

The respiratory arsenate reductase from *Bacillus selenitireducens* strain MLS10

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Abstract

The respiratory arsenate reductase from the Gram-positive, haloalkaliphile, *Bacillus selenitireducens* strain MLS10 was purified and characterized. It is a membrane bound heterodimer (150 kDa) composed of two subunits ArrA (110 kDa) and ArrB (34 kDa), with an apparent K_m for arsenate of 34 μM and V_{\max} of 2.5 $\mu\text{mol min}^{-1} \text{mg}^{-1}$. Optimal activity occurred at pH 9.5 and 150 g l^{-1} of NaCl. Metal analysis (inductively coupled plasma mass spectrometry) of the holoenzyme and sequence analysis of the catalytic subunit (ArrA; the gene for which was cloned and sequenced) indicate it is a member of the DMSO reductase family of molybdoproteins.

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1. Introduction

The diversity and widespread environmental occurrence of prokaryotes capable of utilizing arsenic oxyanions for the generation of energy has only recently been realized [1–4]. At least eighteen species of prokaryotes are now known to respire arsenate anaerobically and these include representatives from the Crenarchaeota, thermophilic bacteria, low G+C Gram-positive bacteria, and Proteobacteria [5–12]. The respiratory dissimilatory reduction of arsenate (AsV) to arsenite (AsIII) is carried out by enzymes very different from those involved in the well-described ArsC system for microbial arsenic resistance [13,14]. Whereas the arsenate reductase ArsC is a small (13–16 kDa), soluble, cytoplasmic enzyme, and requires either

reduced glutathione or thioredoxin [15,16], respiratory arsenate reductase is membrane associated and is coupled to a respiratory electron transfer chain [4]. This was demonstrated by the purification and characterization of the respiratory arsenate reductase from *Chrysiogenes arsenatis*, one of the first species of arsenate respiring bacteria described [17]. A periplasmic enzyme, it is heterodimer with an 87-kDa subunit (ArrA) and a 29-kDa subunit (ArrB) [17]. Specific for arsenate, it has been reported to have a K_m of 300 μM .

Bacillus selenitireducens strain MLS10 is low G+C Gram-positive, non-spore forming rod isolated from the Mono Lake, CA, USA. It grows by coupling the oxidation of lactate (to acetate and carbon dioxide) to the reduction of AsV to As III [8]. A haloalkaliphile, it grows optimally on 10 mM arsenate at pH 9.8 and a salinity of 90 g l^{-1} (1.5 M NaCl). *B. selenitireducens* is not closely related to *C. arsenatis* (part of a deep branch on the bacterial tree) [17] or and, as a Gram-positive bacterium, does not have a true periplasm. The purification and characterization of the respiratory arsenate reductase from *B. selenitireducens* strain MLS10 and the cloning and sequencing of the catalytic subunit (ArrA) are reported here. This is the first

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respiratory arsenate reductase purified from a Gram-positive or a haloalkaliphilic bacterium.

2. Materials and methods

2.1. Organism and growth conditions

B. selenitireducens strain MLS10 was cultivated in the medium as previously described [8] with sodium lactate (20 mM) and sodium arsenate (10 mM). Large scale cultivation was performed using a 14-l fermentor (New Brunswick, Edison, NJ, USA). The average cell yield for a 24-h culture was 0.4 g wet weight l^{-1} . Cells were harvested at an early exponential phase by centrifugation at 9000 rpm for 20 min, resuspended in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA and 10 μ M phenylmethylsulfonylfluoride (PMSF) (Buffer A), and kept at $-20^{\circ}C$ until used.

2.2. Purification of the respiratory arsenate reductase

A pooled pellet of cells (18 g) was suspended in 140 ml of Buffer A. The cells were then disrupted by sonication for 10 min on ice at 150 W and 60 Hz with a Braun Sonic L (B. Braun Biotech, Allentown, PA, USA). After centrifugation (15 min at $7500\times g$) to remove the unbroken cells, the supernatant fluid was centrifuged at $200\,000\times g$ for 2 h. The particulate fractions (i.e., membranes and ribosomes) were suspended in 50 ml of Buffer A containing 0.3 M KCl and centrifuged at $100\,000\times g$ for 1 h. This pellet was suspended in 50 ml of Buffer A, and Triton X-100 was added to 2% (wt vol^{-1}). The suspension was gently stirred for 2 h at $4^{\circ}C$ and then centrifuged at $100\,000\times g$ for 1 h. The supernatant (containing the solubilized respiratory arsenate reductase) was loaded onto a DEAE-Toyopearl (Rohm and Haas, Montgomeryville, PA, USA) ion exchange column (3×14 cm) equilibrated with Buffer A containing 1% Triton X-100 (wt vol^{-1}). The enzyme was eluted with a linear NaCl gradient (0–0.5 M, in Buffer A with 1% Triton X-100). The fractions with arsenate reductase activity were pooled and dialyzed against 2 l of Buffer A containing 1% Triton X-100. The dialyzed fraction was then loaded onto a second DEAE-Toyopearl ion exchange column (1.5×11.5 cm) and again eluted with a NaCl gradient (0–0.5 M, in Buffer A with 1% Triton X-100). The active fractions were pooled and loaded onto a Sephacryl S-300 gel filtration column (65×2.5 cm) equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 1% Triton X-100 and 0.25 M NaCl. The fractions were eluted at a flow rate of 0.2 ml min^{-1} . The fractions containing pure enzyme were pooled and concentrated.

2.3. Activity assays

Reduced methyl viologen was used as the artificial elec-

tron donor in enzyme assays. Spectrophotometric detection of arsenate reductase activity was performed using the methods of Stolz et al. [18] with a Perkin-Elmer Lambda 2 dual-beam spectrophotometer. The reaction mixture, in a sealed 1.5-ml cuvette, contained 50 mM Tris buffer pH 8.5, sodium arsenate (5 mM unless otherwise indicated) and protein and was degassed with oxygen-free nitrogen following standard techniques. The reaction was initiated with the injection of 10 mM reduced methyl viologen. The enzyme was not added to the reference cuvette. Enzyme activity for each substrate (e.g., arsenate) was monitored over 15 min and calculated from the amount of methyl viologen (in μ mol) oxidized protein using the extinction coefficient of $13\text{ mM}^{-1}\text{ cm}^{-1}$ at 600 nm. The kinetic parameters were determined using non-linear regression fit with the Michaelis-Menten equation using the Prism statistical analysis package (GraphPad, San Diego, CA, USA). Substrate specificity was determined by substituting different substrates (5 mM of arsenite, selenate, selenite, nitrate, nitrite, thiosulfate, fumarate, or phosphate) for arsenate. The pH optimum was determined by adjusting the pH of the reaction mixture with either 1 N HCl or NaOH. The salinity optimum was determined by adjusting the final NaCl content of the buffer.

2.4. Protein determination

Protein concentrations were determined using the method described by Lowry et al. [19] with bovine serum albumin as a standard.

2.5. Estimation of molecular mass

The molecular mass of the respiratory arsenate reductase was estimated by gel filtration chromatography using a Sephacryl S-300-HR column (Sigma Chemical, St. Louis, MO, USA). The column was equilibrated with 10 mM Tris-HCl buffer containing 1 mM EDTA, 10 μ M PMSF, 1% Triton X-100 and 0.25 M NaCl, and run at a flow rate of 0.2 ml min^{-1} at $4^{\circ}C$. Dextran blue (MW 200 kDa) was used to determine the void volume of the column. The molecular mass standards (Sigma Chemical, St. Louis, MO, USA) used were thyroglobulin (669 kDa), apoferritin (443 kDa), β -amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), and carbonic anhydrase (29 kDa).

2.6. Protein sequence analysis

An aliquot of sample containing 15 μ g of respiratory arsenate reductase was diluted in sample buffer (BioRad, Hercules CA, USA), run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% Tris-HCl, BioRad, Hercules, CA, USA) and transferred onto PVDF membrane (Immobilon-P^{8Q}, Millipore, Bedford, MA, USA) using a CAPS transfer buffer. The membrane

was then washed several times alternatively with deionized water and 50% ethanol, then stained with 0.05% Coomassie Brilliant Blue R-250, destained with 50% methanol and dried. The individual protein bands were excised from the membrane and the sequence was determined by Edmond degradation at ProSeq Inc. (Boxford, MA, USA).

2.7. Metal analysis

Metal analysis was done at the Center for Environmental Metals Analysis at Duquesne University, using inductively coupled plasma mass spectrometry (HP-4500). The sample was introduced by direct aspiration through Teflon tubing, quartz concentric nebulizer, quartz Scott-type spray chamber and quartz torch. The forward power was 1455 W, the carrier gas flow rate was 0.96 l min⁻¹, the sample depth was 12 mm and the sampling and skimmer cones were nickel. Xanthine oxidase (Sigma Chemical, St. Louis, MO, USA) was used as a control.

2.8. Cloning of *arrA*

The forward and reverse degenerate oligonucleotide primers for polymerase chain reaction (PCR) amplification of *arrA* were designed based on the N-terminal amino acid sequence of the 110-kDa (ArrA) and the 34-kDa (ArrB) subunit, respectively (110JS F1 GARCARGGNGARTG-GATHGC, 34JS R1 TTYTCRTTYTTRCANGTNAC; R = A, G; N = A,C,T,G; H = A,C,T; Y = C,T). The reaction mixture (25 µl) contained 50 ng *B. selenitireducens* DNA, 400 nM each primer, 200 µM dNTPs, 1× reaction buffer, and 1.25 U of PfuTurbo DNA polymerase (Stratagene, La Jolla, CA, USA). The thermocycling profile consisted of a 5 min denaturation step at 95°C, followed by 30 cycles of 95°C, 30 s; 50°C, 30 s; and 72°C, 3 min. The 3' A-extension was added by incubating the reaction product for 10 min at 72°C after the addition of 1 U of Taq DNA polymerase. The PCR product was separated on a 0.8% agarose gel and purified using the QIAquick Gel extraction kit (Qiagen, Valencia, CA, USA). Cloning and transformation was done using the pCR2.1 TOPO kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). After growing transformants overnight on LB (50 µg ml⁻¹) kanamycin agar plates, selected colonies were picked for further analysis. The plasmids were purified using the Qiaprep Spin Minikit (Qiagen) and treated with the restriction endonuclease *EcoRI* to analyze for presence of inserts. Selected clones were initially sequenced using M13 primers and the BigDye Terminator Ready Reaction Mix (Applied Biosystems, Forest City, CA, USA). Additional sequencing primers were designed to complete the sequencing (110JS F2 TGCATGT-GTAGACCCACGAT; 110JS F3 GATGAATGGATGC-CGGTTAT; 110JS R3 ATAACCGGCATCCATTCATC; 34JS F3 CGAAAAGGTGAATTGGCATT; 34JS R3 AATGCCAATTCACCTTTTGC). All sequencing was done

at the DNA sequencing facility at the University of Pittsburgh. The nucleotide sequence (2.4 kb) has been assigned the GenBank accession number AY283639.

3. Results

3.1. Purification of the respiratory arsenate reductase

The bulk of arsenate reductase activity (~92%) was in the membrane fraction (i.e., the KCl washed pellet). This activity was soluble in both CHAPS (1%) and Triton X-100 (2%). Better yields were obtained using 2% Triton X-100 as a solubilizing agent and 1% Triton X-100 was used in the running buffer throughout the purification procedure. The respiratory arsenate reductase was purified through two sequential runs of DEAE ion exchange chromatography followed by size exclusion chromatography. About 64% of the respiratory arsenate reductase activity was recovered from the first DEAE-Toyopearl column. While only 30% of this activity was recovered after the second round ion exchange chromatography, it provided a fraction highly enriched in respiratory arsenate reductase (Fig. 1). After the size exclusion chromatographic step, the enzyme was found to be electrophoretically pure (Fig. 1). SDS-PAGE, in which the sample was heated, revealed subunits of 110 kDa and 34 kDa (Fig. 1). Size exclusion chromatography indicated a native molecular mass of 150 kDa, which suggests that the enzyme is a heterodimer with single large α (ArrA), and a small β (ArrB) subunit.

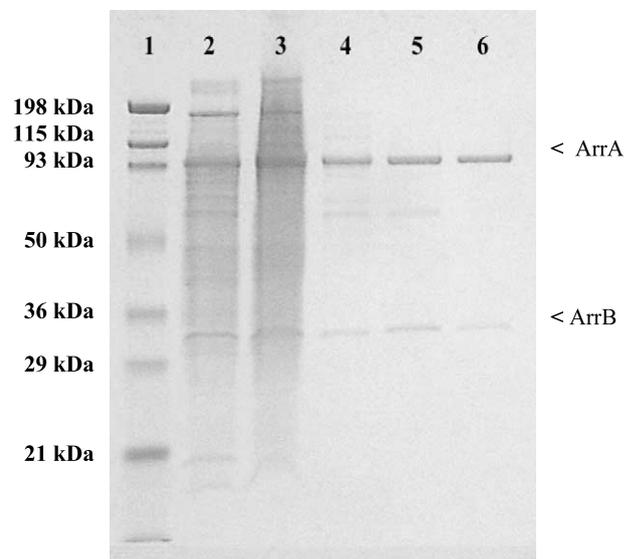


Fig. 1. Purification of the respiratory arsenate reductase. Lane 1: protein standards (198, 115, 93, 50, 36, 29, and 21 kDa), lane 2: cell lysate (prior to ultracentrifugation), lane 3: Triton-X 100 solubilized fraction, lane 4: active fraction from first ion exchange column, lane 5: active fraction from second ion exchange column, and lane 6, the pure enzyme after size exclusion column.

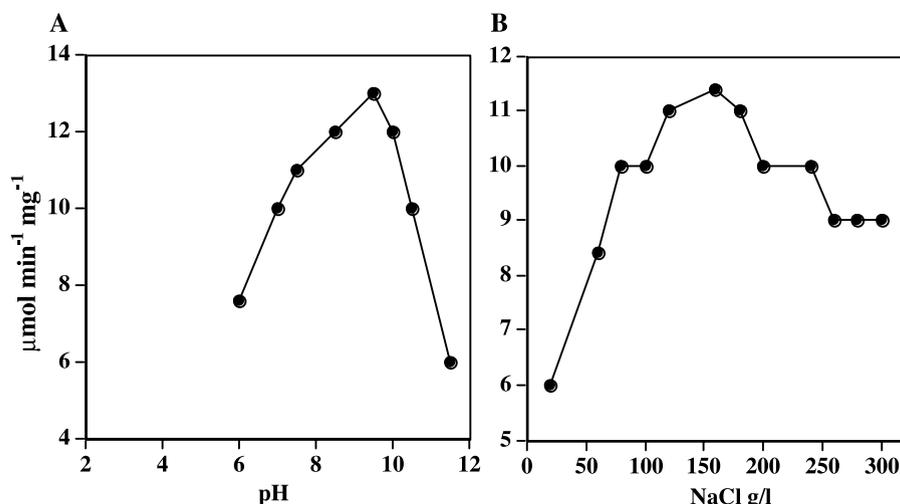


Fig. 2. Optima for (A) pH and (B) salinity of the purified respiratory arsenate reductase. Activity was measured as reduced methyl viologen oxidized ($\mu\text{mol min}^{-1} \text{mg}^{-1}$).

3.2. Kinetics of the purified RAsR

The respiratory arsenate reductase showed the greatest specificity for arsenate with an apparent K_m of $34 \mu\text{M}$ and a V_{max} of $2.5 \mu\text{mol min}^{-1} \text{mg}^{-1}$. It could also, however, couple the oxidation of MVR to the reduction of arsenite (24% of the specific activity for arsenate), selenate (48%), and selenite (29%). No activity was detected when nitrate, nitrite, fumarate, thiosulfate, or phosphate was provided as the electron acceptor. The enzyme was heat sensitive and completely deactivated with heating at 90°C for 1 min. The catalytic subunit maintained its activity in SDS-PAGE gels, however, if the sample was not heated. The enzyme was active over a range of pH (6–12) and salt concentrations (NaCl 25–300 g l^{-1}) tested, but showed clear optima at pH 9.5 and a salinity of 150 g l^{-1} (Fig. 2).

3.3. N-terminal amino acid sequence and metal analyses

Twenty amino acids from the N-terminal of the 110-kDa subunit (SQENKEQGEWIASVCQGCTA) and 31 amino acids from the 34-kDa subunit (AKKNYAM-TIDLQACIGCAGCAVTCKNENSTS) were determined. The 110-kDa subunit shared a 50% identity with the ArrA from *C. arsenatis* while the 34-kDa subunit shared a 57% identity with ArrB [17]. Each also had multiple cysteine residues indicating an iron sulfur binding motif. Metal analysis revealed the presence of both molybdenum and iron; however, the stoichiometry (9 mol of Fe, 0.18 mol Mo) suggests loss of metal.

3.4. Sequence of arrA

A 2.5-kb fragment was amplified using the degenerate forward and reverse primers. It contained all but the immediate 5' end of *arrA*, an intergenic region of 29 nucleotides, and 30 nucleotides of the 5' end of *arrB*. With the

addition of the N-terminal amino acid data, the protein had a calculated molecular mass of 90 980 Da and a pI of 5.12. We compared our sequence with that of *arrA* from *Shewanella* sp. strain ANA-3 [20]. The gene had a 56.7% identity and the inferred amino sequence had a 47.1% identity and 78.8% similarity. BLAST searches with *B. selenitireducens* ArrA showed the closest match with polysulfide reductase (PsrA) from *Wolinella succinogenes* and *Shewanella oneidensis* (23%). The highest degree of identity and similarity, however, was with a hypothetical protein of unknown function from *Desulfitobacterium hafniense* (56% and 60% respectively). Interestingly, ArrA has little homology with the catalytic subunit of arsenite oxidase (13.4% identity, 46% similarity).

4. Discussion

The respiratory arsenate reductase of *B. selenitireducens* strain MLS10 while structurally similar to *C. arsenatis* has significant differences. Unlike *C. arsenatis*, where 80% of the activity is in the periplasmic phase after cell lysis [17], the Respiratory arsenate reductase is tightly associated with the cytoplasmic membrane and must be solubilized with detergent. It has almost an order of magnitude greater affinity for arsenate with a K_m of $34 \mu\text{M}$. In addition, the substrate selectivity is different as arsenite, selenate and selenite served as substrates to oxidize methyl viologen. These activities, however, reflect more the general specificity of mononuclear molybdoenzymes and may not be physiologically relevant as *B. selenitireducens* does not respire arsenite or selenate [8]. Whether it functions as the respiratory selenite reductase is currently under investigation. The inability of the enzyme to reduce fumarate, nitrate, or nitrite (substrates that *B. selenitireducens* is known to also respire) indicates that additional terminal reductases are used for these respiratory pathways.

The respiratory arsenate reductase is adapted to function optimally at high pH and salinity. Preliminary analysis using whole cells indicated a very narrow pH (8–10) range [Kovalyova et al., 2002, ASM meeting abstracts]. While the pure enzyme showed an expanded range (pH 6–11, Fig. 2), the maximum activity was still pH 9.5. This is in marked contrast to *C. arsenatis* which has an optimal activity at pH 6 [17]. The pure enzyme also showed a salinity profile similar to whole cells. These results are interesting when considering that *B. selenitireducens* was isolated from a soda lake with a pH of 9.8 and salinity of about 90 g l⁻¹. The lake also has a high concentration of arsenic (200 µM) that supports a rigorous arsenic biogeochemical cycle [21,22]. *B. selenitireducens* grows optimally at the same pH but can tolerate higher salinity (> 200 g l⁻¹) and concentrations of arsenic (e.g., 10 mM) [8]. The pH and salinity optima could indicate that the enzyme is topologically orientated on the side of the cytoplasmic membrane that faces the peptidoglycan layer of the cell wall, similar to what has been observed in Gram-negative arsenate respiring bacteria [17].

The purified enzyme is a heterodimer composed of single α (ArrA), and β (ArrB) subunits. N-terminal sequence data, in gel activity assays, and metal analysis suggest that ArrA (110-kDa polypeptide) contains the molybdenum catalytic center. The N-terminal sequence shares the greatest identity with ArrA from *C. arsenatis* and the inferred protein from *Shewanella* sp. strain ANA-3 [20] and a hypothetical protein from *D. hafniense*, but also shares a high degree of similarity with other members of the DMSO reductase family of mononuclear molybdenum enzymes [23,24]. These include the polysulfide reductase (PsrA) from *W. succinogenes* [25] and *S. oneidensis* and the thiosulfate reductase of *S. typhimurium* (PhsA). *Alcaligenes faecalis* arsenite oxidase (AsoA) [26] and *E. coli* formate dehydrogenase (FdhH) [27] had only conserved cysteine residues. These enzymes share in common the iron–sulfur cluster binding motif (C-X₂-C-X₃-C) at the N-terminal and a molybdenum atom bound to two guanine dinucleotides (MGD) [27]. The 34-kDa subunit (ArrB) also has an iron–sulfur cluster binding motif C-X₂-C-X₂-C-X₃-C at the N-terminal. The highest degree of identity is with ArrB from all three arsenate respiring bacteria. In addition, significant identity was shared with PsrB from *W. succinogenes* [28], *S. typhimurium* tetrathionate reductase (TtrB), NarH from several species of Gram-positive bacteria including *Staphylococcus carnosus* [28] and the SerB of the selenate reductase from *Thauera selenatis* [29]. This high degree of identity suggests that the 34-kDa polypeptide is an iron–sulfur electron transfer protein.

Determining the sequence of the gene encoding the catalytic subunit (*arrA*) allowed confirmation of the presence of metal binding motifs (i.e., iron–sulfur cluster) and further establish its relatedness to other enzymes of the DMSO reductase family. In addition, the sequence facili-

tated identification of the putative *arr* operon from the arsenic respiring freshwater low G+C Gram-positive bacterium *D. hafniense* [12]. The contig from *D. hafniense* (<http://www.jgi.doe.gov>) that contains the *arrA* homolog also has genes encoding a two-component regulatory system (sensor kinase and response regulator) and an ORF of unknown homology, immediately upstream of the putative *arrA*. Immediately downstream of *arrA* are two genes, *arrB* and *torD*, that encode for an iron sulfur protein (ArrB) and a chaperone protein homolog, respectively. This latter finding is quite intriguing as it possibly suggests a third (γ) subunit (i.e., a membrane anchored cytochrome *b*), such as found in *narGHJI* (nitrate reductase) [28], and *serABDC* (selenate reductase) [29]. Unfortunately, the contig ends a few bases later. A putative Tat signal peptide was found in ArrA, similar to what has been found in *Shewanella* strain ANA-3 [20], indicating that the enzyme is secreted [30]. Thus the respiratory arsenate reductase from Gram-positive bacteria may indeed have a ‘periplasmic’ location. Verification of this topology awaits further DNA sequence determination and localization experiments in *B. selenitireducens*.

Despite its toxicity, it is now evident that arsenate is respired by a variety of prokaryotes and can be an important energetic pathway in arsenic enriched anoxic environments (i.e., hot springs, soda lakes, aquifers) [3]. That this ability is not limited to specific phylogenetic groups or species also suggests that arsenate may have been an important electron acceptor in the development of anaerobic respiration [3]. While the high degree of similarity of respiratory arsenate reductase from phylogenetically distant species suggests that it is a highly conserved enzyme, our study has shown that it may also be adapted to function in specific environmental conditions.

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