *arsC* Rev, respectively. PCR products were then analyzed in 1% agarose gel.

2.10. Determination of the effect of arsenate in expression of *arsC* gene in *Bacillus thuringiensis* KPWP1

To check the influence of arsenate in the expression of *arsC* gene in *Bacillus thuringiensis* KPWP1, cells were grown in NB media in presence of 600 µM, 1200 µM and 5000 µM concentrations of arsenate. Following arsenate treatment, total RNAs were isolated from cells and cDNAs were prepared. The expression of *arsC* gene was determined by RT-PCR using the isolated cDNA preparations as templates and primer set specific for *arsC* gene.

2.11. Cloning and expression of *arsC* gene

To characterize the ArsC protein, *arsC* gene was amplified and cloned. Primers were designed for full length gene based on *Bacillus thuringiensis* serovar konkukian str. 97-27, GenBank Accession no. AE017355.1. After amplification, the products were purified using Qiaquick gel extraction kit and ligated into cloning vector (TA vector: pTZ57R/T). The ligated products were then transformed in *E. coli* XL1-Blue strain. Colonies were obtained and tested for the presence of the desired gene products (**Sambrook and Russel 2001**). The genes were further inserted into expression vector (pET20b which contain C terminal

His tag sequence) after treatment with modifying enzymes (restriction enzyme Nde1 and Xho1), which were then transformed into BL21 (DE3) strains. The cells were induced with 0.5 mM of IPTG for over-expression of the proteins. For ArsC protein, induction was carried out at 37°C at 100 rpm for 4hrs after IPTG addition.

2.12. Phylogenetic analysis arsC gene

The similarity of *arsC* gene sequence of KPWP1 was determined by using the BLAST database search. Eighteen sequences (first 19 hits for each isolate) of the culturable organisms were procured from NCBI-Blast search. Alignment was done by using CLUSTAL X2 software. The alignment was thoroughly checked in software SEAVIEW for any gaps and edited accordingly. A phylogenetic tree was constructed by neighbour joining method (Kimura 2- parameter) using MEGA v4.0 and the tree was subsequently bootstrapped (Random speed 64328, 1000 replicates). Bootstrap values were also calculated to create a pseudo alignment by taking random position of original alignment.

2.13. Purification of ArsC protein

Induction of ArsC protein was carried out at 37°C at 100 rpm for 4 hours after 0.5 mM IPTG addition (**Sambrook and Russel 2001**). The cells were then harvested at 2,300 g for 8 minutes at 4°C, washed with 0.9% NaCl, and then resuspended in 2 ml of lysis buffer (150 mM NaCl, 50 mM Tris- cl, pH 8.0 and Imidazole 5 mM). Cells were then disrupted by sonication and then centrifuged at 16,100 g for 15 minutes at 4°C. Supernatant was taken and added to the equilibrated Ni-NTA agarose beads. Washing of the beads was carried out with lysis buffer followed by three consecutive wash buffers. The desired protein was then eluted with varying the Imidazole concentrations from 50 mM to 250 mM. Purified protein was dialyzed in buffer containing 20mM Tris – Cl, pH 7.5, 150 mM NaCl and 1mM PMSF.

2.14. Arsenate reductase assay using purified protein

ArsC in Gram positive system requires coupling to Thioredoxin and Thioredoxin reductase for proper activity of the protein (**Messens et al. 1999**). So, after protein purification, arsenate reductase assay was performed by using these couplers. ArsC of concentration 200 µM was used for this assay. ArsC enzyme activity was measured in presence of 0.42 µM thioredoxin and 0.14 µM thioredoxin reductase (both the chemicals were purchased from Sigma Aldrich). The assay was performed by using same method used in case of crude extract. The K_M and K_{cat} values of the purified reductase protein was measured by varying the substrate concentration from 0 µM to 150 µM and keeping the enzyme concentration constant at 100 µM. All measurements were recorded in Hidex Chamelion V multimode plate reader at 330 nm.

2.15 Deducing the protein sequence

The sequence for ArsC protein of *Bacillus thuringiensis* were deduced by translating the DNA sequence obtained after sequencing of the amplified gene product by using 'TRANSEQ'.

2.16 Homology modeling and structure validation

Homology models for ArsC protein were constructed using workspace in SWISS-MODEL server (Arnold et al. 2006; Kiefer et al. 2009). X-ray crystallographic structure of *B.subtilis* 1JL3 was obtained from Protein Data Bank (PDB). The homology models constructed were then validated by 'PROCHECK' using the "Structure Assessment" tool in SWISS-MODEL server and ProSA web tool (Wiederstein and Sippl 2007).

2.17. Identification of functional origin

ConSurf server (Celniker et al. 2013) identifies the functional region of the protein by finding out the evolutionary conserved regions based on Multiple Sequence alignment. The functional region of the ArsC protein was identified using the ConSurf server.

2.18. Binding site prediction and docking of arsenate

The binding sites were predicted with Q-Site Finder. The arsenate ion was then docked into the ArsC protein using PatchDock(Duhovny et al. 2002) web-based server. The results obtained were then visualized and analyzed using UCSF Chimera software package (Pettersen et al. 2004).

3. Results

3.1. Salinity, pH and Arsenate tolerance by *Bacillus thuringiensis* KPWP1

Bacillus thuringiensis KPWP1 was found to tolerate a wide range of salt (NaCl) concentrations. KPWP1 grew well up to 3% of NaCl, beyond that KPWP1 showed reduced growth but could tolerate up to 5% of NaCl when grown overnight in nutrient broth (Fig. 1a). Interestingly, KPWP1 showed its ability to grow considerably in a wide range of pH (5.5 to 9) of the growth (Fig. 1b). Further, KPWP1 showed its ability to tolerate arsenate concentration up to 6 mM, although reduced growth was observed for 3 and 6 mM of arsenate in growth media (Fig. 1c).

3.2. Competitive inhibition of arsenate toxicity by phosphate, arsenate accumulation and arsenate reductase activity

To have an initial insight into the mechanism of arsenate tolerance activity, KPWP1 cells were allowed to grow in absence or presence of 3 mM arsenate, 10 mM phosphate and 3 mM arsenate plus 2.5 or 5 mM phosphate. It was observed that 3 mM arsenate had profound growth inhibition towards KPWP1 compared to control growth. Whereas, 10 mM phosphate in growth medium had some inhibitory effect on growth of KPWP1, but it was not considerable. Interestingly, it was perceived that presence of phosphate in growth media could rescue (dose dependent manner) the toxic or inhibitory effect of 3 mM arsenate towards the growth of KPWP1 cells (Fig. 2a). These results indicate competitive inhibition of arsenate transport by phosphate as arsenate is a structural analogue of phosphate and thereby it enters the bacterial system via the phosphate transport pathway.

Transport of arsenate inside the KPWP1 cells was detected by atomic absorption spectroscopy technique. KPWP1 cells were grown in presence or absence of 100 μ M arsenate and amount of arsenic/biomass of KPWP1 was determined. It was observed that 2.2 μ g of arsenate/gm of bacterial biomass of KPWP1 was accumulated inside of bacterial cells. This result signifies that *Bacillus thuringiensis* KPWP1 can transport arsenic inside the cells when present in an arsenic rich environment.

As it was observed that arsenate can be transported inside of KPWP1 cells, the presence of arsenate reductase activity in KPWP1 cells was tested by using crude cell extract. For that, KPWP1 cells were grown in nutrient broth in absence or presence of 600 and 1200 μ M arsenate and arsenate reductase activity was measured in crude extract of KPWP1 cells grown in different conditions. Results (Fig. 2c) showed that KPWP1 cells harbor arsenate reductase activity and more interestingly the arsenate reductase activity of KPWP1 was observed to be inducible in presence of increasing amounts of arsenate in growth media.

3.3. Presence and distribution of *arsR*, *arsB*, and *arsC* genes in *Bacillus thuringiensis* KPWP1

The observed presence and induction of arsenate reductase activity in KPWP1 led the present study to look for the presence of arsenate operon or related genes (reductase gene, *arsC*, arsenite transporter gene, *arsB* and transcriptional repressor, *arsR*) in KPWP1. The presence of genes of interest in KPWP1 was tested by using genomic DNA as a template and respective primer sets (Table 1). 275 bp fragment of putative *arsC* gene, 408 bp fragment of putative *arsB* gene and 233 bp fragment of putative *arsR* gene were found to be amplified (Fig. 3a-c). Sequence analysis confirmed the presence of *arsC*, *arsB* and *arsR* genes in the genome of *Bacillus thuringiensis* KPWP1 (Data not shown).

As the *arsR*, *arsB* and *arsC* genes were detected in the genome of KPWP1, it was further checked whether these genes are present together in the chromosome as a single transcription unit (operon) or the genes are scatterdly distributed. For that, the genomic DNA, isolated from KPWP1, was used as template and PCR were performed by using gene specific primer sets and the combinations of primers as *arsR* Fwd and *arsB* Rev, *arsB* Fwd and *arsC* Rev, and *arsR* Fwd and *arsC* Rev, respectively. No amplification was observed for the selected primer sets. Whereas, PCR products for *arsR*, *arsB* and *arsC* were observed, when gene specific primer sets were used (Fig. 3d). Thus, it may be concluded that the *arsR*, *arsB* and

arsC are present as scatteredly or differentially distributed genes in the genome of *Bacillus thuringiensis* KPWP1.

3.4. Inducible expression of *arsC* in *Bacillus thuringiensis* KPWP1 in presence of arsenate

Arsenate inducible expression of *arsC* gene was determined by normalizing the intensities of *arsC* specific bands in agarose gels with that of *16S rRNA* (Fig. 3e). Results clearly indicated that arsenate in growth media could induce expression of *arsC* gene in *Bacillus thuringiensis* KPWP1. It was observed that 600 μ M, 1200 μ M and 5000 μ M of arsenate in growth media could induce 23%, 40% and 67% of *arsC* over-expression, respectively, compared to the normal expression level.

3.5. Cloning and phylogenetic analysis of *arsC* gene

Full length arsenate reductase (*arsC*) gene comprises of 402 bp was first amplified by using cloning specific primers *arsC* BtK F and *arsC* BtK R (Table 1). *arsC* gene was then cloned in bacterial expression vector pET20b. Digestion of cloned plasmid with restriction enzymes Nde1 and Xho1 yielded a fragment of 402 bp (Fig. 4), thus confirmed insertion of the right size of *arsC* gene. Further, *arsC* gene cloning in pET20b was confirmed by sequencing. The sequence of cloned *arsC* gene was deposited to GenBank (Accession number KM978920). Phylogenetic analysis of KPWP1 *arsC* gene was done by using Kimura2 parameter and Mega4 software (Fig. S1). *arsC* gene sequence phylogeny placed the KPWP1 *arsC* gene in an alignment with other *Bacillus cereus* and *Bacillus thuringiensis* *arsC* sequences, thereby determined that the test sequence is of *Bacillus thuringiensis* origin. Bootstrap values are shown if less than 100%.

3.6. Purification of ArsC protein

For purification of ArsC protein, *arsC* gene was induced in BL21 (DE3) cells, harboring *arsC* gene containing pET20b plasmid, by adding 0.5 mM IPTG in the growth medium. Expressed ArsC protein was purified by using NiNTA agarose beads. Distinct protein band of molecular weight 14.85 kDa for full length ArsC was observed in 14% SDS-PAGE (Fig. 5). Proteins eluted with 50 mM and 100 mM imidazole, represented in Lanes 6 and 7 was used for further experiments.

3.7. Arsenate reductase assay using purified protein

Initially, after expression of *Bacillus thuringiensis* KPWP1 ArsC protein in *E. Coli* BL21 strain, arsenate reductase activity was tested using the crude extract of the clones in BL21. Interestingly, it was found that the full length protein could not exert any activity compared to the control cell extract (data not shown). A probable explanation is that the *arsC* gene was of Gram positive bacterial origin, which uses thioredoxin and thioredoxin reductase as a redox coupler, and this experiment was done using cell free extract of Gram negative bacteria and in the absence of redox coupler. Therefore, the purified full length arsenate reductase protein was tested for its enzyme activity in a coupled thioredoxin, thioredoxin reductase, NADPH oxidation assay. The purified arsenate reductase of *Bacillus thuringiensis* KPWP1 showed a

specific activity of 1819 $\mu\text{M}/\text{min}/\text{mg}$ of protein for 100 μM substrate in reaction. The K_M and K_{cat} values of the purified reductase protein were found to be as 25 μM and 0.00119 s^{-1} , respectively.

3.8. Sequence alignment and sequence comparison of ArsC

The deduced protein sequence of the *B. thuringiensis* KPWP1 was compared with *B. subtilis* and *B. cereus* ArsC protein sequence which showed homology of 73.88% and 84.85%, respectively (Fig. 7). The sequence comparison showed that the *B. thuringiensis* KPWP1 ArsC has the same conserved motifs and residues as found in *B. subtilis* X-ray crystal structure i.e., CX5R motif, Cys10, Cys 82, Cys 89, Arg 16 and Asp 105 which are required for binding and redox-relay reaction during arsenate reduction. The conserved residues are marked in Fig. 6.

3.9. Homology modeling and structure validation

To comprehend the catalytic mechanism of the full length ArsC protein of KPWP1, comparative homology models were prepared using SWISS-MODEL Workspace and refined by energy minimization (Fig. S2). Two models were thus constructed (Fig. 7), one based on the *B. subtilis* 1JL3 PDB (Fig. 7b) and another based on the homology model of *B. cereus* (Fig. 7e). The homology model constructed based on *B. subtilis* 1JL3 PDB contains four beta-sheets and five alpha-helices and model constructed based on *B. cereus* contains four beta-sheets and four alpha-helices, as because the C-terminal part was missing in *B. cereus* template used for modeling (details given in Table S1). The two protein structures modeled were found to be similar to the *B. subtilis* template having the conserved motifs and residues required for the redox relay reaction.

The homology models constructed were validated by checking the stereochemistry of the amino acids in Ramachandran plot and calculating the Z-Score by PROCHECK and ProSA servers, respectively (Fig. S3). Ramachandran plot, calculated by using PROCHECK for the homology model based on *B. subtilis* showed that 92.1% of the residues of KPWP1 ArsC protein lie in allowed region, 7.9% residues are in additional allowed region and none of the residue is present in disallowed region. Homology model based on *B. cereus* showed that 92% of the residues of KPWP1 ArsC protein lie in allowed region, 8% residues are in additional allowed region and none of the residue is present in disallowed region. A good structure should have 90% of its residues in the allowed region. Both homology models here have above 90 % of its residue in the allowed region of Ramachandran plot. Z score for the homology model constructed, based on *B. subtilis* PDB, was found to be -7.16 and that of homology model constructed, based on *B. cereus* PDB, was - 5.85 which falls within the range, calculated based on the known crystal and NMR structures of same amino acid length. The energy plots also showed that the predicted structures are also energetically stable. Based on the Ramachandran plot and the Z-score it can be said that the predicted structures are close to nature.

3.10. Identification of functional region and binding site prediction

The theoretical functional regions of the ArsC protein for *B. thuringiensis*, *B. cereus* and *B. subtilis* were identified by submitting the structures in the ConSurf server. The residues having score 9 are evolutionarily conserved residues and are supposed to play role in the function of the protein. The residues having score 1 represent most variable regions of the protein. The amino acid conservation score along the total length of the ArsC protein for *B. thuringiensis*, *B. cereus* and *B. subtilis* are given in the Table S2. From the comparison it is clear that residues having score of 8 or 9 are conserved in all the three ArsC proteins of *Bacillus* species.

The arsenate binding site in ArsC protein of *B. thuringiensis* KPWP1 was identified by Q-site finder. Top 5 results (based on Q-score) were taken for further analysis and it was found that the first predicted binding site contains all evolutionarily conserved residues based on the ConSurf result (Table S2). The other predicted site does not contain the conserved residues. Details of the five predicted binding site are given in the Table S3.

The arsenate ion was docked in full length homology model and the amino acids interacting with the arsenate ions were analyzed (Fig. 7g). On analyzing the binding site it was observed that the arsenate ion binds and interacts with the same amino acids as it was observed in case *B. subtilis* ArsC protein (Bennett et al. 2001). Hence, it can be assumed that the ArsC from *B. thuringiensis* reduces arsenate in a similar fashion as seen in *B. subtilis* and follows the triple Cys redox relay mechanism.

4. Discussion

Bacterial mechanism of arsenic uptake in the cell has drawn considerable interest of researchers since last few decades (Altowayti et al. 2019). It has been reported back in 1978 about the relationship between phosphate and arsenate transport in arsenate resistant bacteria where two variations of strains were observed and one of which could grow in presence of high phosphate but not in low phosphate content in growth medium, and another could grow in both, but did not allow arsenate to enter into the cell (Alfasi et al. 1979). Reports are also there which showed that *Bacillus cereus* has the ability to uptake phosphate and it gets increased if the cell is starved for almost 2 hours (Rosenberg et al. 1969). In this study, it was observed that *Bacillus thuringiensis* KPWP1 can resist arsenate up to 6mM of concentrations in growth media. In the presence of only arsenate (3mM), growth of KPWP1 cells was observed to be slows down due to the toxic effect of arsenate onto the cells, but when the cells were grown in presence of both arsenate and phosphate, arsenic toxicity was reduced by the presence phosphate in a dose dependent manner (Fig. 2a). This result depicts the rescue nature of phosphate against the toxicity due to the presence of arsenate in growth media and such rescue by phosphates was most likely manifested by competitive inhibition of arsenate transport in *Bacillus thuringiensis* KPWP1 cells. *Bacillus subtilis* is known to possess both the phosphate transporters Pit and Pst, but Pit transporter is only used through which arsenate gets into the cells (Qi et al. 1997). Entry of arsenate inside the KPWP1 cells was further confirmed through inductively coupled plasma mass spectrometry which showed arsenic accumulation in KPWP1 cells when grown in presence of arsenate.

As most of the bacteria can not restrict the entry of arsenate as it is a structural analogue of phosphate, the main step for arsenate tolerance lies on the activity of *arsC* gene encoded ArsC protein which reduces intracellular arsenate to arsenite, which further gets excluded from the cells with the help of ArsB protein. Several studies have undergone on arsenate reductase and it has emerged that two classes of arsenate reductase enzymes are prevalent among the bacterial communities. First, Gram-positive bacteria possessing arsenate reductases which generally use thioredoxin and thioredoxin reductase as redox coupler, and the second class of ArsC proteins of those of Gram-negative bacteria that use glutathione and glutaredoxin as redox coupler (Gladysheva et al. 1994; Messens et al. 1999). Recently, in *Anabaena* sp., a new variety of arsenate reductase has been found which involves in arsenic detoxification. The protein belongs to the thioredoxin superfamily and has functional motifs similar to other arsenate reductase enzymes with respect to the conserved residues present in it (Pandey et al. 2013).

Interestingly, the isolated *Bacillus thuringiensis* KPWP1 was found to be resistant to a wide range of pH and high concentrations of NaCl and arsenate (Fig. 1). Also, the genes (*arsC*, *arsB* and *arsR*) involved in resistance against arsenate toxicity were found to be scatterly distributed in the genome of *Bacillus thuringiensis* KPWP1 (Fig. 3d). Moreover, it was also observed that the expression of *arsC* gene in KPWP1 is inducible in presence of increasing concentrations (0.6 to 5 mM) of arsenate in growth media (Fig. 3e). Thus, it was imperative to clone, express the *arsC* gene and characterize the ArsC protein of *Bacillus thuringiensis* KPWP1. Interestingly, when crude cell extract from induced *E. coli* BL21 (Gram-negative bacteria where the *arsC* gene of *Bacillus thuringiensis* KPWP1 was cloned and expressed) was used, no considerable enzyme activity was observed (data not shown). A probable explanation was the absence of cognate redox coupler needed in Gram positive bacteria (*Bacillus thuringiensis* KPWP1) to perform the reaction efficiently. However, the purified protein showed considerable activity (K_M 25 mM and K_{cat} 0.00119 s^{-1}) in presence of cognate redox coupler. In this study, the structure of arsenate reductase of KPWP1 was modeled by using ArsC proteins of *B. subtilis* and *B. cereus* as templates. Interestingly, it was found that ArsC protein of KPWP1 is structurally similar to both *Bacillus subtilis* and *Bacillus cereus* ArsC proteins. For *Bacillus cereus* ArsC protein (DQ517938), the reported structure was devoid of C-terminal helix region. Therefore, the homology structure of ArsC of *Bacillus thuringiensis* derived by using *Bacillus cereus* ArsC (DQ517938) as template did not contain the C-terminal region. Ramachandran plot construction as well as the Z-score of the predicted structure of KPWP1 ArsC protein validated the stability of the predicted model. Docking of arsenate ion showed the interacting residues with the ion is same for ArsC proteins of KPWP1 and *B. subtilis*.

Declarations

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Availability of data and material: In this study all data generated or analysed are included in the article.

Code availability: Not Applicable

Authors' contributions: PB, AC, PPD and TKS conceived the study and designed the experiments. PB, AC and SJ performed the experiments and analysed the data. NB analysed the data. PB and TKS have written the manuscript.

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Figures

Figure 1

Growth of *Bacillus thuringiensis* KPWP1 under (a) 0 to 5% w/v NaCl, (b) 5.5 to 9.0 pH and (c) 0 to 25 mM concentration of sodium arsenate in Nutrient broth medium. Growth of bacteria was measured as OD at 600 nm and results represent mean $\pm$ SD from three independent experiments ($n = 9$).

(a)

(b)

Figure 2

(a) Phosphate mediated inhibition of arsenic toxicity towards KPWP1. Growth of KPWP1 was measured in absence or presence of 3mM sodium arsenate, 10mM sodium orthophosphate and 3mM sodium arsenate together with varying concentrations (2.5 mM, 5 mM) of phosphate. (b) Evaluation of arsenate reductase activity of *Bacillus thuringiensis* KPWP1 in presence of varying concentrations of arsenate in NB media. Data represent mean $\pm$ SD of three independent experiments (n=9).

Figure 3

Amplification of (a) *arsC* gene: Lane 1- 1 kb DNA ladder, Lane 2 - -ve control, Lane 3 – PCR product of *arsC* gene of KPWP1. (b) *arsB* gene: Lane 1- 1 kb plus DNA ladder, Lane 2 – Blank, Lane 3 - -ve control, Lane 4 – PCR product of *arsB* gene of KPWP1 and (c) *arsR* gene: Lane 1- 1 kb plus DNA ladder, Lane 2 – Blank, Lane 3 - -ve control, Lane 4 – PCR product of *arsR* gene of KPWP1. (d) Agarose gel electrophoresis of PCR products to check the nature of distribution (in operon or scattered) of *arsR*, *arsB*, and *arsC* genes

in *Bacillus thuringiensis* KPWP1. Lane 1 – -ve control of *arsR* F and *arsB* R, Lane 2 – PCR product of *arsR* F and *arsB* R, Lane 3 – -ve control of *arsB* F and *arsC* R, Lane 4 – PCR product of *arsB* F and *arsC* R, Lane 5 – -ve control of *arsR* F and *arsC* R, Lane 6 – PCR pdt. of *arsR* F and *arsC* R, Lane 7 – 1 kb plus DNA ladder, Lane 8 – -ve control of *arsR* gene primers, Lane 9 – *arsR* gene Lane 10 – -ve control of *arsB* gene primers, Lane 11 – *arsB* gene, Lane 12 – -ve control of *arsC* gene primers, Lane 13 – *arsC* gene. (e) Differential expression of *arsC* gene in KPWP1 in presence of varying arsenate concentrations. Differential expression was quantified by measuring the intensity of *arsC* bands over that of 16S rRNA as control.

Figure 4

Agarose gel electrophoresis showing presence of the cloned *arsC* gene. pET20b plasmid, containing the inserted full length *arsC* gene, was double digested with *Nde*I and *Xho*I. Lane 1- 1 kb plus DNA ladder, Lane 2 – Undigested plasmid containing full length *arsC* gene, Lane 3 – Digested plasmid containing full length *arsC* gene, Lane 4 – Plasmid pET 20b. The arrow indicates the band for *arsC* gene in Lane 3.

Figure 7

Energy minimized homology model by using SWISS-MODEL workspace and chimera. (a) *Bacillus thuringiensis* KPWP1 arsenate reductase (ArsC) by using *Bacillus subtilis* 1JL3 ArsC (b) structure as template. (c) Represents superimposed structures of *Bacillus thuringiensis* KPWP1 and *B. subtilis* 1JL3 ArsC proteins. (d) *Bacillus thuringiensis* KPWP1 arsenate reductase (ArsC) by using *Bacillus cereus* DQ517938 ArsC (e) as template. (f) Represents superimposed structures of *Bacillus thuringiensis* KPWP1 and *Bacillus cereus* DQ517938 ArsC proteins. (g) Docking of arsenate ion in arsenate reductase protein, ArsC of *Bacillus thuringiensis* KPWP1 by using “PatchDoc”.

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