Construction and purification of His-tagged staphylococcal ArsB protein, an integral membrane protein that is involved in arsenical salt resistance

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Abstract Bacterial resistance to arsenical salts encoded on plasmid pI258 occurs by active extrusion of toxic oxyanions from cells of *Staphylococcus aureus*. The operon encodes for three gene products: ArsR, ArsB and ArsC. The gene product of *arsB* is an integral membrane protein and it is sufficient to provide resistance to arsenite and antimonite. A poly His-ArsB fusion protein was generated to purify the staphylococcal ArsB protein. Cells containing the His-tagged *arsB* gene were resistant to arsenite and antimonite. The levels of resistance to these toxic oxyanions by the His-tagged construct were greater than the levels obtained with the wild type gene. These data would indicate that the His-tagged protein is functionally active. A new 36 kDa protein band was visualized on 10% SDS-polyacrylamide gel electrophoresis (PAGE), which was confirmed as the His-ArsB protein by immunodetection with polyclonal His-antibodies. The His-ArsB fusion protein was purified by the use of metal-chelate affinity chromatography with a Ni²⁺-nitrilotriacetic acid column and size-exclusion chromatography suggests that the protein was a homodimer.

Keywords Arsenite · Membrane protein · Transport · Affinity chromatography · Arsenic resistance

Introduction

Arsenic is one of the prevalent toxic metals in the environment and it has been used as antimicrobial agent for a long time [1]. Bacteria have developed many mechanisms to overcome the toxicity of arsenic [2, 3]. One of the best characterized microbial arsenic resistance mechanisms is mediated by active efflux of the heavy metals from the cell. Although the most thoroughly studied bacterial arsenical resistance system is the *ars* operon encoded by the conjugative plasmid R773, which confers resistance to arsenate (As^V), arsenite (As^{III}) and antimonite (Sb^{III}) in gram-negative bacteria, analogous *ars* operons have been found on bacterial chromosomes and other transmissible plasmids in gram-positive and gram-negative bacteria. These operons are organized into a single transcriptional unit containing either three (*arsRBC*) or five (*arsRDABC*) genes. The three-gene system is present in several bacterial chromosomes [4] and in *Staphylococcus* plasmids pI258 [5, 6] and pSX267 [7], while the five-gene system is present on plasmids including plasmids R773 and R46 [8, 9].

The five-gene *ars* system consists of three structural genes: *arsA* (ATPase), *arsB* (arsenite permease) and *arsC* (arsenate reductase) [8, 10, 11] and two regulatory genes: *arsR* [12] and *arsD* [13]. The *arsA* and *arsB* gene products are sufficient for resistance to arsenite and antimonite and function as an efficient anion pump (an arsenite-translocating ATPase) [14]. Although the *arsB* gene product alone will provide resistance to these two metalloids, the presence of the ArsA increases the level of resistance [15]. The *arsC* gene product is required in conjunction with *arsA* and *arsB* products to confer arsenate resistance and functions to

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convert arsenate to arsenite prior to efflux. With the three gene system, the ArsB permease functions as a uniporter and uses membrane potential to extrude arsenite and antimonite oxyanions [15, 16].

Although the gene products from R773 and both staphylococcal *ars* operons were identified by use of the T7 expression system [17, 18], only the ArsR, ArsA and ArsC proteins from these systems have been purified [12, 19–23]. The *arsB* gene product has been identified [24], but it has not been purified in any of the *ars* systems. The ArsB protein from plasmid pSX267 [23] is 98% identical to the pI258 ArsB protein [17] differing in only five amino acids. The predicted amino acid sequence of the staphylococcal *arsB* gene product exhibits 58% identity to the *arsB* gene product from plasmid R773. An examination of the hydropathy profiles of the two *arsB* gene products shows a high degree of similarity and suggests that the topology and quaternary structure of the two proteins are similar [25]. The topology of the ArsB protein from R773 has been validated experimentally [26]. The purpose of this study was to generate a functional His-tagged ArsB protein and develop methods for its purification.

Materials and methods

Bacterial strains, plasmids and growth conditions

The following *Escherichia coli* strains and plasmids were used in this study: JM109 [27], M15 [28], C41(DE) [29], pUC19 [27], pGJ104 [17], pQE32 [28], pQE16 [28] and pREP4 [28]. Cells were grown in Luria Bertani (LB) medium [27]. Where required, ampicillin (50 µg/ml) and kanamycin (25 µg/ml) were added to the growth medium, unless otherwise indicated. Cultures were incubated with aeration at 37°C, unless otherwise stated.

Construction of pArsB encoding the staphylococcal ArsB-polyhistidine fusion protein

The *arsB* gene encoding integral membrane ArsB protein from pGJ104 [17] was amplified by PCR using the primers 5'-GTGGGATCCGGTGACTGTTGATGACTATT-3' (N-terminus) and 3'-CCAACCCCTTTCCTTTATCAGCTGCTT (C-terminus). The amplified *arsB* DNA was purified by agarose gel electrophoresis in a 2% seaplaque matrix. The 1387 base DNA fragment was excised from the agarose gel and eluted by the use of a Qiax II gel purification kit according to the manufacturer's recommendations. The resulting 1.38 kb product (with *Bam*HI and *Sal*I sites [underlined] flanking the *arsB* gene) was digested with *Bam*HI and *Sal*I, ligated into a similarly digested vector

(pQE32), and transformed in JM109. Selection was on ampicillin followed by screening for arsenite and antimonite resistance.

Molecular biology

The conditions for plasmid isolation, DNA restriction endonuclease analysis, ligation and transformation have been described [27]. DNA sequencing was performed on an ABI automated sequencer at the University of New Hampshire DNA Sequencing Facility.

Protein assay

Protein content was estimated by a modification of the procedure of Lowry et al. [30].

Measurement of arsenical salt resistance

Arsenical salt resistance was measured by a growth inhibition assay [17] on solid medium or in liquid medium. For the plate assay, cells were streaked onto LB plates containing varying concentrations of freshly-made, filter-sterilized, sodium arsenite or potassium antimony tartrate and incubated at 37°C. The results were recorded for 24 and 72 h.

For the broth assay, cells from a freshly-grown overnight culture were diluted 100-fold into fresh LB broth containing 50 µg/ml ampicillin and different concentrations of arsenite or antimonite. Cells were incubated with aeration at 37°C for 6 h and the turbidity (OD₆₀₀) of the culture was measured by the use of a spectrophotometer. For some experiments, cells were induced with 1 mM isopropyl-B-D-thiogalactoside (IPTG). The OD₆₀₀ versus arsenical salt concentration for each strain was plotted. For comparative purposes, all curves were standardized to the same OD₆₀₀ for the control (0 mM arsenical salts).

Induction of the His-tagged ArsB protein

Fresh overnight cultures were diluted 1:20-fold into pre-warmed LB broth containing the appropriate antibiotics and were incubated at 37°C with aeration. When the cultures reached an OD₆₀₀ between 0.5–0.7, 1 mM IPTG was added and the culture was induced for 6 h.

Cell membrane isolation

Isolation of cell membranes and separation of inner and outer membranes were performed as described previously [24]. Membrane preparations were used immediately or stored at –80°C until further analysis.

Membrane solubilization experiments

The detergents, Triton®X-100, n-octylglucoside, Nonidet® P40, n-dodecylmaltoside, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)], deoxycholic acid, digitonin and Tween® 20, were added to 100 µl [8.5 mg/ml protein] of inner membranes to a final concentration of 1.0%. These suspensions and a control (inner membrane without detergent) were incubated at 0°C (on ice) for 1 h with gentle agitation. To pellet insoluble material, the suspensions were centrifuged at 200,000 × g for 1 h at 4°C. After centrifugation, the supernatant fluids were removed and concentrated by cold acetone precipitation. Both the insoluble pellets and the concentrated supernatant samples were stored at –20°C until further analysis.

Polyacrylamide gel electrophoresis

Samples were prepared by boiling in Laemmli SDS sample buffer for 5 min. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli [31].

Immunoblotting procedures

Immunoblotting was performed as described previously [32, 33]. Rabbit polyclonal IgG anti-His-probe (G-18 Santa Cruz Biotechnology, Santa Cruz, CA) antibodies at a 1:1,000 dilution in Blocking buffer were used as the primary antibodies while the secondary antibody was goat anti-rabbit IgG conjugated to horseradish peroxidase (1:10,000 dilution in Blocking buffer). Other commercially available anti-penta-His, anti-tetra-His and anti-RGS-His primary antibodies were also tested, but were found to be less effective. Alternatively, direct vacuum methods including slot blotter and microtiter top blotter were also used to transfer proteins onto nitrocellulose.

Purification of the His-tagged ArsB protein

The His-ArsB protein was purified by the use of metal-chelate affinity chromatography [34]. A volume (1 ml) of purified inner membranes was incubated in 3.0 ml lysis buffer 1 (8 M urea; 0.1 M NaH₂PO₄; 0.01 M Tris-Cl; 1% Triton®X-100; pH 8.0) at 0°C for 1 h with gentle agitation. After solubilization, the cleared lysate was incubated with 1 ml of the column matrix, nickel-nitrilotriacetic acid (Ni-NTA) agarose for 1 h at 4°C with agitation. The protein-Ni-NTA agarose slurry was carefully loaded into a 20 ml column. Chromatography was performed and 3 ml fractions were collected. The column was equilibrated and washed with 15 ml of wash buffer 1 (8 M urea; 0.1 M

NaH₂PO₄; 0.01 M Tris-HCl; 0.1% Triton®X-100; pH 6.3) at a rate of 0.5 ml/min. The proteins that remained bound to the column were eluted with 25 ml of the elution buffer 1 (8 M urea; 0.1 M NaH₂PO₄; 0.01 M Tris-Cl; 0.1% Triton®X-100; pH 4.5) at a rate of 0.5 ml/min. After the elution step, the column was washed with 10 ml of wash buffer. The fractions collected were analyzed by immunoblotting to identify those fractions containing His-ArsB protein. The fractions containing a strong signal were pooled and further purified by size exclusion chromatography.

Size exclusion chromatography

Fractions from Ni-NTA chromatography that contained His-ArsB protein were pooled and subjected to further purification by size exclusion chromatography with a sephadex G-200 column. The G-200 column was calibrated by the use of MW-GF-200 gel filtration molecular weight markers and a standard curve was generated (molecular weight range 12,400–200,000). After 1 ml crudely purified ArsB (fraction 8 eluted from Ni-NTA column) was loaded onto the top of the gel bed, the column was continuously hydrated and eluted with column buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM KCl and 0.1% Triton X-100. During the entire elution process, 1 ml fractions were collected and analyzed. The molecular weight of the protein eluted off in a fraction was determined by the gel filtration standard curve and the following equation:

$$\text{Log molecular weight} = \text{Elution volume/void volume}$$

The void volume of blue dextran was 18 ml.

Results and discussion

Formation and identification of the His-tagged ArsB construct

Our approach for the purification of the ArsB protein centered the construction of a His-tagged ArsB fusion protein and the use of Ni-chelate chromatography for the purification process. PCR amplification of plasmid pGJ104 resulted in a single amplified product of the predicted size, 1.38 kb. The purified PCR product was digested with *Bam*H I and *Sal* I and ligated into pQE32. The resulting construct was called pArsB.

The construct plasmid pArsB was initially transformed into *E. coli* JM109 as described in Materials and methods. Transformants were selected on growth medium containing ampicillin for the vector and either arsenite or antimony for functional *arsB* gene activity. Large numbers of suspect transformants (approximately 1200 colony forming units)

grew on both media. Three clones from the arsenite plates were isolated and purified for further studies. Restriction analysis of the purified plasmids indicated that a properly sized insert was subcloned into the His-tagged vector and DNA sequencing confirmed that the identity of inserted DNA (data not shown). These results indicate that the construct contained a His-tagged *arsB* gene in the appropriate reading frame.

Functional expression of the His-tagged ArsB protein

To test the functional expression of the His-ArsB fusion protein, the level of arsenical salt resistance of the cells containing the construct was measured. Since the construct was initially selected on a LB plate containing arsenite, it was expected that the fusion protein would confer resistance to arsenical salts. Cells harboring the pArsB plasmid had a higher level of resistance to arsenite and antimonite than cells expressing the entire staphylococcal *ars* operon encoded on pGJ104. Control cells were sensitive to both oxyanions; growth of cells containing either pUC19 or pQE32 was inhibited by 0.5 mM arsenite or 0.1 mM antimonite. The minimum inhibitory concentration (MIC) values for these oxyanions with cells containing pGJ104 were 1.5 mM for arsenite and 0.2 mM for antimonite, which was similar to those reported previously [17]. Cells containing the construct, pArsB, showed increased resistance and had MIC values of 2 mM for arsenite and 0.4 mM for antimonite. These data show that the His-tagged ArsB protein is functionally active and suggest that the His-tag does not interfere with protein-metalloid interactions.

Although cells (strains JM109 and M15) containing this construct were resistant to arsenical salts, the fusion protein

was not expressed at a level to allow for protein purification. To facilitate protein purification, the pArsB plasmid was transformed into *E. coli* C41(DE3), which was developed to allow over-expression of membrane protein [29]. Transformants containing the plasmid were selected on LB medium containing 50 µg/mL ampicillin and the presence of the plasmid in C41 transformants was confirmed by restriction analysis. C41/pArsB cells also had a 2-fold increase in their MIC for arsenite on solid medium compared to the control C41/pQE32 cells (data not shown).

A broth growth inhibition assay was used to obtain more accurate measurements of the MIC values for arsenite and antimony. The results are shown in Fig. 1. Under uninduced conditions (without IPTG), the MIC values for arsenite and antimony with strain C41 cells containing the pArsB construct were 4.0 and 0.3 mM, respectively. The strain C41 containing the control plasmid (pQE32) the MICs were 1.0 and 0.2 mM for arsenite and antimony, respectively. The addition of 1 mM IPTG increased the MIC values for arsenite and antimony to >5.0 mM and 0.4 mM, respectively. With the control cells, the inducer did not alter the MIC value for antimony and showed a slight increase for the MIC for arsenite. Cell growth was not affected by the addition of IPTG (data not shown). These results suggest that the fusion protein was expressed to a higher level under induced conditions which resulted in the increased resistance to the arsenical salts.

Immunodetection of the His-tagged ArsB protein

One advantage of His-tag purification methods is the commercial availability of antibodies that are specific to the histidine residues. Although several different antibodies were

Fig. 1 Growth inhibition of *E. coli* C41/pQE32 and C41/pARSB by arsenite and antimonite. Overnight cultures were diluted 100-fold into fresh LB broth containing ampicillin and increasing concentrations of arsenite (Panel A) or antimony (Panel B). The cultures were incubated at 37°C with aeration for 6 h and the optical density (A_{600nm}) was measured. At time zero 1mM IPTG was added to one set of cultures. Symbols represent: IPTG induced C41/pArsB (Δ), Uninduced C41/pArsB (○), IPTG induced C41/pQE32 (▼) and uninduced C41/pQE32 (●)

tested for the immunodetection of the poly-His fusion protein, one polyclonal anti-His antibody preparation produced the best results and the others generated a faint signal at the same position. Figure 2 shows a strong cross-reactive band at about the 36 kDa range for the inner membrane fraction of cells containing the construct. No cross-reactivity was detected with inner membranes from cells without the construct. A total membrane preparation from cells containing the construct failed to show a strong response. The failure to detect the fusion protein may suggest that the His-tag may be buried in the total membrane preparation because of the hydrophobic nature of ArsB protein. These results confirm that the fusion protein is expressed and indicate that inner membrane preparations should be used for further purification work.

Effect of solubilization treatment on the His-tagged ArsB protein

Since the His-tagged ArsB protein was an integral membrane protein, its purification required an effective solubilization treatment. To identify the best method, inner membranes were treated with eight different detergents as described in Materials and methods. The presence of His-ArsB protein was detected immunologically (data not shown). Membranes treated with 1% Triton®X-100, Nonidet® P40, or Tween® 20 released the protein into the supernate and had no detectable protein in the detergent insoluble material.

Fig. 2 Immunodetection of the His-ArsB protein. Total, inner and outer membranes were isolated from *E. coli* C41/pQE32 and C41/pArsB. The solubilized proteins were resolved on an 8–16% SDS-PAGE gel and electrophoretically transferred onto nitrocellulose membrane as described in Materials and methods. The resulting immunoblot was developed as described in Materials and methods. Lanes: (1) molecular markers; (2, 4, 6) C41/pArsB, (3, 5, 7) C41/pQE32 (2, 3) total membrane, (4, 5) inner membrane, (6, 7) outer membrane, (8) pQE16 (positive control) expressing His-tagged murine DHFR

Triton® X-100 produced the quickest and strongest signal, followed by Tween® 20 and Nonidet®, respectively. After n-octylglucoside treatment, only a small amount of His-ArsB was released while the majority of the protein remained with the detergent insoluble material. Thus, n-octylglucoside did not appear to be an effective solubilizing agent for this protein. CHAPS, deoxycholate and digitonin were ineffective (data not shown). Dodecylmaltoside was not selective and solubilized all of the membrane proteins. These results indicate that Triton® X-100 was the best detergent for the removal of His-ArsB from the inner membrane and for further purification by metal-chelate chromatography.

Purification of the His-tagged ArsB protein

After detergent solubilization, metal-chelate affinity chromatography was used as the next major step in the purification of His-ArsB. Triton® X-100 solubilized inner membranes of *E. coli* C41/pArsB were added to Ni-NTA agarose slurry. Proteins were eluted off the column step-wise where buffers dropped in pH in two stages from 8.0 to 6.3 and finally to 4.5. Figure 3A shows the chromatogram of the procedure and a peak was observed in fraction 8. The protein profile of the chromatogram (Fig. 3B) shows that many non-specific proteins were washed off the column and were contained in fractions 4, 5 and 6. The fractions of this chromatogram were analyzed for the presence of the His-ArsB protein by immunoblotting (Fig. 3C). A strong signal was detected in fraction 8, which corresponded to the peak found in the chromatogram, and a weak signal was detected in fraction 9.

Based on the immunoblotting data (Fig 3C), the protein of interest was found in Fractions 8 and 9, which contained the His-ArsB protein and a few other proteins that differed based on size. The arrow indicates a protein band corresponding to about 36 kDa present in Fraction 8 (Fig. 3B).

Exclusion chromatography

To further purify His-ArsB after Ni-NTA metal chelate chromatography, Fraction 8 was subjected to size exclusion chromatography on a sephadex G-200 column as described in Materials and Methods. Fractions collected were analyzed by immunoblotting and a strong signal was detected in fraction 23 (data not shown). Based on the gel filtration standard curve, the molecular weight of the fraction 23 was approximately 89,000–90,000 Da (data not shown), which was a higher molecular weight than predicted from the sequence data. The resulting 89–90 kDa size of this cross-reactive fraction is the size expected for dimerization of the ArsB protein. The ArsA protein is known to exist as a homodimer that is catalytically active [14] and a trimeric form in solution with arsenite [35]. The effects of arsenite

Fig. 3 Purification of the ArsB protein. Panel A, Nickel chelate affinity chromatography of His-tagged ArsB protein. The peak found in fraction 8 indicates elution of the His-tagged ArsB protein. The left vertical axis measures absorbance units at 280 nm. The right vertical axis measures conductivity of the elution buffer (milliSiemens/cm). The fraction number is shown at the top of the chromatogram, while the elapsed time for the chromatography run is displayed at the bottom of the chromatogram. Panel B, Protein profiles of the fractions collected from nickel chelate affinity chromatography. Fractions were collected from column and handled as described in the Methods. Samples were resolved on an 8–16% SDS-PAGE that was silver stained. Lanes: (1–3) fractions 4, 5 and 6, respectively; (4) 1:50 dilution of low molecular weight markers; (5–9) fractions 7, 8, 9, 10 and 11, respectively; (10) untreated *E. coli* C41/pArsB inner membrane. Panel C, Immunological identification of the fractions from nickel chelate affinity chromatography containing the His-tagged ArsB protein. Fractions 4–11 were blotted onto a nitrocellulose membrane using a slot-blot microfiltration apparatus and were analyzed by chemiluminescent immunodetection as described in Materials and methods. Rows B and D and columns 2, 4 and 6 were left blank to minimize cross reactivity between wells. Wells A1, A3 and A5 contain fractions 4, 5 and 6, respectively. Wells C1, C3 and C5 contain fractions 7, 8 and 9, respectively. Wells E1 and E3 contain fractions 10 and 11, respectively.

and other anion substrates on the elution profile of ArsB in size exclusion chromatography were not tested in this study. Alternatively, the size discrepancy may be due to the nature of membrane proteins, which are known to show anomalous sizes on SDS-PAGE.

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