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Localization of the Dissimilatory Arsenate Reductase in *Sulfurospirillum Barnesii* Strain SeS-3

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Abstract: Problem statement: Sulfurospirillim barnesii strain SeS-3 is one of the recently known bacterial isolates that can obtain energy to support growth by respiring the toxic oxyanions of arsenic and selenium under anaerobic conditions. **Approach:** The ultimate goal of this investigation is to localize the active sites for the reduction of arsenate arsenite as well as selenate selenite in S. barnesii strain SeS-3. **Results:** The ability of the type strain Sulfurospirillium barnesii strain SES-3 (ATCC 700032) to reduce selenate and arsenate were tested using cell grown anaerobically with 20 mM lactate as the carbon source and either 10 mM selenate or 5 mM arsenate as the terminal electron acceptors were harvested after the turbidity reached 0.3, 0.4 absorption units at 600 nm, cell density 4gm wet cells. The results of this study showed that the mobilization of the toxic oxyanions of arsenic and selenium by Sulfurospirillum barnesii strain SES-3 is linked to the membrane. The enzyme is specific for the reduction of arsenate, selenate, selenite, nitrite, thiosulfate and phosphate. Whereas, no specificity was detected for arsenite nitrate, fumarate, when they served as the final electron acceptor. **Conclusion/Recommendations:** This study concluded that the mechanism of arsenate reduction by S.barnesii strain SeS-3 is connected into the membrane. The environmental significance of this bacterium and its impact to the bioremediation potential in the underground water and sedimentary environment is also discussed.

Key word: World Health Organization (WHO), arsenate reduction, environmental conditions, toxic oxyanions, terminal electron, dissimilatory arsenate, *Sulfurospirillum Barnesii*

INTRODUCTION

Why bacteria respires Arsenic: Bacteria respires the toxic oxyanions of arsenic for several reasons; (i) Arsenic come in to the cell via phosphate-transport system (phosphorylation mechanism); since it is very toxic cells needs to dump it out again trough detoxification mechanism (Arsenic detoxification), (Afkar *et al*.,1999; 2003; Herbel *et al*., 2002; Hoeft *et al*., 2004; Krafft and Macy 1998; Macur *et al*., 2004; Mukhopadhyay and Rosen, 2002; Oremland and Stolz, 2005; Frankenberger, 2002; Stolz *et al*., 2002); (ii) some bacteria has ArsC system like E. coli, S. aureus, S.xylosus and other Enteric bacteria once arsenic comes into the cell it reduces it by forwarding some of its reducing equivalents (arsenic reduction), (Macur *et al*., 2004; Messens *et al*., 2002; Oremland *et al*., 2003; Rosen, 2002a; Srini *et al*., 2004; Sun, 2004). ArsC gene, a part of the ars operon that encodes the enzyme Arsenate reductase that carries out the first step in arsenate metabolism in living organisms before its efflux from the cell, reduce arsenate As (V) to arsenite (III) (Rosen, 2002b; Saltikov *et al*., 2003; Frankenberger, 2002; Srini *et al*., 2004), (iii) Cyanobacteria that use light for photosynthesis, until certain limit, excess light energy become toxic, so, they generates high electronegative power inside the cell to balance the high electropositive charge for O^2; this happens in case of O^+ toxicity for example (Li *et al*., 2003; Yoshitaka *et al*., 2004). Bacteria usually try to regulate and create equilibration inside the cell and the surrounding environmental conditions through dumping off or even creates excess charges in/or outside the cell to survive. Arsenic is found in four oxidation states: Arsenate (As^{+5}), Arsenite (As^{+3}), Arsenic (As°) and Arsine (As^{-3}). The two highest oxidation states are the most common and the two lowest are rare (Kulp *et al*., 2004; Lazaridis *et al*., 2002 Oremland and Stolz, 2005). Many microbes reduce As (V) to As (III) as means of resistance. These Arsenate-Resistant Microbes (ARMs) do not gain energy from the process, but use it as a means of coping with the high arsenic in their environment (Anderson and Cook, 2004; Ford *et al*., 2005; Mukhopadhyay *et al*., 2002; Oremland and Stolz, 2003; Messens *et al*., 2002; Saltikov *et al*., 2003; (Silver and Phung, 1996). Arsenate that has entered the

microbe's cytoplasm is converted into As (III) through a process mediated by small polypeptide (ArsC) and expelled out of the cell by detoxification mechanism via As (III)-specific transporter (ArsB) (Meng *et al*., 2004; Rosen, 2002a; Saltikov *et al*., 2003; Wange *et al*., 2004).

It is hypothesized that bacteria capable of either oxidizing As (III) or reducing As (V) coexist and are ubiquitous in soil environment, suggesting that the relative abundance and metabolic activity of specific microbial populations plays an important role in the speciation of inorganic as in soil pore water. Recent investigation by (Macur *et al*., 2004) at Montana State University on bacterial populations associated with the oxidation and reduction of arsenic in an unsaturated soil (aerobic) have proved that microbial communities responsible to the oxidation and reduction of As coexist under the same environmental conditions; environmental samples manipulated with 75 µM arsenite [As (III)] or 250 µM arsenate [As (V)], when the rate of oxidation and/or reduction of Arsenate Arsenite was monitored; Arsenite (III) was rapidly oxidized to As (V) via microbial activity, whereas no apparent reduction of As(V) was observed in the column experiments. However, eight aerobic heterotrophic bacteria with varying redox phenotypes were isolated from the same column. Three isolates were as oxidizers and five isolates were as reducers based on 16s rRNA analysis (Anderson and Cook, 2004; Macur *et al*., 2004). The poisonous properties of arsenic compounds have been known since antiquity (Frankenberger, 2002) arsenic trioxide (As_2O_3) gained much favor as a homicidal agent it was once referred as to inheritance powder in the mid-19th century James Marsh devised the first chemical test for the presence of arsenic in tissue thereby advancing forensic science while putting such nefarious heirs on notice. The health of tens of millions of people worldwide is at risk from drinking arsenic-contaminated well water (Hadi and Parveen 2004; Lu *et al*., 2004; Macur *et al*., 2004; Mandal and Biswas, 2004; Rahman *et al*., 2005; Sun *et al*., 2004). In most cases this arsenic occurs naturally within the subsurface aquifers, rather than being derived from identifiable sources of pollution. The mobilization of arsenic into the aqueous phase is the first crucial step in a process that eventually leads to the human arsenicosis, increasing evidence suggests that this is a microbiological phenomenon. Sulfurosprillum barnesii sp. nov. Strain SeS-3 and Sulfurosprillum arsenophilum strain MIT-13 sp. nov. Were assinged to be new members of the Sulforosprillum clad of the-roteobacteria based on the 16S rRNA analysis (Stolz *et al*., 1999). They are two trains of the dissimilatory arsenate-reducing vibrioid to sprillium-shaped cells, 0.3µm wide and 1-2µm in length, motile by polar flagellum, growth optimum 33°C, pH 7.5 and 0.05% NaCl. Selenate, arsenate, thiosulfate, elemental sulfur,trimethylamine oxide, trimethylamine oxide. Fe (III), nitrate, fumarate, aspartate and manganese dioxide can all be used as the terminal electron acceptor. Capableof microaerobic growth and gain energy to support growth from the dissimilatory reduction of arsenate to arsenite. This study reporting that the arsenate reductase from Sulfurosprillum barnesii strain SeS-3 is linked to the membrane and some of its physiological and biochemical features have been characterized.

MATERIALS AND METHODS

Organism, growth conditions and preparation of the cell free-extract: The ability of the type strain Sulfurospirillium barnesii strain SES-3 (ATCC 700032) to reduce selenate and arsenate were tested using the medium described by (Stolz *et al*., 1997), grown anaerobic ally with 20 mM lactate as the carbon source and either 10 mM selenate or 5 mM arsenate as the terminal electron acceptors previously described; cells were harvested after the turbidity reached 0.3~0.4 absorption units at 600 nm, cell density 4 gm wet cells L. The harvested cells were concentrated by centrifugation at 10000 rpm for 30 min, by using SORVALL RC 5B plus SN 9500466,USA USA, then washed and suspended, in 80 ml 10 mM Tris-HCl buffer pH 8.0, 1 mM EDTA pH 8.0, 10 µM PMSF, after DNase treatment for overnight at 4°C, the cells were disrupted by the sonication (Braun-Sonic L, purchased from B. Braun Biotech, Inc., USA Ser. Nr. G-0194364; Type 853963 4, Hz 50 60, V 120, on ice under aerobic conditions with output power of 200+10 Watts over three min total time at 30 sec time intervals. Unbroken cells were separated by low speed centrifugation at 7500 xg for 20 min at 4°C. Membrane pellet is separated from the soluble fraction with high-speed centrifugation 200,000 xg for 1hr at 4°C. The pelleted membrane suspended in 10 mM Tris-HCl buffer pH 8.0, I mM EDTA, 10 µM PMSF and stored at -80°C until use. The protein concentration is determined spectrophotometrically with the assay reagents (Lowry *et al*., 1951) with bovin serum albumin used as the standard. The membrane pellets (4.5mg mL^{-1} protein) were solubilized by the zwitterinonic detergent Chaps (1%) overnight at 4°C, followed by centrifugation at 7500 xg for 10 min to remove the unsolubilized pellets. The solubilized membrane as well as unsolubilized pellets were examined for the selenate, selenite, arsenate and arsenite reductase activities by MV as previously described by (Stolz *et al*., 1997) using a Perkin-Elmer Lambada 2 dual-beam spectrophotometer (made in Germany). Enzyme activity was calculated as umol MV oxidized min^{-1} mg^{-1} protein using an

extension coefficient of 13 mM^{-1} cm^{-1}. In this assay, the reduced MV (20 mM) served as the electron donor and the As (V) (5-10) mM as the electron acceptor, suspended in 50 mM Tris-HCl buffer pH 8.0, the decrease in the absorbtion at 604 nm is measured as the function of the AsR activity using the extinction coefficient of the reduced MV is 13 mM^{-1} cm^{-1}. The subunit composition of the solubilized membrane was examined by the SDS-PAGE in 12% resolving gel and 4% stacking gel, running at 100 constant volts for 1.5 hrs, at room temperature.

Gel electrophoresis and western blot analysis: For gel electrophoresis and western blot analysis the fraction showed the senelat, selenite, arsenat and arsenite reductase activities (10-20 ug each) were digested in the suspended in sample buffer (Laemmili, 1970) in ratio 1:2 sample to sample buffer (provided from Bio-RAD laboratories. USA), heated for 5 min at 100°C and charged on to 4% stacking gel and 12% resolving to test the subunit composition. The relative molecular mass of the proteins was determined using the relative mobility of the following standards; myosin (206 kDa), B-mgalactosidase (119 kDa), bovine serum albumin (91 kDa), ovalbumin (51.4 kDa), carbonic anhydrase (34.7 kDa), soybeantrypsin inhibitor (28.1kDa), lysozyme (20.4 kDa) and aprotinin (7.2 kDa). The large format SDS-PAGE was performed using the Maxi-Protean II apparatus (BIO-RAD) according to, using a 4% stacking and a 12.5% resolving gel, each lane contained 100 ug of total protein. For N-terminal amino acid sequence analysis, proteins were transferred directly to the PVDF membrane and sequenced via Edmond degradation (ProSeq, Boston MA). Antibody were develop to detect the and b subunit of the enzyme via a western blot protocol.

PAGE and activity staining: For detection of arsenate and selenate reductase activity in the Gel-PAGE, the SDE- PAGE or Non-denaturationg gel electrophoresis were performed under heating and non-heating conditions, the gels were soaked in 50 mM Tris-HCl buffer pH 8.0, containing 10 mM Methyl Viologen (MV) reduced with sodium hydrosulfite (dithionite) Lot No.38H3652 (0.1%). The violet-stained gels were then rinsed and exposed to 5 mM As (V) and monitored. Aresenate-reductase activity appeared as a clear band of the oxidized methyl viologen.

Reduction of Nitrate in S. barnesii strain SeS-3 compared with other different electron acceptors: Sulfurosprillum barnesii strain SES-3 is routinely cultivated under growth conditions as described above except that the final electron acceptor is replaced by, 20 mM selenate, or 5 mM fumarate, or 20 mM nitrate, after the cells reached the appropriate growth phase. The cells harvested by centrifugation at 12000 rpm for 20 min at 4°C, then digested by SDS-PAGE sample buffer for 10 min and loaded on to the SDS-PAGE. After electrophoresis the gel-page is soaked in the reduced Methyl Viologen (100 mM) as the artificial electron donor for about10 min and followed by reduction the stained gel with the addition of the appropriate electron acceptor.

Determination of the sites of arsenate reduction in S. barnesii strain SeS-3: In order to localize the dissimilatory arsenate reductase in the S. barnesii strain SeS-3, about 40 mL of 10 mM Tris-HCl buffer pH 8.0, 1mM EDTA pH 8.0 and 10 u M PMSF containing 1gm of the freshly harvested cells (264 mg total protein), were stirred at 4°C for 2 h after the addition of 1mg mL^{-1} protease inhibitor deoxyribonuclease (DNase). The cell homogenate is subjected to a series of fractionations by passing it through the French pressure machine or by sonic treatment to separate the cytoplasmic fractions from the membranes. All the steps are carried out at 4°C unless otherwise noted. Aliquots of each of the intact cells (6.6 mg protein mL^{-1}), cell homogenate plus DNase (6.0 mg protein $m^{-1}L$), French pressure resulting cell free extract (5.2 mg protein mL^{-1}), sonic treatment fraction (4.6 mg protein mL^{-1}),The cytoplasmic fraction (2.4 mg protein mL^{-1}), the unsolubiliozed membrane pellets (3.6 mg mL^{-1}), in addition to the 1% CHAPS solubilized membrane (4.4 mg mL^{-1}), all are examined for their ability to reduce arsenate to arsenite by MV assay, the reaction mixture contained 1.3 ml of 50 mM Tris-HCl buffer pH 8.0, 30 ul reduced methyl viologen (provided from anaerobic stock 10 mM to produce a final concentration 200 uM in a total volume 1.5 mL which is the volume of the anaerobic cuvettes set used in the assay, arsenate 5 mM was used as the electron acceptor, 150 ul is added from previously prepared anaerobic stock solution (50 mM sod. arsenate suspended in 50 mM Tris-HCl buffer pH 8.0) to provide final concentration 5 mM, in addition to 20 uL each of the tested samples. The reaction mixture initiated by bubbling the reactants with the gas phase, a mixture of Nitrogen (N_2) and carbon dioxide (CO_2) in ratio of 80: 20 for 5 min, then sealing the top of the anaerobic cuvette with a screw rubber cap and changing the head space for one min with the same gas phase before introducing the cuvettes to the spectrophotometric measurements. Two sets are prepared every assay; one for the sample and the other one used as a control that is containing the same reactants under the same conditions except that the examined sample is replaced by the assay buffer. The assay started by introducing the cuvettes to the main body of the dual-beam spectrophotometer, the control cuvette is placed in the reference beam side and

the sample cuvette is placed in the sample side and the decrease in the absorbance at 604 nm over 10 min is monitored as a function of the arsenate reductase activity. The extension coefficient of the oxidized methyl viologen at 604 mm is 13 mM^{-1} cm^{-1} (Stolz *et al*., 1997).

Measurement of the arsenate reductase activity in the s. barnesii strain ses-3 spheroplasts prepared using ionic and non-ionic detergents:

Preparation of the Spheroplast: The spheroplasts were obtained according to (Hepel *et al*., 1969), 2 gm of freshly harvested and washed cells were suspended in 15 mL 30 mM Tris-HCl buffer pH 7.6, containing 0.1 mM EDTA, 0.5 M sucrose and incubated on a magnetic stirrer for 15 min. After centrifugation (8000 xg for 15 min, 4°C), the sediments were resuspended in ice-cold solution of 15 mL of 0.5 mM $MgCl_2$ and vigorously stirred for 15 min. After centrifugation at (8000 xg for 15 min, 4°C); the supernatant (periplasm) is separated and the pellets (spheroplasts) were assayed for the arsenate reductase activity. The spheroplasts is divided into two halves one of them is solubilized with 1% Chaps and the other half is solubilized with 1% Triton X-100, for 1 h each, followed by centrifugation at 7500 xg, 4°C for 20 min to separated the unsolubilized membrane pellets. The resulting solubilized membrane as well as the unsolubilized membrane pellets are assayed for their arsenate reductase activity by MV protocol as described above, using the untreated spheroplasts as a negative control.

Effect of sonic treatment on the AsR activity of the S. bernesii strain SeS-3 spheroplasts: To investigate the effect of sonication on the arsenate reductase activity of the spheroplasts treated with 1% Chaps and 1% Triton X-100; 1mL of the spheroplast suspension from each preparation is placed in 1.5 mL eppendorf tubes at 4°C, sonicated for 5 min at 120 Watts, followed by centrifugation at 10000 rpm for 15 min, the cytoplasmic fractions from the total membrane pellets, MV assay applied to access the arsenate and selenate reductase activities as mentioned above.

Chemicals and reagents: The chemicals and reagents used in this investigation are the highly available commercial grades, purchased from SIGMA, Fisher Scientific, Fisher Biotech and J.T. Baker Inc., USA. The SDS-PAGE electrophoresis reagents and apparatus purchased from the BIO-RAD, USA. The chromatography resins and buffers used are the finest known trusted qualities.

RESULTS AND DISCUSSION

Sulfurosprillium barnesii strain SeS-3 is among the first to be described with its unique physiology; couple the oxidation of organic matter to the reduction of the toxic oxyanions of arsenic and selenium under anoxic conditions. The genus name Geosprillium was previously proposed and its physiology and biochemistry was published using that genus name (Logerman *et al*., 1996; Winkelmann, 1997; (Stolz *et al*., 1997). S. barnesii strain SeS-3 isolated from a selenite contaminated freshwater marsh in western Nevada (Oremland *et al*., 1989; 1994). The intact cell suspension was assayed for the arsenate reductase activity (AsR) by monitoring the rate of the oxidation of the membrane-impermeable reduced Methyl Viologen as the artificial electron donor with the reduction of sodium arsenate as the terminal electron acceptor. The highest percentage of arsenate reduction rate was found to be 7mM^{-1} min^{-1} (representing 100% of AsR activity). Whereas (80%) of arsenate reduction rate was found in the membrane pellets (5.6 $mM^{-1}min^{-1}$) (Table1). The solubilized membrane using 1% Chaps showed lesser arsenate reductase activity (4 mM^{-1} min^{-1}) compared to the unsoulbilized membrane (5.6 $mM^{-1}min^{-1}$). No arsenate reductase activity was detected in the periplasmic fractions of S. barnesii indicating that the arsenate reductase of S. barnesii is linked to the membrane. Arsenic reduction mechanism inside the cell is considered to be the first phase in the arsenic resistance; operons which encode arsenic resistance have been found in multicopy plasmids from both gram-negative and gram-positive bacteria.

The resistance mechanism is encoded from a single operon, which typically consists of an arsenite ion-inducible repressor that regulates expression of an arsenate reductase and inner membrane-associated arsenite export system. In Escherichia coli chromosomal ars operon homolog is functional in arsenic detoxification and is conserved in gram-negative (Dioro *et al*., 1995). One of the most frequently employed strategies to gain resistance to cytotoxic compounds in both eukaryotes and prokaryotes is the active extrusion of these compounds from the cell to reduce the intracellular concentration to subtoxic levels (Georgopapadakou, 1995).

Table 1: Localization of the dissimilatory Arsenate Reductase Activity (AsR) S. bernesii strain SeS-3. IC (intact cells), CF (cytoplasmic fraction), CDFP (cells disrupted with French pressure machine), CDS (cells disrupted with sonication), Memb. pellets (membrane pellets) and sol. memb. (solubilized membrane)

Sample identity	Corresponding activity $mM^{-1}min^{-1}$	[a] recovery (%)
IC	7.0	100
CDFP	5.3	76
CDS	6.1	87
CF	0.0	0
memb. pellets	5.6	80
chaps sol.memb 1%	4.0	57

Table 2: Comparison of the AsR activity in S. bernesii spheroplasts treated with and ionic and non-ionic detergents. The arsenate reductase activity was assayed in the presence of 10 mM reduced MethyleViologen as the electron donor and 5 mM Arsenate as the electron acceptor and the assay mixture were suspended in 50 mM Tris-HCl buffer pH, 8.0. The AsR activity was calculated as μmol MV oxidized min^{-1} mg^{-1} protein using an extension coefficient of 13 mM^{-1} cm^{-1}. In this assay, the reduced MV (20 mM) served as the electron donor and the As (V) (5-10) mM as the electron acceptor, suspended in 50 mM Tris-HCl buffer pH 8.0, the decrease in the absorption at 604 nm is measured as the function of the AsR activity using the extinction coefficient of the reduced MV is 13 mM^{-1} cm

Sample identity Spheroplasts	AsR activity mM^{-1} min^{-1}	Recovery (%)
1% Chaps pellets	4	100
1% Chaps supernatant	0	0
1% Triton X-100 pellets	3.4	85
1% Triton X-100 supernatant	0	0

Table 3: Effect of sonic treatment on the S. bernesii strain SeS-3 AsR activity of spheroplasts treated with 1% Chaps and 1% Triton X-100. The percentage of the recovery is refereed to the total activity of the samples both of 1% Chaps and 1% Triton x-100 solubilized membrane pellets. The recorded values are the average mean of triplicate measures ± S.D

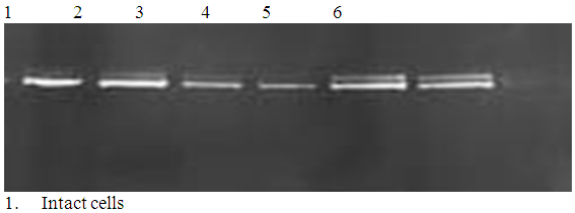
Sample identity	ASR mM^{-1} min^{-1}
1% chap supernatant	4.0
1% chaps pellets	4.0
1% Triton X-100 supernatant	0.0
1% Triton X-100 pellet	3.4

Protein pumps that span the membrane catalyze this extraction process; many of them belong to the ATP Binding Cassette $(ABC)^{-1}$ superfamily (Ambudkar *et al*., 1999). Generally, These ABC transporters are composed of two homologs halves, each containing two parts as follows: a trans-membrane domain putatively arranged in to 6-helices and a Nucleotide Binding Domain (NBD). When the intact cells of S. barnesii strain SeS 3 disrupted with either French pressure or sonic treatments, there were a remarkable decrease in the arsenate reduction (5.6 and 6.1 $mM^{-1}min^{-1}$ respectively compared to the arsenate reduction rate of the intact cells; 7 $mM^{-1}min^{-1}$), this albiet decrease in the activity of arsenate reduction could be attributed to oxygen toxicity during the fractionation processes. Similarly, lysozyme treatment of the intact cells resulted in a remarkable inhibition of the arsenate reductase activity; only 43% of the total activity of the arsenate reductase is retained in the spheroplast. In the contrary, osmotic shock does not affect the rate of arsenate reduction in S. barnesii strain SeS-3. Spheroplasts prepared by osmotic shock according to the method of (Heppel, 1969) showed an arsenate reduction value very similar to the one reported for the intact cells, that means that spheroplast prepared by this protocol does not disrupt the integrity of the enzyme. In order to investigate the effect of the different ionic and non-ionic detergents on the arsenat reductsae activity; the spheroplasts prepared by osmotic shock subjected to two different detergents Chaps as an ionic detergent and Triton X-100 as a non-ionic detergent to solubilized the intact membrane. The membrane pellets were solubilized for 1h at 4°C, unsolubilized membrane separates by ultracentrifugation at 100000 xg for 1h, both the solubilized and unsolbilized membrane assayed for the arsenate reducing activity using the 10 mM reduced Methyl Viologen (MV) as the electron donor and 5 mM arsenate as the electron acceptor as described above. Aliquot of the freshly prepared spheroplasts, which is not subjected to any of the detergents, was used as the negative control (-ve). No activity was found in the supernatants obtained either form 1% Chaps or 1% Triton X-100 solubilization, most of the arsenate reduction activities were observed in the unsolubilized membrane (4 $mM^{-1}min^{-1}$ and 3.4 $mM^{-1}min^{-1}$) form 1% Chaps and Triton X-100 treatments respectively and the AsR values were very close to the total AsR activity of the negative control (4 $mM^{-1}min^{-1}$) (Table 2). This is considered strong evidence that the arsenate reductase of S. barneii strain SeS-3 is a membrane bound enzyme. Furthermore; exposure of the spheroplasts prepared from the osmotic shock for sonic treatment at 120 Wattes for 5 min resulted in partial disruption of the arsenate reductase from the membrane. After sonic treatment the Arsenate Resuctase activity (AsR) for both 1 Chaps and 1% Triton X-100 were measured. The reported AsR activity for the membrane fraction solubilized with 1% Chaps was 3 $mM^{-1}min^{-1}$ compared to 4 mM $mM^{-1}min^{-1}$ for the unsolubilized membrane, about 2 $mM^{-1}min^{-1}$ AsR activity is detected in the solubilized membrane fraction with 1% Triton X-100 compared with 3.3 mM $mM^{-1}min^{-1}$ for the unsoubilized membrane (Table 3). Hence, it is clear that the exposure of S. barnesii strain SeS-3 spheroplasts for sonic makes the arsenate reductase more accessible to the action of these soulbilizing agents, i.e., provides more surface area to be reacted with the functional groups in Chaps and Triton X-100. The higher value for the AsR obtained by Chaps solubilization (50% more) compared to TritonX-100 could be attributed to the nature of this detergent since Chaps is zwitterinonic detergent carrying both positive and negative charges that binds different charged groups on the protein surface. This result evaluates Chaps as a perfect choice for the soulbilozation and purification of the AsR from the membrane of S. barneii strain SeS-3. The arsenate reductase from S. barnesii strain SeS-3 has been purified with two successive steps of iron exchange chromatography followed by one single step of gel

filtration on sephacryl S-300. SDS-PAG gel revealed that the putative respiratory arsenate reductase from the S. barnesii is a multimeric complex containing a major subunit of 47 kDa that readily degrades into two fragments of 26 and 19 kDa. This latter fact that resulted in continued loss of the activity both during the purification and the subsequent storage (Afkar *et al*., 2005). Although the arsenate reduction activity of S. barnesii strain SeS-3 is detected by MV assay spectrophotometrically, it was difficult to measure the activity on the Gel-PAGE, this may be due to the denaturing of the active sites for the arsenate reductase during the electrophoresis. Aliquots from the intact cells of S. barnesii st SeS-3, soluble fraction, solubilized membrane, 1st ion exchange chromatography, 2nd ion exchange chromatography and gel filtration were digested in SDS-PAGE sample buffer and resolved in 12% polyacrylamide gel. The Zymogram developed with 10 mM reduced methyl viologen as the electron donor and 5 mM Na-arsenate as the electron acceptor to express the arsenate reduing activity on the gel page (Fig. 1). Alternatively, the nitrate reductase activity in S. barnesii strain SeS3 was detected clearly on the SDS-PAGE (data not shown), however, no nitrate reducing activity could be detected. spectrophotometrically, this results show that the putative nitrate reductase of S. barnesii strain SeS-3 is distinct from the arsenate reduactase. Moreover, S. barneii strain SeS-3 was able to grow with either sodium arsenate (5-10 mM), sodium selenate (5-10 mM) or sodium selenite (5-10 mM) and reach the optical density within 24 h, reduce the arsenate to arsenite and selenate/selenite to elemental selenium (Fig. 2). However, it was difficult to purify the selenate/selenite reductase due to technical problem to handle the sticky selenium that from clumps with the cells in the growth medium. However, in Bacillus selenatireducens strain MLS10, a gram-positive bacterium that respires arsenic and selenium microrerophilically (Afkar *et al*., 2003), it was easy to detect the arsenate respiring activity on the Gel-PAGE gel regardless the denaturing effect of SDS.

No wondering, since it is well known that gram positive bacterial spp. can persist in the environment over decades and withstand the sever environmental conditions compared to the gram-negative spp. due to spore formation that may help the bacteria to be resistant to the sever fluctuations in environmental conditions and exposure to several toxic and denaturing effect.

Environmental significance of *Sulfurosprillium spp*: Considering the toxicity of arsenic for both prokaryotes and eukaryotes, the discovery that As (V) serves as a nutrient to certain anaerobes by functioning as their respiratory oxidant came as a surprise.



1. Intact cells
2. Soluble fraction
3. Soubilized menbrane
4. 1st iron Exchange chromatography
5. 2nd ion exchaneg chromatography
6. Gel filtration

Fig. 1: Detection of the AsR activity on the Gel-PAGE of Gram negative bacterium *S. barnesii* strain SeS-3. Aliquots from the intact cells (lane 1), soluble fraction (lane 2), solubilized membrane (lane 3), 1st ion exchange chromatography (lane 4), 2nd ion exchange chromatography (lane 5) and gel filtration (lane6), were digested in SDS-PAGE sample buffer and resolved in 12% polyacrylamide gel. The Zymogram developed with 10 mM reduced methyl viologen as the electron donor and 5 m M Na-arsenate as the electron acceptor

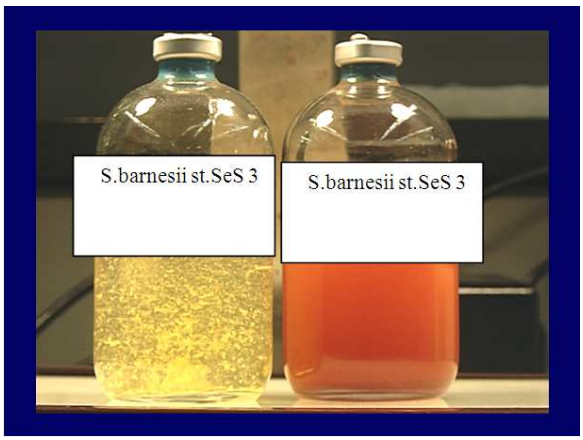


Fig. 2: Mobilization of the toxic oxyanions of Arsenic and Selenium by *S. barnesii* strain SeS-3. 10% of freshly growing cells were transferred in 100 ml basal medium under anaerobic conditions. Either 5-10 mM sodium arsenate or sodium selenate were introduced to the medium from anaerobic stock solution serving as the terminal electron acceptor. After 18 hrs of incubation at 30°C, yellow precipitate of elemental Arsenic (1) or orange precipitate (2) from elemental Selenium are visualized

The reaction is energetically favorable when coupled with the oxidation of organic matter because the As (V)/As (III) oxidation/reduction potential is ±134 mV.

Two closely related representatives of the ε-proteobacteria, Sulfurrospirillum barnesii and Sulfurrospirillum arsenophilum were the first microbes reported that could achieve this feat. Both conserve energy by linking the oxidation of lactate to the reduction of As (V) to As (III) [Gibbs free energy (ΔG^0) = -295 Kj/mol lactate]. Arsenic toxicity in both natural and humanly impacted environments is an important issue of current public health. Recently problem sites include (a) drinking water in Taiwan where arsenic poisoning is known as Black foot because of the necrotic destruction of tissue, (b) well water in West Bengal India and Bangladesh (Hadi and Parveen 2004; Lu *et al*., 2004; Mandal and Biswas, 2004) where world health organization (WHO) efforts to provide pathogen-free drinking water by placing shallow tube wells has replaced a problem of Cholera with one of arsenic toxicity, (c) release of arsenic from burning coal into foods in Southwest China (Sun *et al*., 2004) and (d) within the USA where arsenic in drinking water from residential wells in Michigan and Wisconsin (Brasier, 1997; Ryker, 2000), as well as industrially impacted mining waters in western USA and recreational waters in north of Boston (Ahmann *et al*., 1994) are far above the WHO recommended levels. Therefore, it is of interest to design biochemical and molecular probes to target microorganisms that have unique metabolism similar to Sulfurosprillium spp. and likely organisms that can serve as Arsenic scavengers from the sites of pollution and can be employed to serve in the environmental restoration.

CONCLUSION

No wondering, since it is well known that gram positive bacterial spp. can persist in the environment over decades and withstand the sever environmental conditions compared to the gram-negative spp. due to spore formation that may help the bacteria to be resistant to the sever fluctuations in environmental conditions and exposure to several toxic and denaturing effect.

Environmental significance of *Sulfurosprillium* spp: Considering the toxicity of arsenic for both prokaryotes and eukaryotes, the discovery that As (V) serves as a nutrient to certain anaerobes by functioning as their respiratory oxidant came as a surprise. The reaction is energetically favorable when coupled with the oxidation of organic matter because the As (V)/As (III) oxidation/reduction potential is ±134 mV. Two closely related representatives of the ε-proteobacteria, Sulfurrospirillum barnesii and Sulfurrospirillum arsenophilum were the first microbes reported that could achieve this feat. Both conserve energy by linking the oxidation of lactate to the reduction of As (V) to As (III) [Gibbs free energy (ΔG^0) = -295 Kj/mol lactate]. Arsenic toxicity in both natural and humanly impacted environments is an important issue of current public health. Recently problem sites include (a) drinking water in Taiwan where arsenic poisoning is known as Black foot because of the necrotic destruction of tissue, (b) well water in West Bengal India and Bangladesh (Hadi and Parveen 2004; Lu *et al*., 2004; Mandal and Biswas, 2004) where world health organization (WHO) efforts to provide pathogen-free drinking water by placing shallow tube wells has replaced a problem of Cholera with one of arsenic toxicity, (c) release of arsenic from burning coal into foods in Southwest China (Sun *et al*., 2004) and (d) within the USA where arsenic in drinking water from residential wells in Michigan and Wisconsin (Brasier, 1997; Ryker, 2000), as well as industrially impacted mining waters in western USA and recreational waters in north of Boston (Ahmann *et al*., 1994) are far above the WHO recommended levels. Therefore, it is of interest to design biochemical and molecular probes to target microorganisms that have unique metabolism similar to Sulfurosprillium spp. and likely organisms that can serve as Arsenic scavengers from the sites of pollution and can be employed to serve in the environmental restoration.

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REFERENCES

Afkar, E. and Y. Fukumori, 1999. Purification and characterization of triheme cytochrome c7 from the metal-reducing bacterium, *Geobacter metallireducens.* FEMS Microbiol. Lett., 175: 205-210. DOI: 10.1111/j.1574-6968.1999.tb13621.x

Afkar, E., J. Lisak, C. Saltikov, P. Basu and R.S. Oremland *et al*., 2003. The respiratory arsenate reductase from Bacillus selenitireducens strain MLS10. FEMS Microbiol. Lett., 226: 107-112. DOI: 10.1016/S0378-1097(03)00609-8

Afkar, E.D., K. Newman, C. Saltikov, B. Kail, R.S. Oremland and J.F. Stolz, 2005. A novel metalloid reductase from the dissimilatory arsenate-reducing bacterium Sulfurospirillum barnesii strain SES-3. J. Applled En. Microbi.

Ahmann, D., A.L. Roberts, L.R. Krumholz and F.M. Morel, 1994. Microbe grows by reducing arsenic. Nature, 371: 75. DOI: 10.1038/371750a0

Ambudkar, S.V., S. Dey, C.A. Hrycyna, M. Ramachandra and I. Pastan *et al*., 1999. Biochemical, cellular and pharmacological aspects of the multidrug transporter. Ann. Rev. Pharm. Toxic., 39: 361-398. DOI: 10.1146/annurev.pharmtox.39.1.361

Anderson, C.R. and G.M. Cook, 2004. Isolation and characterization of arsenate-reducing bacteria from arsenic-contaminated sites in New Zealand. Curr. Microbiol., 48: 341-347. DOI: 10.1007/s00284-003-4205-3

Brasier, L.L., 1997. Water making many ill and oakland to map arsenic sities. Detroit Free Press.

Dioro, C., J. Cai, J. Marmor, R. Shinder and M.S. DuBow, 1995. An Escherichia coli chromosomal ars operon homolog is functional in arsenic detoxification and is conserved in gram-negative bacteria. J. Bacteriol., 177: 2050-205.

Frankenberger, W.T., 2002. Environmental Chemistry of Arsenic. 1st Edn., Marcel Dekker, New York, ISBN-10: 0824706765 pp: 391.

Georgopapadakou, N.H., 1995. Drug Transport in Antimicrobial and Anticancer Chemotherapy. Dekker, New York, ISBN-10: 0824793994, pp: 644.

Hadi, A. and R. Parveen, 2004. Arsenicosis in Bangladesh: Prevalence and socio-economic correlates. Public Health, pp: 559-64. PMID: 15530935

Heppel, L.A., 1969. The effect of osmotic shock on release of bacterial proteins and on active. J. Gen. Phys., 54: 95-109. DOI: 10.1085/jgp.54.1.95

Herbel, M.J., J. Swotzer Blum, S.E. Hoeft, S.M. Cohen and L.L. Arnold, *et al*., 2002. Dissimilatory arsenate reductase activity and arsenate-respiring bacteria in bovine rumen fluid, hamster feces and the termite hindgut. J. FEMS Microbi. Ecol., 41: 59-67. DOI: 10.1016/S0168-6496(02)00266-0

Hoeft, S.E., T.R. Kulp, J.F. Stolz, J.T. Hollibaugh and R.S. Oremland, 2004. Dissimilatory arsenate reduction with sulfide as electron donor: Experiments with mono lake water and Isolation of strain MLMS-1, a chemoautotrophic arsenate respirer. J. Applied Environ. Microbiol., 70: 27-41. DOI: 10.1128/AEM.70.5.2741-2747.2004

Krafft, T. and J.M. Macy, 1998. Purification and characterization of the respiratory arsenate reductase of Chrysiogenes arsenatis. J. Biochem., 255: 647-53. DOI: 10.1046/j.1432-1327.1998.2550647.x

Kulp, T.R., S.E. Hoeft and R.S. Oremland, 2004. Redox transformations of arsenic oxyanions in periphyton communities. J. Applied Eng. Microbiol., 70: 6428-34. DOI: 10.1128/AEM.70.11.6428-6434.2004

Laemmili, U.K., 1970. Cleavage of structural proteins during an assembly of the head of bacteriophage T4. Nature, 227: 680-685.

Lazaridis, N.K., A. Hour and K.A. Matis, 2002. Flotation of metal-loaded clay anion exchangers. Part II: the case of arsenates. Chemosphere, 47: 319-324. DOI: 10.1016/S0045-6535(01)00218-1

Li, X., Y. Liu, L. Song and J. Liu, 2003. Responses of antioxidant systems in the hepatocytes of common carp (Cyprinus carpio L.) to the toxicity of microcystin-LR. Toxicon, 42: 85-89. DOI: 10.1016/S0041-0101(03)00104-1

Logerman, D.J., H.L. Jenter, J.D. Coates, E.J. Phillips and T.M. Schmidt *et a1*., 1996. Phylogenetic analysis of dissimilatory Fe(III)-reducing bacteria. J. Bacteriol., 178: 2402-2408.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193: 265-75.

Lu, S.N., N.H. Chow, W.C. Wc, T.T. Chang and W.S. Huang *et al*., 2004. Characteristics of hepato cellular carcinoma in a high arsenicism area in Taiwan: a case-control study. J. Occup Eng. Med. 46: 437-441. PMID: 15167390

Macur, R.E., C.R. Jackson, L.M. Botero, T.R. McDermott and W.P. Inskeep, 2004. Bacterial populations associated with the oxidation and reduction of arsenic in an unsaturated soil. Eng. Sci. Technol., 38: 104-111. DOI: 10.1021/es034455a

Mandal, N.K. and R. Biswas, 2004. A study on arsenical dermatosis in rural community of West Bengal. Indian J. Public Health, 48: 30-33. PMID: 15704724

Meng, Y.L., Z. Liu and B.P. Rosen, 2004. As(III) and Sb(III) Uptake by GlpF and Efflux by ArsB in Escherichia coli. J. Biol. Chem., 279: 18334-18341. Messens, J., J.C. Martins, E. Brosens, K. Van Belle and D.M. Jacobs *et al*., 2002. Kinetics and active site dynamics of Staphylococcus aureus arsenate reductase. J. Biol. Inorg. Chem., 7: 146-156. DOI: 10.1007/s007750100282

Mukhopadhyay, R. and B.P. Rosen, 2002. Arsenate reductases in prokaryotes and eukaryotes. En. Health Perspect 110 Suppl, 5: 745-748.

Mukhopadhyay, R., B.P. Rosen, L.T. Phung and S. Silver, 2002. Microbial arsenic: From geocycles to genes and enzymes. FEMS Microbiol. Rev., 26: 311-325. DOI: 10.1111/j.1574-6976.2002.tb00617.x

Oremland, R.S. and J.F. Stolz, 2003. The ecology of arsenic. Science, 300: 939-344. DOI: 10.1126/science.1081903

Oremland, R.S. and J.F. Stolz, 2005. Arsenic, microbes and contaminated aquifers. Trends Microbi., 13: 45-49. DOI: 10.1016/j.tim.2004.12.002

Oremland, R.S., J. Switzer Blum, C.W. Culberston, P.T. Visscher and G. Miller *et al*., 1994. Isolation, growth and metabolism of an obligately anaerobic, selenate-respiring bacterium, Strain SES-3. J. Applled Eng. Microbiol., 60: 3011-3019.

Oremland, R.S., J.T. Hollibaugh, A.S. Macur, T.S. Presser and L.G. Miller *et al*., 1989. Selenate reduction to elemental selenium by anaerobic bacteria in sediments and culture: biogeochemical significance of a novel, sulfate-independent respiration. J. Applied Eng. Microbiol., 55: 2333-2343.

Rahman, M.M., M.K. Sengupta, S. Ahamed, U.K. Chowdhury and D. Lodh, 2005. Arsenic contamination of groundwater and its health impact on residents in a village in West Bengal, India. Bull. World Health Organ., 83: 49-57. DOI: 10.1590/S0042-96862005000100014

Rosen, B.P., 2002a. Biochemistry of arsenic detoxification. FEBS Lett., 529: 86-92. DOI: 10.1016/S0014-5793(02)03186-1

Rosen, B.P., 2002b. Transport and detoxification systems for transition metals, heavy metals and metalloids in eukaryotic and prokaryotic microbes. Comp. Biochem. Physiol. A Mol. Integr. Phys., 133: 689-693. DOI: 10.1016/S1095-6433(02)00201-5

Ryker, S., 2000. Mapping arsenic in ground water. Geotimes, 46: 34-38.

Saltikov, C.W., A. Cifuentes, K. Venkateswaran and D.K. Newman, 2003. The ars detoxification system is advantageous but not required for As(V) respiration by the genetically tractable shewanella species strain ANA-3. J. Applied Eng. Microbiol., 69: 2800-2809. DOI: 10.1128/AEM.69.5.2800-2809.2003

Silver, S. and L.T. Phung, 1996. Bacterial heavy metal resistance: New surprises. Ann. Rev. Microbiol., 50: 753-789. DOI: 10.1146/annurev.micro.50.1.753

Srini, D., J. Shi, P. Martin, B.P. Rosen and B.F.P. Edwards, 2004. Arginine 60 in the ArsC arsenate reductase of E. coli plasmid R773 determines the chemical nature of the bound As(III) product. J. Protein Sci., 13: 2330-2340. DOI: 10.1110/ps.04787204

Stolz, J., P. Basu and R. Oremland, 2002. Microbial transformation of elements: The case of arsenic and selenium. Int. Microbiol., 5: 201-207. DOI: 10.1007/s10123-002-0091-y

Stolz, J.F., D.J. Ellis, J.S. Blum, D. Ahmann and D.R. Lovley *et al.*, 1999. Sulfurospirillum *barnesii* sp. nov. and *Sulfurospirillum arsenophilum* sp. nov., new members of the Sulfurospirillum clade of the epsilon Proteobacteria. Int. J. Syst. Bacteri., 49: 1177-1180. PMID: 10425777

Stolz, J.F., T. Gugliuzza, J.S. Blum, R. Oremland and F.M. Murillo, 1997. Differential cytochrome content and reductase activity in Geospirillum barnesii strain SeS3. Arch. Microbi., 167: 1-5. DOI: 10.1007/s002030050408

Sun, G., 2004. Arsenic contamination and arsenicosis in China. Toxicol. Applled Pharmacol. 198: 268-271. DOI: 10.1016/j.taap.2003.10.017

Sun, Y., E.A. Polishchuk, U. Radoja and W.R. Cullen, 2004. Identification and quantification of arsC genes in environmental samples by using real-time PCR. J. Microbiol. Methods 58: 335-349. DOI: 10.1016/j.mimet.2004.04.015

Winkelmann, G., 1997. Transition Metals in Microbial Metabolism. 1st Edn., CRC Press, ISBN-10: 9057022192, pp: 516.

Wange, G., S.P. Kennedy, S. Fasiludeen, C. Rensing and S. DasSarma, 2004. Arsenic resistance in halobacterium sp. strain NRC-1 examined by using an improved gene knockout system. J. Bacteri., 186: 3187-94. DOI: 10.1128/JB.186.10.3187-3194.2004

Yoshitaka, N., I.A. Suleyman, Y. Hiroshi, H.H. and M. Norio, 2004. Singlet oxygen inhibits the repair of photosystem II by suppressing the translation elongation of the D1 protein in synechocystis sp. PCC 6803. J. Biochem., 43: 11321-11330. DOI: 10.1021/bi036178q