ABSTRACT

Background: Shewanella oneidensis MR-1 is a gram negative facultative anaerobe that uses soluble and insoluble terminal electron acceptors for respiration. These include Fe(III) oxides, DMSO, fumarate, elemental sulfur, and sulfite. Previous work on sulfite reduction have identified SirA as a terminal sulfite reductase in Shewanella oneidensis MR-1. However not much is known about other genes that contribute to sulfite reduction process. In this study we isolated transposon mutants in twin arginine translocation system and identified their role in sulfite reduction process.

Objective: To identify the role of twin arginine secretion system in sulfite reduction of Shewanella oneidensis MR-1.

Methods: Transposon mutagenesis of Shewanella oneidensis MR-1 with pMini Himar RB1 was used to isolate mutants deficient in sulfite reduction. These consisted of mutants deficient in twin arginine transport (tatC, tatA). Further the wild type and the mutant’s strains were analyzed by qualitative and quantitative sulfite reduction assay.

Result: The wildtype strain of Shewanella oneidensis MR-1 reduced 10 mM of sulfite within 3 days with resultant production of H₂S. Sulfite reduction was also coupled to growth as indicated by increase in total protein content from 38 to 703 ugs by the end of 72 hrs of incubation. On the other hand the tatA and tatC mutant strains were completely deficient in sulfite utilization and they did not produce any detectable amount of H₂S.

Conclusion: This study suggest that the terminal sulfite reductase (or components of the sulfide reduction system) may be transported to the periplasmic space through the twin arginine transport system. Based on sequence analysis it may be involved in transport of SirC protein. Additional studies are required to confirm the secretion of SirC protein by twin arginine translocation system and its role in sulfite reduction in Shewanella oneidensis MR-1.

Key Words: Sulfite reduction, Shewanella oneidensis MR-1, TAT secretion system

INTRODUCTION

Shewanella oneidensis MR-1 is a metal reducing Gram-negative bacterium that belongs to the γ- group of the Proteobacteria. This bacterium, formerly referred to as S. putrefaciens MR-1, was originally isolated in 1988 from Lake Oneida sediments as a manganese reducer (17). Shewanella species that are closely related to MR-1 have since been isolated from many aquatic environments, including the Black Sea, the Baltic Sea and Lake Michigan (9). S. oneidensis MR-1 is best known for its respiratory versatility. This bacterium uses O₂, insoluble metal oxides, fumarate, dimethyl sulfoxide (DMSO), trimethyl amine-N-oxide (TMAO), nitrate, nitrite and selenite (18, 14) as terminal electron acceptors for respiration. S. oneidensis MR-1 is also able to reduce U(VI) and Cr (IV), and is thus of interest for use in bioremediation (9). In addition, S. oneidensis MR-1, and other closely related species such as Shewanella putrefaciens MR-4, can use thiosulfate (S₂O₃²⁻), tetrathionate (S₄O₆²⁻), sulfite, and elemental sulfur as terminal electron acceptors (6,16,19). The S. oneidensis genome is predicted to encode several terminal reductases and 42 c cytochromes (15). This large number of genes dedicated to respiration provides the bacterium with the ability to survive in diverse environments consisting of a variety of electron acceptors.

Secretion systems play an important role in transport of many terminal reductases and enzymes involved in anaerobic respiration in S. oneidensis MR-1. There are 6 major export systems present in Gram-negative bacteria. S oneidensisMR-1 contains all 6 secretions systems including TAT (twin-arginine translocation pathway) secretion system. The twin-arginine translocation pathway exports proteins across the cytoplasmic membrane in a folded form. Proteins
secreted by TAT secretion pathway typically contain an S/T-R-R-X-F-L-K consensus motif in their amino-terminal region (1, 2). The two arginine residues are especially important and mutagenesis of one or both of these residues severely impairs transport of proteins via the TAT secretion system (4).

As mentioned earlier secretion system play a crucial role in anaerobic respiration in *Shewanella oneidensis* MR-1. Previous studies on this bacterium had identified SirA to be an important protein involved in sulfite reduction process (21). In the current work we have identified that the TAT secretion system play an important role in sulfite reduction process in *S. oneidensis*. Our finding imply that component involved in sulfite reduction are transported by TAT secretion system.

MATERIALS AND METHODS

**Bacterial strains and growth conditions:**

The bacterial strains and plasmids used in this study are described in Table 1. LB was routinely used for aerobic growth of *S. oneidensis* MR-1 and *E. coli* strains. Anaerobic cultures of *S. oneidensis* strains were grown in basal medium (pH 7.4) supplemented with 50 mM lactate and 0.02% casamino acids (20). Electron acceptors were used at 10 mM unless noted otherwise. Growth and reduction of sulfite (10 mM) was performed anaerobically in a coy anaerobic chamber using biometer flasks. The side arm of the flasks contained 10 ml of 40% KOH to trap H$_2$S (10). Kanamycin (25 µg/ml) was added for selection and growth of transposon mutant strains. MR-1 and mutant cells were grown overnight in basal medium and 250 µl of each culture was used as inoculum. Total protein concentration of the inoculum was determined using the BCA protein assay kit (PIERCE). The flasks were sealed and incubated in the coy anaerobic chamber. Samples were removed every 24 hrs for up to 120 hrs to measure H$_2$S and sulfite.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
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<tbody>
<tr>
<td><em>S. oneidensis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MR-1</td>
<td>Lake Oneida isolate</td>
<td>(17)</td>
</tr>
<tr>
<td>SS201</td>
<td>MR-1 with transposon insertion in <em>tatA</em></td>
<td>This work</td>
</tr>
<tr>
<td>SS202</td>
<td>MR-1 with transposon insertion in <em>tatA</em></td>
<td>This work</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC100D+</td>
<td><em>E. coli</em> EC100 derivative, <em>pir</em>+</td>
<td>Epicenter Technologies</td>
</tr>
<tr>
<td>β2155</td>
<td><em>pir</em>:RP4, Kmr</td>
<td>(8)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMini-himar</td>
<td>mini-himar transposon, oriT ,Km R</td>
<td>(5)</td>
</tr>
</tbody>
</table>

**Transposon mutagenesis:**

Transposon mutants deficient in the twin arginine translocation system (TAT system) were isolated and identified as described previously (5). Wild type MR-1 cells were mixed with *E. coli* cells harboring the pMiniHimar RB1 plasmid in 1:1 ratio and spotted on an LB- agar plate. Following a 4-hr incubation at room temperature, the cells were scraped off the agar and spread on basal medium agar plates containing lactate (50 mM), casamino acids and 40 mM polysulfide in an attempt to isolate mutants deficient in sulfur reduction. Isolated mutants with transposon insertions in *tat* genes were analyzed for sulfite reduction.

**Analytical methods:**

*S. oneidensis* wild-type and mutant strains were screened qualitatively for the ability to reduce sulfite using H$_2$S detection agar medium. H$_2$S detection medium was prepared with 1.0% basal agar medium supplemented with 0.02% casamino acids, 50 mM lactate, 10 mM sodium sulfite and 0.015% FeSO$_4$. 5.0 ml aliquots of molten detection medium were dispensed into glass culture vials. Once solidified, the detection medium was stab-inoculated with overnight
cultures of wild type and mutant strains grown on LB plates, and incubated at 30°C for 24 hours. Production of H₂S as a result of sulfite reduction was visualized by the formation of a black precipitate (FeS).

Quantitative analysis of H₂S produced and sulfite remaining was performed as described. H₂S was measured using the methylene blue assay (7). Briefly, 0.5 ml of the KOH trap was transferred to 25 ml of dH₂O, and 1 ml of mixed diamine reagent (20 g N, N-dimethyl-p-phenylenediamine sulfate and 30 g FeCl₃·6H₂O in 500 ml of cool 50% hydrochloric acid) was added. The color was allowed to develop for 20 minutes, and the optical density was measured at 670 nm. Hydrogen sulfide concentrations were determined using sodium sulfide as a standard. Sulfite concentrations were determined using the fuchsin assay as described previously (13) using sodium sulfite as standard. Briefly, 100 μl of the reagent (40 mg of fuchsin dye dissolved in 87.5 ml double distilled water and 12.5 ml concentrated H₂SO₄) and 50 μl of sample were mixed with 840 μl of water. After a 10 min incubation at RT, 10 μl of formalin was added followed by a 90 min incubation. The samples were diluted when needed, and the absorbance was measured at 570 nm.

RESULTS

Sulfite reduction by the tatA and tatC mutant:

The mutation in tatA and tatC gene in strains SS201 and SS201 respectively were confirmed by sequencing. Quantitative H₂S detection assay was used to test the ability of these mutants to reduce sulfite. As represented in fig 1 the wild type strain MR-1 was able to utilize sulfite and produce H₂S as visualized by formation of black precipitate by reaction of H₂S to form FeS. However strains SS201 and SS201 did not show any formation of black precipitate suggesting that they are deficient in sulfite reduction. Further these mutants and wild type strain MR-1 were analysed by quantitative sulfite reduction assay.

Anaerobic growth of \textit{S. oneidensis} MR-1 with sulfite was monitored in biometer flasks, with potassium hydroxide in the sidearm as a H₂S trap to prevent accumulation of toxic H₂S concentrations in the culture medium. In these cultures, sulfite concentrations decreased to undetectable levels after 3 days of incubation (Fig.2A). Total protein in MR-1 cultures increased during this time period from 38 ± 3 to 703 ± 56 μg indicating that sulfite reduction was coupled to growth. Sulfite reduction resulted in slow accumulation of H₂S in the sidearm of the biometer flask. Roughly 300 μmoles of H₂S (30%) were detected at the time of SO₃ depletion (Fig. 2B). By the end of 6 day accumulation of H₂S increased till about 680 μmoles of H₂S (70%)(Fig. 2B). On the other hand the tatA and tatC mutant strains SS201 and SS202 respectively were completely deficient in sulfite utilization and they did not produce any detectable amount of H₂S(Fig 2A, 2B). These results suggest that TAT secretion system is important for transport of proteins involved in anaerobic sulfite reduction in \textit{Shewanella oneidensis} MR-1.

DISCUSSION

Previous studies on sulfite reduction in \textit{Shewanella oneidensis} MR-1 have identified SirA as the terminal sulfite reductase (20). The \textit{SirA} locus in \textit{S. oneidensis} MR-1 contains 10 genes (Table 2). Of these two genes, \textit{sirCD}, showed elevated expression in the presence of thiosulfate (3). The exact function of these genes has not been determined, but due to their high degree of similarity to \textit{nrfCD}(12) we predict that they encode proteins involved in electron transfer from menaquinones to SirA. SirC contains two CxxCxxCxxx motifs characteristic of ferredoxins, but does not contain the cysteine clusters (CxxxxxC and CxxxC) that bind siroheme (11). SirD is predicted to have 8 transmembrane domains suggesting that it is an inner membrane protein. Sequence analysis of SirC revealed the presence of ERRFLK at the N terminal region of the polypeptide. This sequence is similar to the S/T-R-R-X-F-L-K twin arginine translocation (TAT) system consensus sequence. The TAT pathway functions to export folded proteins across the cytoplasmic membrane. To determine if a functional TAT secretion system is
required for sulfite reduction, we tested two TAT mutants that we have generated by transposon mutagenesis. The mutants had himar insertions in tatA or tatC and were deficient in sulfite reduction. This suggests that at least some components of the sulfite reductase system are transported by the TAT system. Based on sequence analysis, we predict that this component is SirC. Further analysis on SirC and SirD genes are required to characterize their role in sulfite respiration in *Shewanella oneidensis* MR-1.

**CONCLUSION**

Secretion systems play an important role in transport of many terminal reductases and enzymes involved in anaerobic respiration in *Shewanella oneidensis* MR-1. From the study we conclude that twin arginine translocation system plays an important role in sulfite reduction process. Based on sequence analysis it may be involved in transport of SirC protein. Additional studies are required to confirm the secretion of SirC protein by twin arginine translocation system and its role in Sulfite reduction in *Shewanella oneidensis* MR-1.

**Table 2. List of genes in sirAGCD locus and their function**

<table>
<thead>
<tr>
<th>Locus tag or gene name</th>
<th>Predicted or identified function</th>
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<tbody>
<tr>
<td>SO_0479</td>
<td>periplasmic sulfite reductase octaheme cytochrome c, SirA</td>
</tr>
<tr>
<td>SO_0480</td>
<td>putative cytochrome c maturation system sulfurtransferase</td>
</tr>
<tr>
<td>SO_0481</td>
<td>cytochrome c maturation system peptidyl-prolyl cis-trans isomerase</td>
</tr>
<tr>
<td>SO_0482</td>
<td>Heme lyase component, SirG (NrfG)</td>
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<tr>
<td>SO_0483</td>
<td>Ferredoxin, SirC (NrfC)</td>
</tr>
<tr>
<td>SO_0484</td>
<td>Menaquinol oxidase, SirD (NrfD)</td>
</tr>
<tr>
<td>SO_0485</td>
<td>NosL</td>
</tr>
<tr>
<td>SO_0486</td>
<td>NosD</td>
</tr>
<tr>
<td>SO_0487</td>
<td>NosF</td>
</tr>
<tr>
<td>SO_0488</td>
<td>NosY</td>
</tr>
</tbody>
</table>

**Figure 1: Qualitative sulfite reduction by *S. oneidensis* strains.** SO₃ reduction is detected by the formation of FeS (black precipitate) as evident in the wild type strain MR-1. Strains and SS202 strains with transposon insertion in tatA and tatC genes respectively appear to be deficient in sulfite reduction.
Figure 2A

![Graph showing sulfite reduction over time for different strains](image)

Figure 2B

![Graph showing accumulation of H₂S over time for different strains](image)

**Figure 2.** Sulfite reduction by wild type and strains SS201 (*tatA* mutant) and SS202 (*tatC* mutant). 2A Wild type strain MR-1 utilized about 900 umoles of Sulfur species completely within 3 days, while the SS201 and SS202 mutants strains accumulated all of the sulfite. 2B. The wild type strain MR-1 kept accumulating H₂S till about 680 umoles by the end of 6 days. H₂S was not detected in the mutants strains SS201 and SS202 indicating their inability to reduce sulfite. Error bars indicate standard deviations of three biological replicates.

**ACKNOWLEDGEMENT**

This work was conducted in Daad Saffarini’s lab at University of Wisconsin Milwaukee, USA and supported by MCB grant from the National Science Foundation and GTL grant from the Department of Energy. Author will also gratefully acknowledge Dr. Ashok Chauhan and Dr Atul Chauhan (Amity University) for their guidance.
Sulfite Reduction in Shewanella oneidensis MR-1 Requires TAT... 

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How to cite this article: Shirodkar S. Sulfite Reduction in Shewanella Oneidensis MR-1 Requires TAT Secretion System. Indian J Microbiol Res 2015;2(1):14-19.