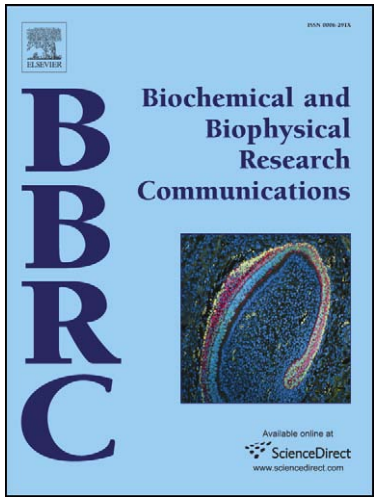

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PII: S0006-291X(09)00463-X
DOI: 10.1016/j.bbrc.2009.03.045
Reference: YBBRC 22718

To appear in: *Biochemical and Biophysical Research Communications*

Received Date: 20 February 2009
Accepted Date: 4 March 2009

Please cite this article as: C. Richey, P. Chovanec, S.E. Hoeft, R.S. Oremland, P. Basu, J.F. Stolz, Respiratory Arsenate Reductase as a Bidirectional Enzyme, *Biochemical and Biophysical Research Communications* (2009), doi: 10.1016/j.bbrc.2009.03.045

Respiratory Arsenate Reductase as a Bidirectional Enzyme

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Abstract

The haloalkaliphilic bacterium *Alkalilimnicola ehrlichii* is capable of anaerobic chemolithoautotrophic growth by coupling the oxidation of arsenite (As(III)) to the reduction of nitrate and carbon dioxide. Analysis of its complete genome indicates that it lacks a conventional arsenite oxidase (Aox), but instead possesses two operons that each encode a putative respiratory arsenate reductase (Arr). Here we show that one homolog is expressed under chemolithoautotrophic conditions and exhibits both arsenite oxidase and arsenate reductase activity. We also demonstrate that Arr from two arsenate respiring bacteria, *Alkaliphilus oremlandii* and *Shewanella* sp. strain ANA-3, is also biochemically reversible. Thus Arr can function as a reductase or oxidase. Its physiological role in a specific organism, however, may depend on the electron potentials of the molybdenum center and [Fe-S] clusters, additional subunits, or constitution of the electron transfer chain. This versatility further underscores the ubiquity and antiquity of microbial arsenic metabolism.

Keywords: arsenite oxidase, arsenate reductase, *Alkalilimnicola ehrlichii*, principle of microscopic reversibility

Introduction

Arsenic, in spite of its toxicity, is readily metabolized by microorganisms and has a robust biogeochemical cycle [1]. Over the past two decades key components of this cycle have begun to emerge [1,2]. Oxidation/reduction reactions in particular are central as they are linked to organic matter mineralization and carbon fixation (e.g., autotrophy). In Mono Lake, CA for example, 11-14% of organic carbon mineralization is coupled with respiratory arsenate reduction [3]. Chemolithoautotrophic As(V) reducing bacteria, that oxidize hydrogen sulfide in the process,

also contribute such that the combined rates of As(V) reduction exceed the geothermal sources of As(V) [4]. The balance is provided by significant rates of biologic As(III) oxidation even in the anoxic zone, where nitrate serves as the terminal electron acceptor in place of oxygen [5]. More remarkably, As(III) has recently been shown to serve as an electron donor in anoxygenic photoautotrophy for several of the lake's microbial mat communities [6]. Organisms have been isolated and characterized from the lake including As(V) reducing heterotrophs [7] and chemolithoautotrophs [4], as well as photoautotrophic[6] and chemolithoautotrophic As(III) oxidizers [5].

As(V) reduction is employed for resistance and energy generation but accomplished by two very different mechanisms. Arsenic resistance is conferred by a small (13-15 kDa) arsenate reductase ArsC and an As(III)-specific efflux pump (e.g., ArsB, ACR3) [8]. Additional components may include an ArsA (an ATPase that forms a complex with ArsB), the regulatory components ArsR and ArsD, as well as ArsH and ArsM (a methylase) [2]. Dissimilatory As(V) reduction generates energy through oxidative phosphorylation with electron donor and acceptor complexes. The respiratory arsenate reductase, Arr, is a heterodimer with the catalytic subunit (ArrA) containing a molybdenum center and a [4Fe-4S] cluster, and a smaller subunit (ArrB) that contains at least three, possibly four, [4Fe-4S] clusters [2,9]. The enzyme is periplasmic, with the twin arginine-containing leader sequence at the N-terminus of the catalytic subunit. Arsenite oxidation whether involved in resistance or to generate energy, is carried out by arsenite oxidase (Aox) [8]. Aox is similar to Arr in that belongs to the same family of molydoenzymes, but has several characteristics that are different [2]. A heterodimer, the catalytic subunit (AoxB) contains the molybdenum center and a [3Fe-4S] cluster [10]. The smaller subunit, AoxA, harbors a Rieske-type iron-sulfur [2Fe-2S] cluster and contains the twin arginine motif that targets the

enzyme for transport to the periplasm [2,10]. Although common to most arsenite oxidizing bacteria, recent investigations using molecular approaches have failed to detect *aoxB* in a number of species suggesting an alternative mechanism may be operable [11,12] One such organism, *Alkalilimnicola ehrlichii*, is capable of anaerobic chemolithoautotrophic growth by coupling the oxidation of arsenite (As(III)) to the reduction of nitrate and carbon dioxide [12]. Analysis of its complete genome indicates that it lacks Aox, but instead possesses two operons that each encodes a putative respiratory arsenate reductase (Arr), thus raising the question of how this organism grows on As(III). We report here that only one of the homologs is expressed under chemolithautotrophic conditions and exhibits both arsenite oxidase and arsenate reductase activity. We also demonstrate the reversible nature of Arr from two As(V) respiring bacteria, *Alkaliphilus oremlandii* and *Shewanella* sp. ANA-3.

Materials and methods

Culture and cell preparation. Cultures of *A. ehrlichii* strain MLHE-1^{T} were grown heterotrophically (aerobic on 10 mM acetate) or chemolithautotrophically (anaerobic on 10 mM As(III) and 10 mM sodium nitrate) on basal salts medium with 60 g/L NaCl and pH 9.8 as described in Hoeft et al. [12]. Cultures of *Alkaliphilus oremlandii* were grown anaerobically on lactate (20 mM) and sodium arsenate (10 mM) as described in Fisher et al. [13]. Cultures of *Shewanella* sp. ANA-3 also grown anaerobically on sodium arsenate [14] were provided courtesy of Dr. C. Saltikov (University of California, Santa Cruz). The cultures were harvested by centrifugation, cells were lysed by French pressure cell (cell lysate), and the particulate (membrane) fractions were obtained by ultracentrifugation [9].

Gel electrophoresis, activity assays, and proteomic analyses. Native gels were done as reported in [15] but used a 4% acrylamide stacking gel and 10% acrylamide resolving gel and Coomassie

blue was left out of all solutions. The samples were incubated on ice in buffer containing 1% Triton X-100 for 30 min then centrifuged (12,000 x g) before loading. SDS-PAGE was done as modified in Fisher et al. [13]. Specifically, no reducing agent such as β-mercaptoethanol or dithiothreitol was used, nor were the samples heated, in order to maintain enzyme activity. Activity assays were done as described using 2,6-dichlorophenolindolephenol (DCIP) [10], methyl viologen or benzyl viologen [16]. In gel enzyme assays were done under nitrogen atmosphere in a Nexus One anaerobic dry box (Vacuum Atmosphere Co. Newburyport MA) at pH 9.5 in 10 mM Tris buffer. For arsenate reductase activity gels were stained with a 10 mM solution of reduced methyl viologen (blue color) or benzyl viologen (purple color); upon addition of sodium arsenate (20 mM) the active band was revealed as a clearing. For arsenite oxidase activity, the gels were incubated with either oxidized methyl viologen (clear, 10 mM), oxidized benzyl viologen (clear, 10 mM) or oxidized DCIP (blue, 5 mM). Upon addition of sodium arsenite (20 mM), the active band turned blue (methyl viologen), purple (benzyl viologen) or cleared (DCIP). The gels were placed into zip-lock bags, then removed from the dry box and photographed using a GS-800 Calibrated Densitometer (BioRad, Hercules CA). The presence of dithionite in the reductase activity assay stabilized the color of the reduced methyl and benzyl viologen providing better contrast with the clearing of the active band. However, for arsenite oxidase activity no reducing agent was used. The gels were stained with Coomassie blue, and selected bands were excised, trypsin digested, and subjected to either LC/MS/MS or MALDI-TOF MS analyses as described [17] using a ThermoFinnigan LCQ Deca XP or an Applied Biosystems Voyager DE-Pro mass spectrometer, respectively (Genomics and Proteomics Core Facility, University of Pittsburgh). Peptide fingerprint matching was achieved with Mascot using a value of 0 for missed cleavage and a mass tolerance of 0.5 Da.

Genomic and phylogenetic analysis. The annotated genome was available through the Joint Genome Institute's Integrated Microbial Genomes (IMG) website (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi). BLAST analysis of the genome was done using the IMG site (http://genome.ornl.gov/microbial/mlg_mlhe1). Additional sequences were obtained from NCBI, sequence alignments were done using CLUSTAL X[18], and phylogenetic trees constructed using PAUP[19].

Results and discussion

A. ehrlichii strain MLHE-1^T was isolated from the anoxic bottom waters of Mono Lake [5]. It was found to be capable of both aerobic respiration (with acetate as the electron donor and carbon source) and anaerobic chemolithoautotrophic growth, coupling the oxidation of As(III) to the reduction of nitrate to nitrite [5]. The genome (which has been sequenced to closure) does not include any genes annotated as arsenite oxidase. Direct BLAST analysis of the genome using the amino acid sequence of AoxB from *Alcaligenes faecalis* identified two pyranopterin-containing proteins, assimilatory nitrate reductase (Mlg_1702), and a putative tungsten-containing formate dehydrogenase (Mlg_2513). Neither, however, had a high degree of similarity to AoxB and both lacked the Rieske subunit. A more in depth search using the catalytic subunits of assimilatory nitrate reductase (NasA) and dissimilatory nitrate reductase (NarG), located the NarG homolog (Mlg_1003), as well as a tetrathionate reductase (TtrA, Mlg_0633), DMSO reductase (DorA, Mlg_1675), formate dehydrogenase (FdhA, Mlg_1286), and two putative homologs of respiratory arsenate reductase (ArrA, Mlg_0216, Mlg_2426)(Fig. 1). A similar search using AoxA from *A. faecalis* revealed one Rieske-type iron-sulfur cluster protein. While this homolog (Mlg_1558) did show 32% identity and 42% similarity with AoxA, none of the associated genes

in the operon encoded a molybdoprotein. Thus it was concluded that *A. ehrlichii* lacks an Aox homolog, providing an explanation for the previous failure to amplify *aoxB* [5].

Enzyme activity assays and proteomics were used to identify the putative arsenite oxidase. Activity assays, done under strict anoxic conditions using the redox mediators 2,6-dichlorophenolindolephenol (DCIP), methyl viologen, and benzyl viologen as electron acceptors, revealed arsenite oxidase activity in cell lysates (containing both cytoplasm and membrane components) and membrane fractions of the chemolithoautotrophically grown cells. The reactions could also be done in reverse with As(V) as the substrate and reduced methyl viologen or benzyl viologen as the electron donor (data not shown). Native polyacrylamide gels were then used to compare the protein composition in the cell lysates of cells grown heterotrophically to those grown chemolithoautotrophically. A prominent protein complex with high mobility was seen only in the membrane fraction of the chemolithoautotrophic cells (Fig. 2A). Mass spectrometric (LC-MS/MS) analysis revealed that the band contained only two proteins, Mlg_0215 and Mlg_0216, in a stoichiometry of 1:1 based on the number of peptide fragments detected. It was then determined that the protein complex remained active in the native gels, and using the membrane fraction both arsenite oxidase (Fig. 2B, lane 1) and arsenate reductase activity (Fig. 2B, lane 2) could be demonstrated depending on the direction of the assay. The enzyme was also found to maintain its activity in SDS-PAGE provided the sample was not heated or exposed to reducing agents such as β-mercaptoethanol. Subsequently, the active band from a native gel was excised and run on SDS-PAGE (Fig. 2C). Under these conditions, three protein bands were observed (Fig. 2C, lane 2), however, only the middle band exhibited activity (Fig. 2C, lane 1). Mass spectrometric (MALDI-TOF MS) analysis identified the top band as Mlg_0216 (ArrA, 91 kDa), the lower band as Mlg_0215 (ArrB, 28 kDa), and the middle band as

a mixture of both (Supplementary Fig. 1). This indicates that the presence of both subunits, ArrA and ArrB, is required for active enzyme consistent with the findings for the respiratory arsenate reductase from *Shewanella* sp. strain ANA-3 [20]. We attribute the greater mobility of the ArrAB complex in SDS-PAGE to the general property of unreduced proteins with multiple disulfide bonds to migrate further than their reduced/relaxed form [21]. More importantly, these results revealed that Arr is a reversible enzyme. As *A. ehrlichii* is incapable of respiratory growth on As(V), the results also suggest that the enzyme functions as the arsenite oxidase in this organism.

ArrA (Mlg_0216) from *A. ehrlichii* shares greater total amino acid sequence identity (~30%) and similarity (~65%) to other ArrA homologs than to AoxB (~14% identity, ~50% similarity), and has a similarly constituted catalytic pocket (Fig. 3A). Although no crystal structure for ArrA is yet available, sequence alignment suggests the molybdenum is coordinated to the highly conserved cysteinato sulfur (Fig. 3A) similar to that shown for periplasmic nitrate reductase [22]. In addition it contains the motif for a [4Fe-4S] cluster (C-X_2-C-X_3-C-X_{27}-C) rather than the [3Fe-4S] cluster of AoxB (Fig. 3B) [8]. The *arr* operon from *A. ehrlichii* also has genes predicted to encode a larger (45 kDa) [4Fe-4S] cluster-containing subunit (ArrB'), a 44 kDa membrane anchoring subunit that has homology with NrfD (ArrC), and a chaperone TorD (33 kDa, ArrD). These additional genes are found in *arr* operons from other arsenate respiring bacteria but not *aox* operons [2]. Interestingly, the closely related phototroph *Halorhodospira halophilum* and the heterotroph *Magnetospirillum magnetotacticum* MS-1 have similar operons with the same five genes (*arrB'ABCD*) suggesting that these two organisms may also be capable of arsenite oxidation. Furthermore, the two *Ectothiorhodospira* species (e.g., PHS-1, MLP2)

178 shown to use arsenite as the electron donor for anaerobic photosynthesis also possess the unique
179 *arrA* (but not *aoxB*) [6].

180 To understand whether the reversibility is a general feature of Arr, we probed the arsenite
181 activity of the same enzyme from two well characterized arsenate respiring bacteria *Alkaliphilus*
182 *oremlandii* [13] and *Shewanella* sp. strain ANA-3 [14]. Gel assays of membrane fractions of *A.*
183 *oremlandii* revealed that Arr exhibited As(III) oxidation coupled to DCIP, methyl viologen, or
184 benzyl viologen, as well as As(V) reduction coupled to methyl viologen oxidation (the latter is
185 shown in Fig. 4A). Mass spectrometric analysis confirmed that the active protein band was Arr.
186 Similar results were found for *Shewanella* sp. strain ANA-3 (Fig. 4B). Of the three organisms,
187 only the Arr from *Shewanella* sp. strain ANA-3 has been shown through mutational studies to
188 function as respiratory arsenate reductase [14].

189 The ability of Arr to function as both a reductase and an oxidase suggests that the
190 oxidation/reduction reaction involving arsenic oxyanions follows the principle of microscopic
191 reversibility. Conceptually this means that in a reversible reaction, both the forward and the
192 reverse reaction pass through the same intermediate steps [23]. Interestingly, a related enzyme,
193 DMSO reductase, whose physiological function is to reduce dimethyl sulfoxide (DMSO) to
194 dimethyl sulfide (DMS), can also catalyze the oxidation of DMS to DMSO [24]. For arsenite
195 oxidation, the electrons are shuttled from the metal center via a high potential [3Fe-4S] cluster in
196 AoxB to the lower potential Rieske [2Fe-2S] cluster in AoxA [22-27]. For arsenate reduction,
197 the direction is reversed with electrons from ArrB shuttled to the molybdenum center via the
198 [4Fe-4S] in ArrA. ArrB is predicted to contain four iron-sulfur clusters (C-X_2-C-X_2-C-X_3-C; C-
199 X_2-C-X_4-C-X_3-CP; C-X_2-C-X_2-C-X_3-CP; C-X_2-C-X_{27}-C-X_2-CP), but whether they are all [4Fe-
200 4S] remains to be determined [2]. That ArrAB exhibits both reductase and oxidase activity

indicates that ArrB can shuttle electrons in both directions. The redox potential of the molybdenum center may also play an important role in determining the direction of electron flow as it can be altered by subtle perturbations such as the protonation state or the relative geometry of the cofactor [28,29].

It has been proposed that the widespread occurrence of Aox throughout the bacterial and archaeal domains suggests that it preceded Arr, appearing before the time of the Bacteria/Archaea divergence [30,31]. Our discovery that Arr is bidirectional and may function as an arsenite oxidase in some organisms presents the alternate possibility [6]. The physiological role of Arr as a reductase or oxidase may depend on the redox potentials of the molybdenum center and [Fe-S] clusters, additional subunits (e.g., ArrC), as well as other components and organization of the electron transfer chain. In the case of *A. ehrlichii*, arsenite oxidation is coupled to nitrate reduction. Thus the flow of electrons is from Arr (donor complex) to respiratory nitrate reductase (Nar, acceptor complex) via the quinone pool. In As(V) respiring organisms, the flow is reversed with electrons generated by a different donor complex (e.g., formate dehydrogase, NADH dehydrogenase) and Arr functioning as the terminal reductase [8,14]. This reversibility in function further underscores the utility of the "redox protein construction kit" [32] and the versatility of the molybdenum enzymes.

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Acknowledgements We thank the JGI for sequencing and annotating the genomes of *Alkalilimnicola ehrlichii* and *Alkaliphilus oremlandii*. C. Saltikov is thanked for providing arsenic grown cells of *Shewanella* sp. strain ANA-3. This work was supported in part by a NASA grant to R.S.O. and J.F.S.

346 **Figure Legends**

347 **Figure 1** Phylogenetic tree (Neighbor Joining) showing relatedness of molybdoproteins
348 (catalytic subunit) from *A. ehrlichii* (Mlg_0216, Mlg_0633, Mlg_1003, Mlg_1286, Mlg_1675,
349 Mlg_1702, Mlg_2426, Mlg_2513) with members of the DMSO reductase family. Mlg_0216
350 (bolded) and Mlg_2426 form a sub branch of respiratory arsenate reductase (ArrA). BisC - biotin
351 sulfoxide reductase (*Escherichia coli*), DorA - DMSO reductase (*E. coli*), FdhG- formate
352 dehydrogenase (*E. coli*), NapA - periplasmic nitrate reductase (*E. coli*), NarG - respiratory
353 nitrate reductase (*E. coli*), NasA - assimilatory nitrate reductase (*Klebsiella pneumonae*), PsrA -
354 polysulfide reductase (*Wolinella succinogenes*), SerA - respiratory selenate reductase (*Thauera*
355 *selenatis*), TorA - trimethylamine oxide reductase (*E. coli*), TtrA - tetrathionate reductase
356 (*Salmonella typhimurium*).
357
358 **Figure 2** Arr functioning as an arsenite oxidase in *A. ehrlichii*. A) Native gel of cell lysates from
359 heterotrophically (aerobic) grown cells (lane 1) and chemolithoautotrophically (anaerobic)
360 grown cells (lane 2). B) Native gel of the membrane fraction from chemolithoautotrophically
361 grown cells developed for arsenite oxidase activity with DCIP as electron acceptor (lane 1),
362 arsenate reductase activity with methyl viologen as electron donor (lane 2), and stained with
363 Coomassie blue to visualize the proteins (lane 3). C) SDS-PAGE gel of the protein band
364 exhibiting arsenite oxidase activity excised from the native gel. Lane 1, gel developed for
365 arsenite oxidase activity using benzyl viologen as the electron acceptor. Lane 2, same gel stained
366 with Coomassie blue. MALDI-TOF MS analysis of the three bands indicated that the top band is
367 ArrA, the bottom band is ArrB and the middle band, which exhibits the activity in lane 1, is the

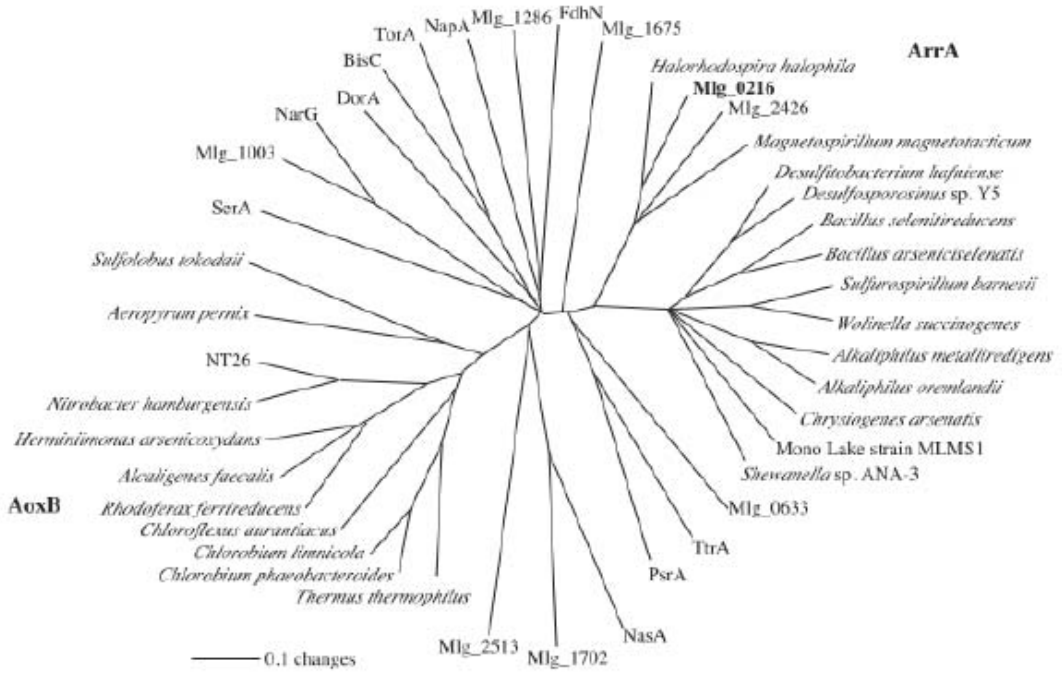
ArrAB complex. Double arrow indicates the position of ArrAB in the native gels. Molecular weight standards (in kDa) are to the left of lanes 1 in A and C.

Figure 3 Conserved binding domains in respiratory arsenate reductase (ArrA) and arsenite oxidase (AoxB). A) The Mo binding domain. Conserved amino acids are shaded in grey, the cysteine in ArrA predicted to coordinate to the molybdenum and the alanine in AoxB (which does not), are bolded. B) The [Fe-S] binding domain showing conserved cysteine residues (shaded in grey) that coordinate to the Fe. Note the absence of a fourth cysteine residue in the [3Fe-4S] domain of AoxB.

Figure 4 Oxidoreductase activity of Arr from arsenate respiring bacteria. A) *A. oremlandii*, arsenite oxidase activity with methyl viologen as electron acceptor (left panel) and arsenate reductase activity with methyl viologen as electron donor (right panel). B) *Shewanella* sp. ANA-3 arsenite oxidase activity with benzyl viologen as electron acceptor.

Supplementary Information

Figure S1. Identification by MALDI-TOF peptide mass finger printing of the three proteins bands associated with arsenite oxidase and arsenate reductase activity in *Alkalilimnicola ehrlichii*. Identities were made with MASCOT (www.matrixscience.com) using a value of 0 for missed cleavage and a mass tolerance of 0.5 Da. Score values over 75 were positively assigned. Lines indicate corresponding bands from Fig. 2C lane 2. A) upper band ArrA, B) middle (active) band, ArrAB, and C) lower band ArrB.



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394 Fig 1

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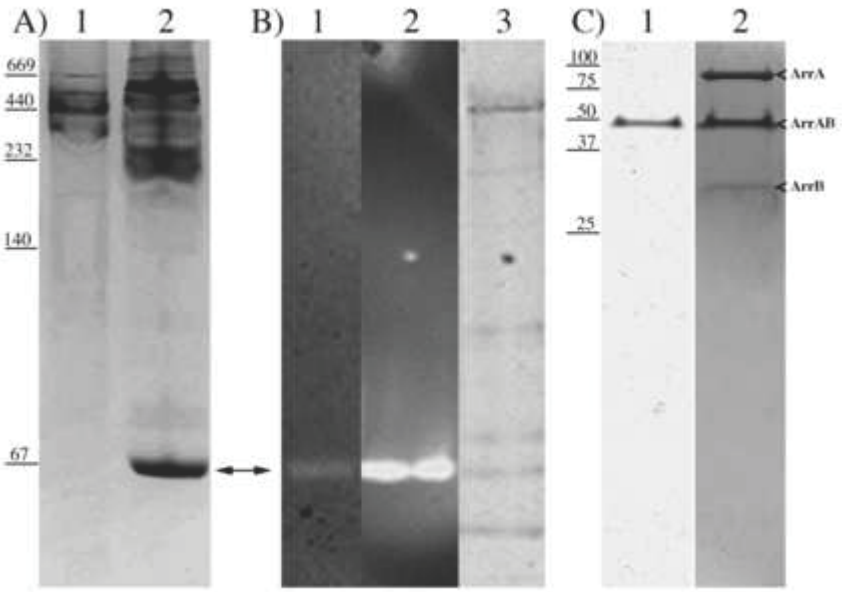

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397 Fig 2

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402 Fig 3

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