Analysis of RarA, a Unique Metalloid Reductase from *Sulfurospirillum barnesii* SES-3

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Introduction

Arsenic contamination of soil and groundwater is a major public health threat faced by millions of people around the world. According to the World Health Organization, arsenic concentrations in drinking water should remain at levels below 10 µg/L\(^1\). However, in West Bengal and Bangladesh, over 50 million people have been exposed to groundwater with levels of arsenic ranging from 50 to 1,000 µg/L\(^1\). Frequent consumption of arsenic polluted water can lead to arsenicosis, a chronic condition characterized by cutaneous skin lesions\(^2\). If untreated, arsenicosis can also lead to the development of severe respiratory distress, peripheral neuropathy, carcinomas of the skin, urinary bladder, liver, and lungs, as well as an arsenic-induced myocardial infarction\(^2\). Anthropogenic sources of arsenic contamination include heavy use of insecticides and fertilizers, combustion of fossil fuels, and wastewater from the mining of precious metals\(^3\)-\(^4\). Contamination also occurs naturally through volcanic eruptions, forest fires, and wind erosion\(^4\). In nature, arsenic is usually found as arsenate or arsenite\(^5\). By acting as an analog to enter energy-generating systems via phosphate transporters, arsenate disrupts oxidative phosphorylation\(^6\). Arsenite’s toxicity, however, occurs as a result of its ability to bind to sulfhydryl groups, disrupting cysteine residues crucial to protein structure\(^6\).

While arsenic is frequently toxic to both prokaryotes and eukaryotes, it has been observed that arsenate can function as a respiratory oxidant for certain anaerobic microbes\(^5\). Among these microorganisms is our bacterium of interest, *Sulfurospirillum barnesii* SES-3. SES-3 is a gram negative, motile vibrioid strain of epsilon-proteobacteria isolated from selenate-contaminated freshwater wetlands in Nevada\(^7\). Our interest is in SES-3’s unique reductase, RarA, which allows it to reduce a variety of metal and metalloids, such as arsenate and selenate\(^8\). Due to its ability to reduce oxyanion substrates, RarA could be valuable to the bioremediation of contaminated water.
through the mobilization of inorganic arsenic\(^5\). Therefore, this study focuses on the affinity of RarA’s reductase activity for oxyanion substrates when introduced to them based on different fractions of enzyme and whole cells of SES-3.

**Methods**

**Cell Culture and Extraction of Particulate Fractions**

In order to resuscitate stock cultures of pure SES-3 from \(-80^\circ\text{C}\), the optimal medium for *Sulfurospirillum* was prepared following the protocol from Stolz et al (1997). After the addition of all ingredients, the pH of the media was adjusted to 7.3 through the addition of 1 M HCl. In order to create anaerobic conditions, each liter of medium was degassed with oxygen-free CO\(_2\) and N\(_2\) gas for 45 minutes and sealed. 20 mM lactate, 10 mM fumarate, and 5mM arsenate were added to the media following it being autoclaved to prevent the arsenate from reducing during sterilization. Pure cultures of SES-3 were grown at 30\(^\circ\text{C}\) for 72 hours in 100 ml bottles, then transferred to 1 liter bottles to increase cell growth for harvest and protein extraction. To extract the membrane protein RarA, cells were then harvested and lysed with a French Press, spun at 5,000\(^\times\)g to remove unbroken cells and debris, and then ultracentrifuged at 100,000\(^\times\)g for an hour. All fractions were stored at \(-80^\circ\text{C}\) until further analysis.

**In-Gel Activity Assay**

A native acrylamide gel was prepared with a 4\% stacking gel and 7\% resolving gel. The protein samples were solubilized in 0.5\% CHAPs overnight at 4\(^\circ\text{C}\) and then run at 85V at 10\(^\circ\text{C}\) until the dye front reached the bottom. Activity assays were performed in an SL Bactron IV anaerobic glove box. The gel was soaked in a solution of 10mM reduced methyl viologen in 10mM HEPES buffer (pH 7.5) then developed with a solution of 20mM arsenate in 10 mM
HEPES buffer for 10 minutes. Activity was observed as a band of clearing within the gel. The gels were stained with Coomassie Brilliant blue and de-stained with 10% glacial acetic acid.

**Cuvette Activity Assay**

For kinetics and specificity, activity assays were performed in 2 ml volumes in 10 ml spectrophotometric tubes corked with rubber bungs. Anaerobic conditions were maintained through bubbling with oxygen-free N₂ gas. All solutions involved were also degassed and stored in sealed bottles. The reaction mixture contained 1.68 ml HEPES buffer (pH 7.5), 100µL of SES-3 whole cells (730 µg protein), 100µL of electron acceptor (i.e. 10 mM arsenate, selenate, selenite, nitrite, thiosulfate, phosphate) and 100µL of 10mM reduced methyl viologen, all in HEPES buffer. The reaction was initiated with the addition of 20 µL of a 1 mg/ml solution of sodium dithionite. Data was collected at 600 nm for 15 minutes on a UV 1800 Shimadzu dual beam spectrophotometer. Enzymatic activity was observed by the change in the solutions’ absorbances as RarA coupled the reduction of its substrate to the oxidation of blue reduced methyl viologen, turning the solution colorless. The reference cuvette contained all components except protein.

**SDS-PAGE and Western Blot**

All fractions were run on an SDS-PAGE gel (5% stacker, 10% resolving) to analyze the extracted protein content. In addition to the known molecular mass (55kDa), the presence of RarA in the different fractions was confirmed by Western blot using the polyclonal antibodies (i.e., 30-kDa antibody from rabbit) developed previously and a fluorescent secondary antibody.
Results

1: Protein ladder (kDA)  
2: Whole cells  
3: Lysed cells  
4: Mixed fractions  
5: Particulate fractions  
6: Cytosolic fractions  
7: Particulate CHAPS solubilized  
8: Particulate insolubilized

Figure 1: SDS-PAGE with fractions of protein extracted from SES-3.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Methyl Viologen Oxidized (µmoles/minute)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arsenate</td>
<td>0.009</td>
</tr>
<tr>
<td>Selenate</td>
<td>0.011</td>
</tr>
<tr>
<td>Selenite</td>
<td>0.008</td>
</tr>
<tr>
<td>Nitrite</td>
<td>0.007</td>
</tr>
<tr>
<td>Thiosulfate</td>
<td>0.011</td>
</tr>
<tr>
<td>Phosphate</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Table 1: Activity for substrates tested with 100µL of SES-3 whole cells.

Figure 2: Native-PAGE with arsenate activity and Coomassie stained gel.

Figure 3: Western Blot.

Analysis of harvested fractions through the SDS-PAGE confirmed that our protein of interest had been extracted, demonstrated by the presence of bands at RarA’s molecular weight of 55 kDA (Figure 1). This was additionally confirmed in the Western Blot through the binding of our extracted protein to the polyclonal and fluorescent antibodies developed specifically for RarA (Figure 3). Cuvette activity assays demonstrated that RarA has reductase capabilities for the oxyanion substrates of arsenate, selenate, selenite, nitrite, and thiosulfate (Table 1). This was demonstrated by spectral data indicating changes in the solutions’ absorbances as RarA’s activity changed them from blue to colorless. Additionally, in-gel activity assays exhibited reductase
activity occurring in both the particulate and particulate solubilized fractions. This was demonstrated by the formation of clear bands as RarA coupled the reduction of arsenate to the oxidation of the reduced methyl-viologen in which the gel had been soaked (Figure 2).

**Conclusion and Future Directions**

In this study, pure SES-3 was successfully resuscitated from -80°C stocks in media containing 20 mM lactate, 5mM arsenate, and 10 mM fumarate. Growth studies demonstrated that SES-3 grew exponentially in the first 24 hours and reached maximum growth at 84 hours. Not only was RarA successfully extracted from *Sulfurospirillum barnesii* SES-3, but its ability to reduce a variety of metal and metalloid substrates in aqueous environments was also confirmed. RarA’s affinity was greatest for selenate and thiosulfate, followed by arsenate. This most likely occurred as a result of the stock culture of SES-3 originally being isolated from selenate-contaminated waters. While pure cultures of SES-3 were maintained, successful extraction of the protein of interest required significant cell mass to have been accumulated. Thus, one source of improvement would be to optimize growth conditions for SES-3 in order to yield a higher concentration of RarA in relation to cell mass harvested. To gain a greater understanding of the activity of this reductase, future studies could include carrying out kinetic analyses to determine the Km, Vmax, and turnover rate of RarA on various oxyanion substrates. Additionally, the purification of this active protein from native whole cells of SES-3 could be conducted using anion exchange chromatography. Because this study confirmed RarA’s affinity for a variety of oxyanions, including arsenate, future efforts should be directed toward developing strategies to incorporate it into bioremediation technology. Purification of this active reductase will be key to the utilization of RarA in modern solutions for arsenic-contaminated water.
References


