SULFITE REDUCTION IN *SHEWANELLA ONEIDENSIS* MR-1 REQUIRES TAT SECRETION SYSTEM

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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 transocation 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 H_2S. 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 H_2S.*
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 O_2, 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 (S_2O_3), tetrathionate (S_4O_6), 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.

Secretions 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 oneidensis*MR-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_2S (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 ul 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 to120 hrs to measure H_2S and sulfite.

Table 1. List of strains and plasmids used in this study.

Strain	Description	Source
S. oneidensis		
MR-1	Lake Oneida isolate	(17)
SS201	MR-1 with tranposon insertion in *tatA*	This work
SS202	MR-1 with tranposon insertion in *tatA*	This work
E. coli		
EC100D+	*E. coli* EC100 derivative, *pir*+	Epicenter Technologies
β2155	*pir*::RP4, Kmr	(8)

Plasmid	Description	Source
pMini-himar	mini-himar transposon, oriT ,Km R	(5)

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 pMini*Himar* 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_2S detection agar medium. H_2S 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 H_2S as a result of sulfite reduction was visualized by the formation of a black precipitate (FeS).

Quantitative analysis of H_2S produced and sulfite remaining was performed as described. H_2S was measured using the methylene blue assay (7). Briefly, 0.5 ml of the KOH trap was transferred to 25 ml of dH_20, and 1 ml of mixed diamine reagent (20 g *N, N*-dimethyl-*p*-phenylenediamine sulfate and 30 g $FeCl_3{\cdot}6H_20$ 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 H_2SO_4) 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 H_2S 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 H_2S as visualized by formation of black precipitate by reaction of H_2S 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 quantitaive sulfite reduction assay.

Anaerobic growth of *S. oneidensis* MR-1 with sulfite was monitored in biometer flasks, with potassium hydroxide in the sidearm as a H_2S trap to prevent accumulation of toxic H_2S 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 H_2S in the sidearm of the biometer flask. Roughly 300 µmoles of H_2S (30%) were detected at the time of SO_3 depletion (Fig. 2B). By the end of 6 day accumulation of H_2S increased till about 680 umoles of H_2S (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 H_2S(Fig 2A, 2B). These results suggest that TAT secretion system is important for transport of proteins involved in anaerobic sulfite reduction in *Shewanella oneidensis* MR-1.

DISCUSSION

Previous studies on sulfite reduction in *Shewanella oneidensis* MR-1 have identified SirA as the terminal sulfite reductase (20). The *sirA* locus in *S. oneidensis* MR-1 contains 10 genes (Table 2). Of these two genes, *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 *nrfCD*(12) we predict that they encode proteins involved in electron transfer from menaquinones to SirA. SirC contains two CxxCxxCxxxC 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 ERRRFLK 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 onedensis* 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

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

Figure 1: Qualitative sulfite reduction by *S.* oneidensis strains. SO_3 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

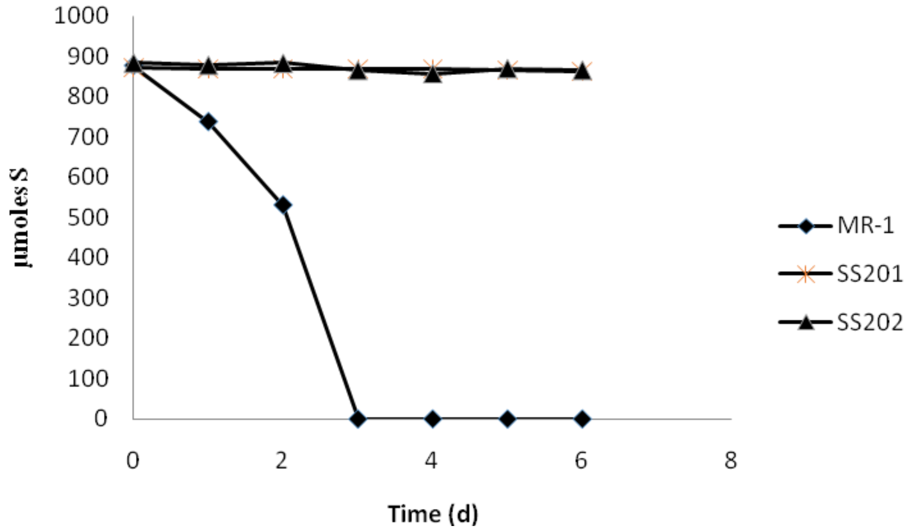

Figure 2B

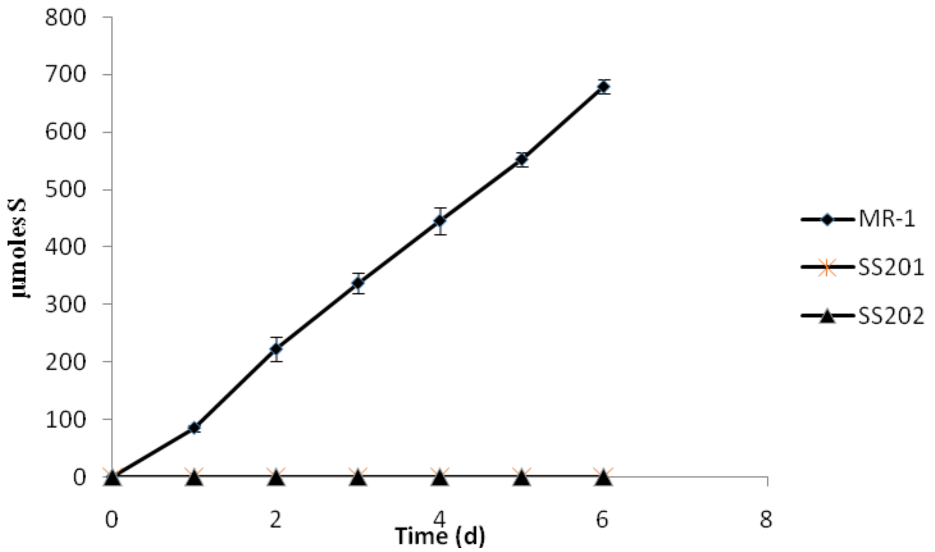

Figure 2. Sulfite reduction by wild type and strains SS201 (*tatA* mutant) and SS202 (*tatC* mutant). 2A Wild type strain MR-1 utilized about 900umoles of Sulfur species completely within 3 days, while the SS201 and SS202 mutants strains accumulated all of the sulfite. 2B. the wild type strain MR-1 kept accumulating H_2S till about 680 umoles by the end of 6 days. H_2S 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.

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