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Anaerobic Respiration of Elemental Sulfur and Thiosulfate by *Shewanella oneidensis* MR-1 Requires *psrA*, a Homolog of the *phsA* Gene of *Salmonella enterica* Serovar Typhimurium LT2▽†

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Shewanella oneidensis **MR-1, a facultatively anaerobic gammaproteobacterium, respires a variety of anaerobic terminal electron acceptors, including the inorganic sulfur compounds sulfite (SO_3^{2-}), thiosulfate ($S_2O_3^{2-}$), tetrathionate ($S_4O_6^{2-}$), and elemental sulfur (S^0). The molecular mechanism of anaerobic respiration of inorganic sulfur compounds by *S. oneidensis*, however, is poorly understood. In the present study, we identified a three-gene cluster in the *S. oneidensis* genome whose translated products displayed 59 to 73% amino acid similarity to the products of *phsABC*, a gene cluster required for S^0 and $S_2O_3^{2-}$ respiration by *Salmonella enterica* serovar Typhimurium LT2. Homologs of *phsA* (annotated as *psrA*) were identified in the genomes of *Shewanella* strains that reduce S^0 and $S_2O_3^{2-}$ yet were missing from the genomes of *Shewanella* strains unable to reduce these electron acceptors. A new suicide vector was constructed and used to generate a markerless, in-frame deletion of *psrA*, the gene encoding the putative thiosulfate reductase. The *psrA* deletion mutant (PSRA1) retained expression of downstream genes *psrB* and *psrC* but was unable to respire S^0 or $S_2O_3^{2-}$ as the terminal electron acceptor. Based on these results, we postulate that PsrA functions as the main subunit of the *S. oneidensis* $S_2O_3^{2-}$ terminal reductase whose end products (sulfide [HS^-] or SO_3^{2-}) participate in an intraspecies sulfur cycle that drives S^0 respiration.**

Microbial reduction of inorganic sulfur compounds is central to the biogeochemical cycling of sulfur and other elements such as carbon and metals (29). The ability to reduce elemental sulfur (S^0) is found in members of both prokaryotic domains (20), including mesophilic deltaproteobacteria (*Desulfovibrio vulgaris*, *Pelobacter carbinolicus*, *Geobacter sulfurreducens*) (6, 9, 36, 51), thermophilic deltaproteobacteria (*Desulfurella acetivorans*) (39), gammaproteobacteria (*Shewanella putrefaciens*) (41), epsilonproteobacteria (*Wolinella succinogenes*) (49), cyanobacteria ("*Oscillatoria limnetica*") (45), and hyperthermophilic archaea (1, 53). Partially reduced inorganic sulfur compounds such as tetrathionate ($S_4O_6^{2-}$), thiosulfate ($S_2O_3^{2-}$), and sulfite (SO_3^{2-}) are also important electron acceptors in the biogeochemical cycling of sulfur (29, 51). $S_4O_6^{2-}$-reducing bacteria, for example, may produce $S_2O_3^{2-}$ as a metabolic end product of $S_4O_6^{2-}$ reduction, while $S_2O_3^{2-}$ disproportionation is a key reaction catalyzed by sulfate-reducing bacteria, resulting in the formation of sulfate (SO_4^{2-}) and sulfide (S^{2-}) (26).

Shewanella oneidensis MR-1, a facultatively anaerobic gammaproteobacterium, respires a variety of compounds as an anaerobic electron acceptor, including the inorganic sulfur compounds S^0, SO_3^{2-}, $S_2O_3^{2-}$, and $S_4O_6^{2-}$; transition metals [e.g., Fe(III) and Mn(IV)]; and radionuclides [e.g., U(VI) and Tc(VII)] (8, 21, 41, 44, 50, 55, 56). The majority of studies of anaerobic respiration by *S. oneidensis* have focused on the mechanism of electron transport to transition metals and radionuclides (11, 14, 34, 46, 58, 59), while the mechanism of electron transport to inorganic sulfur compounds has not been thoroughly examined.

Microbial S^0 respiration is postulated to occur via two pathways, both of which are based on an intraspecies sulfur cycle. In the first pathway (catalyzed by members of the genus *Salmonella* [20]), $S_2O_3^{2-}$ is reduced, yielding HS^- and SO_3^{2-} (24). SO_3^{2-} diffuses from the cell and reacts chemically with extracellular S^0 to form $S_2O_3^{2-}$, which reenters the periplasm and is rereduced, thereby sustaining an intraspecies sulfur cycle. In the second pathway (catalyzed by *W. succinogenes* [24]), water-soluble polysulfides (S_n^{2-}; $n > 2$), formed by chemical interactions of S^0 at pHs >7 (52), are reduced stepwise in the periplasm to S_{n-1}^{2-} and HS^-. Similarly to what occurs with the first pathway, microbially produced HS^- diffuses from the cell and reacts chemically with S^0 to produce additional S_n^{2-}, which reenters the periplasm and is rereduced to sustain an analogous intraspecies sulfur cycle (24).

Genetic analyses of $S_2O_3^{2-}$ reduction-deficient mutants of *Salmonella enterica* serovar Typhimurium have demonstrated that *phsA* (denoting *p*roduction of *h*ydrogen *s*ulfide) is required for HS^- production during $S_2O_3^{2-}$ respiration (10, 17, 22). In addition, *phsA*-deficient mutants are unable to reduce S^0 as an electron acceptor (24). The *phsA* homolog of *W. succinogenes* (annotated as *psrA*, for *p*olysulfide *r*eduction) is required for S^0 respiration (32, 37). *W. succinogenes psrA* is the first gene of a three-gene cluster (including *psrA*, *psrB*, and *psrC*) whose products encode a polysulfide reductase, a quinol oxidase, and a membrane anchor, respectively (15). In addi-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Feature(s)	Source or reference
Strains		
Shewanella oneidensis		
MR-1	Wild-type strain	ATCC
PSRA1	In-frame deletion mutant	This study
PSRA+	"Knock-in" complementation mutant	This study
Escherichia coli		
EC100D *pir*-116	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara leu*)*7697 galU galK* λ$^-$	Epicentre
β2155 λ pir	*thrB1004 pro thi strA hsdS lacZ*ΔM15 (F9 *lacZ*ΔM15 *lacI*^{q} *traD36 proA1 proB1*) Δ*dapA*::*erm pir*::RP4 Km^r	12
Plasmids		
pKO2.0	4.5-kb γR6K, *mob* RP4 *sacB* Gm^r *lacZ*	This study
pKNOCK-Gm	1.6-kb γR6K, *mob* RP4 Gm^r	2
pBBR1MCS	Cm^r *lacZ*	31
pJQ200	ColE1 *sacB* Gm^r	48
pKOPSRA	pKO2.0 containing in-frame deletion of *psrA*	This study
pKOPSRA+	pKO2.0 containing wild-type *psrA*	This study

TABLE 2. Primers used in this study

Primer	Sequence
PSRAD1	GACTGGATCCCACAGCTTATTTGGTCG
PSRAD2	ATATCTTTTCGTCATTTGAGCCTCCAAGGTTACC TCCATCACACTCA
PSRAD3	TGAGTGTGATGGAGGTAACCTTGGAGGCTCAAA TGACGAAAAGATAT
PSRAD4	GACTGTCGACACAAGCCAAGCCTAAGCTGATGG
PSRADTF	GCGTGCATTTTGAACGACAG
PSRADTR	GCTTTCTAAAGGGCATAAGCAGC
RT-PSRAF	GTCGCCGATAAAGCCGATGAATGGTA
RT-PSRAR	GGCATATCTTTTACGCCGGGCTTAC
RT-PSRBF	TATGGCGAAATGCCCAATCTGC
RT-PSRBR	AAGCGGGTGTCCTTACAGAAGT
RT-PSRCF	AGTCACGACAAGACGTTAGCCA
RT-PSRCR	GCTTTGGCCCGCATACACAATA
RT-RPOAF	GTTCAACACGAGCTGCTTCTA
RT-RPOAR	GGCTTAGCAGTGACTATCGAG

tion, the structure of the polysulfide reductase complex (PsrABC) from *Thermus thermophilus* has recently been solved, and results indicate that PsrC acts as a quinol oxidase that transfers electrons stepwise via PsrB and PsrA to S_n^{2-} during anaerobic S^0 respiration (27). The main objectives of the present study were to (i) identify the *S.* Typhimurium *phsA* homolog in the *S. oneidensis* genome, (ii) employ a newly constructed suicide cloning vector for in-frame gene deletion mutagenesis in *S. oneidensis* to delete the *S.* Typhimurium *phsA* homolog of *S. oneidensis*, and (iii) test the *S. oneidensis psrA* deletion mutant for respiratory activity on a combination of two electron donors and 11 electron acceptors, including the inorganic sulfur compounds $S_4O_6^{2-}$, $S_2O_3^{2-}$, and S^0.

MATERIALS AND METHODS

Growth media and cultivation conditions. *S. oneidensis* MR-1 was cultured at 30°C in Luria-Bertani (LB) medium (10 g liter^{-1} NaCl, 5 g liter^{-1} yeast extract, 10 g liter^{-1} tryptone) for genetic manipulations. For anaerobic growth experiments, cells were cultured in a defined salts medium (44) supplemented with lactate (18 mM) or formate (30 mM) as the carbon/energy source. Anaerobic growth experiments were carried out with 13-ml Hungate tubes (Bellco Glass, Inc.) filled with 10 ml of salts medium and sealed with black butyl rubber stoppers under an N_2 atmosphere. Growth experiments were performed in two parallel yet independent incubations. In a second set of anaerobic growth experiments with S^0 and $S_2O_3^{2-}$, *S. oneidensis* cultures were incubated with continual flushing of the headspace with N_2 to remove bacterially produced HS^- (37). Electron acceptors were added from filter-sterilized stocks (synthesized as described previously [13]), except where indicated, at the following final concentrations: O_2 (atmospheric); NO_3^-, 10 mM; Fe(III) citrate, 50 mM; hydrous ferric oxide and Mn(III)-pyrophosphate, 40 mM (30) and 10 mM, respectively; trimethylamine-*N*-oxide (TMAO), 25 mM; $S_2O_3^{2-}$, 10 mM; $S_4O_6^{2-}$, 2 mM; fumarate, 30 mM; dimethyl sulfoxide (DMSO), 25 mM; and S^0, 20 mM (41). When required, antibiotics were supplemented at the following final concentrations: gentamicin (Gm), 15 μg ml^{-1}, and chloramphenicol, 25 μg ml^{-1}. For growth of *Escherichia coli* β2155 λ *pir* (12), diaminopimelate was supplemented at a final concentration of 100 μg ml^{-1}.

Analytical techniques. Cell growth was monitored by direct cell counts via epifluorescence microscopy and by measuring terminal electron acceptor depletion or end product accumulation. Acridine orange-stained cells were counted (Carl Zeiss AxioImager Z1 microscope) according to previously described procedures (35). Cell numbers at each time point were calculated as the average of 10 counts from two parallel yet independent anaerobic incubations. NO_2^- was measured spectrophotometrically with sulfanilic acid-*N*-1-naphthyl-ethylenediamine dihydrochloride solution (40). Fe(III) reduction was monitored by measuring Fe(II) production with the ferrozine technique (54). Mn(III)-pyrophosphate concentration was measured colorimetrically as previously described (30). $S_2O_3^{2-}$ and $S_4O_6^{2-}$ concentrations were measured by cyanolysis as previously described (28). Growth on O_2, TMAO, DMSO, and fumarate was detected by monitoring cell growth only. Control experiments consisted of incubations with cells that were heat killed at 80°C for 30 min prior to inoculation or by omission of the terminal electron acceptor.

Nucleotide and amino acid sequence analyses. Genome sequence data for *S. oneidensis* MR-1 (21), *S.* Typhimurium LT2 (38), and *W. succinogenes* (5) were obtained from the comprehensive microbial resource (J. Craig Venter Institute; http://cmr.jcvi.org). *S. oneidensis* proteins that displayed significant similarity to *S.* Typhimurium PhsA (designated PsrA in the *S. oneidensis* genome), tetrathionate reductase (TtrA), and anaerobic sulfite reductase (AsrA) were identified via BLAST analysis (3). Functional motifs were analyzed via Pfam (http://pfam.sanger.ac.uk) and ClustalW (33). The *S. oneidensis* PsrA sequence was used as the query sequence for BLAST analysis of several other recently sequenced *Shewanella* genomes, including *S. putrefaciens* strain 200, *S. putrefaciens* CN32, *S. putrefaciens* W3-18-1, *S. amazonensis* SB2B, *S. denitrificans* OS217, *S. baltica* OS155, *S. frigidimarina* NCIMB400, *S. pealeana* ATCC 700345, *S. woodyi* ATCC 51908, *Shewanella* sp. strain ANA-3, *Shewanella* sp. strain MR-4, *Shewanella* sp. strain MR-7, *S. loihica* PV-4, *S. halifaxensis* HAW-EB4, *S. piezotolerans* WP3, *S. sediminis* HAW-EB3, and *S. benthica* KT99. Preliminary genome sequence data for these strains were obtained from the Department of Energy, Joint Genome Institute (http://www.jgi.doe.gov).

Construction of suicide vector pKO2.0. The strategy for construction of the suicide vector pKO2.0 (Table 1) is outlined in Fig. S1 in the supplemental material. The R6K *oriV* and the RP4 origin of transfer (*oriT*) genes of pKNOCK-Gm (2) were PCR amplified with NheI restriction sites engineered onto the ends of the *oriV-oriT* fragment. The chloramphenicol acetyltransferase (*cat*) gene and the LacZ-containing multiple-cloning site (MCS) were PCR amplified from pBBR1MCS (31) with NheI sites also engineered onto the ends (see Table S1 in the supplemental material for corresponding primers). The resulting PCR products were ligated to form pKOCat-MCS. The open reading frames encoding SacB and Gm acetyltransferase (conferring Gm resistance [Gm^r]) were PCR amplified from pJQ200 (48) as a contiguous fragment with ApaLI and MunI restriction sites engineered onto the ends. The *cat* gene was removed from pKOCat-MCS by inverse PCR, resulting in a fragment containing R6K *oriV*, RP4 *oriT*, and the LacZ-MCS with ApaLI and MunI sites on the ends. These two fragments were ligated to form pKO2.0 (R6K *oriT* LacZ-MCS Gm^r *sacB*).

In-frame gene deletion mutagenesis. *psrA* was deleted from the *S. oneidensis* genome by the following method. Regions corresponding to ~750 bp upstream and downstream of the *psrA* open reading frame were PCR amplified using iProof ultrahigh-fidelity polymerase (Bio-Rad, Hercules, CA). Primers used for construction of the *psrA* deletion mutant are listed in Table 2. PCR cycling routines consisted of 98°C for 30 s, 35 cycles of 98°C for 15 s, 60°C for 30 s, and 72°C for 30 s, concluding with a final extension step at 72°C for 7 min. PCRs (50-μl total mixture volume) were performed on a Bio-Rad iCycler (Bio-Rad, Hercules, CA), and each mixture contained 20 ng of *S. oneidensis* genomic DNA (DNAzol; Invitrogen), 250 μM of the deoxynucleoside triphosphates, and 50 ng of each oligonucleotide. The resulting fragments were separated by agarose gel

TABLE 3. Identities and similarities between PsrA, PsrB, and PsrC homologs

Protein	*Shewanella* spp.[a]			*S.* Typhimurium LT2			GenBank[b]			
	Sim	%ID	E value	Sim	%ID	E value	Best hit	Sim	%ID	E value
PsrA	85–98	75–96	0.0	73	56	0.0	*W. succinogenes*	65	47	0.0
PsrB	90–99	79–98	10^{-87}–10^{-107}	71	54	10^{-57}	*W. succinogenes*	80	65	10^{-63}
PsrC	66–99	51–96	10^{-57}–10^{-137}	59	40	10^{-38}	*W. succinogenes*	67	45	10^{-62}

[a] Percentages of sequence similarity (Sim), percentages of identity (%ID), and expect values (E values) between *S. oneidensis* Psr predicted amino acid sequences obtained from TIGR. Ranges were determined by pairwise comparison with translated sequence data from draft genome sequences of 17 recently sequenced *Shewanella* strains, including *S. putrefaciens* 200, *S. putrefaciens* CN32, *S. putrefaciens* W3-18-1, *S. amazonensis* SB2B, *S. denitrificans* OS217, *S. baltica* OS195, *S. frigidimarina* NCIMB400, *S. pealeana* ATCC 700345, *S. woodyi* ATCC 51908, *Shewanella* strain ANA-3, *Shewanella* strain MR-4, *Shewanella* strain MR-7, *S. loihica* PV-4, *S. halifaxensis*, *S. piezotolerans*, *S. benthica*, and *S. sediminis.*

[b] Organism outside the genus *Shewanella* or *Salmonella*, with the ortholog of highest similarity (best hit) determined by BLASTP analysis of the nonredundant GenBank database.

electrophoresis, isolated from the gel (QIAquick gel extraction kit; Qiagen), and subsequently joined using overlap extension PCR (25) to generate a DNA fragment containing regions homologous to the regions flanking *psrA*. This region was cloned into the newly constructed knockout vector pKO2.0, and the resulting plasmid (pKOPSRA) was electroporated into *E. coli* EC100D *pir*-116 (Epicentre) (Bio-Rad Xcell electroporation system; Bio-Rad, Hercules, CA). Recipients were detected on LB agar medium containing Gm and 40 μg ml^{-1} X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The recombinant plasmid was confirmed by detection of an ~1.5-kb insertion following digestion with BamHI. Following confirmation, pKOPSRA was electroporated into *E. coli* β2155 λ *pir* (12) (Bio-Rad Xcell electroporation system; Bio-Rad, Hercules, CA), and the resulting donor strain was mated biparentally with *S. oneidensis* on LB agar medium supplemented with diaminopimelate. Following an 18-h incubation, the mating mix was resuspended in LB liquid medium and subsequently plated onto LB agar medium containing Gm.

S. oneidensis MR-1 colonies with pKOPSRA integrated into the genome were screened by PCR using primers flanking the recombination region. The resulting strain (MR-1::pKOPSRA) was grown in LB medium with NaCl omitted and subsequently transferred to LB agar medium with NaCl omitted and containing 10% (wt/vol) sucrose. Colonies were patched to LB agar medium containing Gm to confirm the loss of pKO2.0, and the deletion in *psrA* was confirmed in the Gm-sensitive colonies via PCR using primers flanking the recombination region. One mutant strain (PSRA1) was also confirmed by direct DNA sequencing of the targeted region (University of Nevada, Reno Genomics Center).

Confirmation of in-frame deletions. Liquid cultures of *S. oneidensis* wild-type and deletion mutant strains were grown to mid-log phase (corresponding to 1.8×10^9 cells ml^{-1}) in LB medium, and total RNA was extracted with PureZol reagent (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. RNA (~150 ng μl^{-1}) was further purified (RNeasy Kit; Qiagen) with on-column DNase I treatment according to the manufacturer's instructions. RNA was reversely transcribed with a high-capacity cDNA reverse transcription (RT) kit (Applied Biosystems). RT reaction mixtures (20 μl) consisted of 150 ng RNA, 50 U MultiScribe reverse transcriptase, 4 mM deoxynucleoside triphosphates, and random hexamers. Cycling routines were 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. The resulting cDNA was PCR amplified with primers specific for internal regions of *psrA*, *psrB*, and *psrC* (Table 2). RNA polymerase factor *rpoA* served as a positive control. Negative controls consisted of identical reactions with reverse transcriptase omitted.

Knock-in complementation analysis of PSRA1. PSRA1 was complemented via a strategy analogous to that followed in the gene deletion protocol. Wild-type *psrA* was PCR amplified from MR-1 genomic DNA with *psrA*-specific primers PSRAD1 and PSRAD4. The resulting amplicon contained the entire open reading frame and ~750 bp of upstream and downstream DNA for subsequent recombination into PSRA1. The amplicon was cloned into pKO2.0 using identical restriction sites, and the resulting construct was subsequently transformed into *E. coli* strains as described above. "Knock-in" complementation was performed as described for in-frame deletion above, except that PSRA1 was used as the recipient strain. Insertion into the proper location of the genome was confirmed via PCR amplification with flanking primers PSRADTF and PSRADTR (Table 2) followed by DNA sequencing (University of Nevada, Reno Genomics Center). The resulting "knock-in" complementation strain was designated PSRA+.

RESULTS

Construction of new suicide vector pKO2.0. Suicide vector pKO2.0 was constructed in a stepwise fashion, integrating the hallmark components of previously constructed vectors. The R6K *oriV* and RP4 *oriT* origins from pKNOCK-Gm and the chloramphenicol resistance marker (*cat*) and the LacZ-containing polylinker (MCS) from the broad-host-range vector pBBR1MCS were independently PCR amplified and joined to form pKOCat-MCS (See Fig. S1 in the supplemental material). The *cat* gene was subsequently removed via inverse PCR, and the resulting product (containing R6K*ori*, *oriT*, and MCS) was ligated with the PCR product containing the Gm^r gene and *sacB*, encoding levansucrase (see Fig. S1 in the supplemental material).

Identification of *S. oneidensis* gene products that display similarity to the PhsA homolog of *S.* Typhimurium. BLAST analysis revealed that the *S. oneidensis* MR-1 genome contained a three-gene cluster (annotated as *psrABC*) whose translated products displayed 59 to 73% and 65 to 80% amino acid similarity to the *phsABC* (*psrABC*) clusters of *S.* Typhimurium LT2 and *W. succinogenes*, respectively (Table 3; see also Fig. S3 in the supplemental material). The three-gene *psr* cluster in *S. oneidensis* MR-1 has the same genomic organization as the three-gene *psr* clusters in both *S.* Typhimurium and *W. succinogenes* (data not shown). ClustalW multiple alignments of PsrA functional domains with those of other proteobacteria indicated that *S. oneidensis* PsrA contains the conserved 4Fe-4S cluster and molybdopterin guanine-dinucleotide (MGD) binding motifs postulated to contribute to PsrA activity (Fig. 1). PsrA contained the predicted Pfam domains, consistent with other members of the polysulfide (thiosulfate) reductase family of proteins, including the 4Fe-4S cluster (Pfam accession no. PF04879) and MGD binding motifs (accession no. PF01568) (Fig. 1). Proteins homologous to *S.* Typhimurium TtrA (tetrathionate reductase) or AsrA (anaerobic sulfite reductase) were not detected in the *S. oneidensis* genome (data not shown).

$S_2O_3^{2-}$-reducing members of the genus *Shewanella* contain Psr homologs with high amino acid sequence similarity. The *psrABC* cluster was also identified in the recently sequenced genomes of *Shewanella* strains that respire S^0 and $S_2O_3^{2-}$, including *S. putrefaciens* CN32, *S. putrefaciens* 200, *S. putrefaciens* W3-18-1, *S. amazonensis* SB2B, *S. frigidimarina* NCIMB400, *S. baltica* OS155, *S. pealeana*, *Shewanella* sp. strain ANA-3, *Shewanella*

FIG. 1. Identification of conserved Pfam domains in the PsrA family of proteins. ClustalW multiple alignment of PsrA family proteins containing conserved Pfam domains. (A) Multiple alignment of Pfam domain PF04879 (4Fe-4S binding domain) with *S. oneidensis* MR-1 PsrA (PsrA_MR1), *S.* Typhimurium LT2 PhsA (PhsA_LT2), and *W. succinogenes* PsrA (PsrA_Ws). (B) Multiple alignments of Pfam domain PF01568 (bis-MGD binding domain) with PsrA and PhsA proteins. Identical residues are shaded.

loihica PV-4, *Shewanella* sp. strain MR-4, *Shewanella* sp. strain MR-7, *S. benthica*, *S. halifaxensis*, *S. piezotolerans*, and *S. sediminis*. The *psr* gene cluster was missing from the genomes of *S. denitrificans* OS217 and *S. woodyi*, a finding that correlated with the inability of these two strains to respire $S_2O_3^{2-}$ (57). PsrA (85 to 98%), PsrB (90 to 99%), and PsrC (66 to 99%) amino acid sequence similarities were highly conserved among $S_2O_3^{2-}$-respiring *Shewanella* species (Table 3).

Construction and confirmation of in-frame deletion mutant strain PSRA1 and knock-in complementation strain PSRA+. *S. oneidensis psrA* was deleted in frame via application of the newly developed pKO2.0-based gene deletion system. Regions flanking *psrA* were PCR amplified and joined to construct a region used for gene deletion. This region was cloned into pKO2.0 (creating plasmid pKOPSRA) and subsequently mobilized into *S. oneidensis* via conjugal transfer. Single integrants were selected on the basis of their resistance to Gm, and their sequences were confirmed via PCR using test primers flanking the recombination region. The second step involved resolution of the single integration by counterselection on medium containing sucrose. Several sucrose-resistant colonies were screened by PCR and selected for further study. A single strain (designated PSRA1) displaying a PCR product corresponding to an in-frame *psrA* deletion was chosen and tested for expression of downstream genes *psrB* and *psrC* via RT-PCR. Transcripts for *psrB* and *psrC* were detected in PSRA1, while transcripts for *psrA* were not detected (Fig. 2).

To avoid problems associated with in *trans* complementation (e.g., inadvertent overexpression), the newly constructed suicide vector pKO2.0 was used to generate a "knock-in" genetic complementation strain, designated PSRA+. *psrA* and regions upstream and downstream (identical to those used to construct

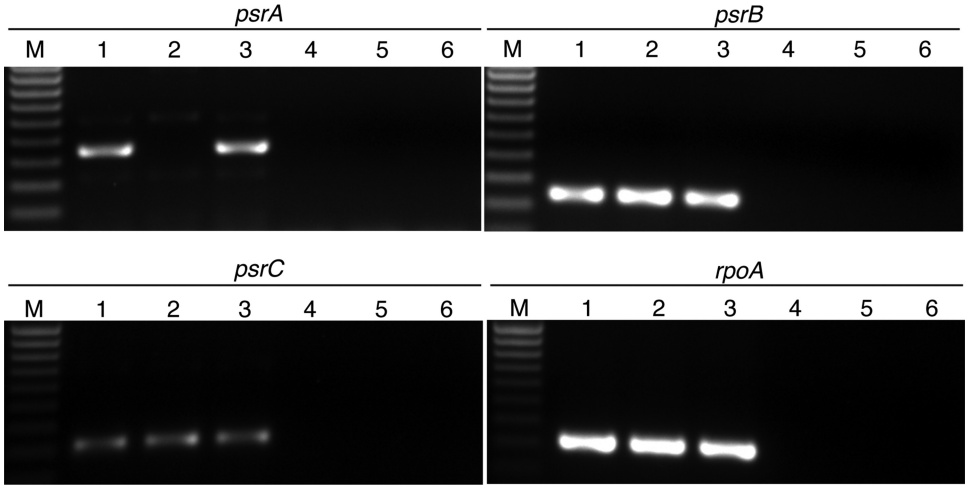

FIG. 2. RT-PCR of genes in the *psr* operon. PCR products generated from cDNA from the wild type (lane 1), PSRA1 (lane 2), or PSRA+ (lane 3) were separated electrophoretically and visualized via staining with ethidium bromide. Transcripts for *psr* or housekeeping (*rpoA*) genes are indicated above the gel lanes. Negative-control lanes 4, 5, and 6 include cDNA from the wild type, PSRA1, and PSRA+, respectively, with reverse transcriptase omitted from the cDNA synthesis reaction. Lanes M, molecular size markers.

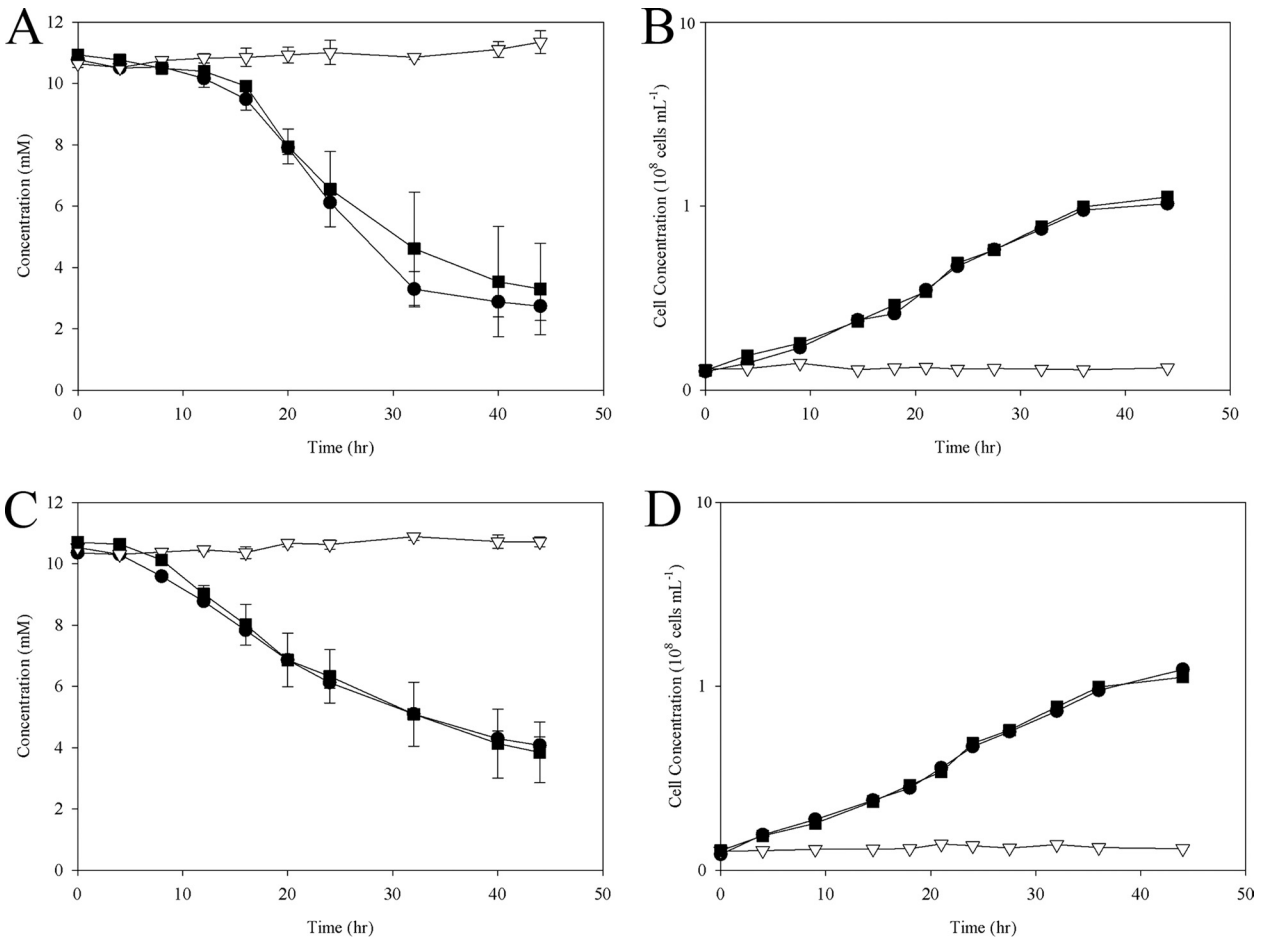

FIG. 3. $S_2O_3^{2-}$ reduction phenotypes of the wild-type (MR-1) (filled circles), *psrA* deletion mutant (PSRA1) (open triangles), and "knock-in" complementation (PSRA+) (filled squares) strains. (A) $S_2O_3^{2-}$ concentration as a function of time for the wild-type, PSRA1, and PSRA+ strains with lactate (18 mM) as the electron donor. (B) Cell growth as a function of time with lactate as the electron donor. (C) $S_2O_3^{2-}$ concentration as a function of time with formate (30 mM) as the electron donor. (D) Cell growth as a function of time with formate as the electron donor.

PSRA1) were PCR amplified and cloned into pKO2.0 as described above. pKOPSRA+ was used to reinsert the *psrA* gene into PSRA1 as described for construction of the in-frame deletion, with the exception that PSRA1 was used as the recipient strain. Insertion of wild-type *psrA* back into PSRA1 was confirmed via DNA sequencing and RT-PCR analyses as described above (Fig. 2).

Anaerobic growth capabilities of *S. oneidensis* strains PSRA1 and PSRA+. Wild-type *S. oneidensis*, deletion mutant PSRA1, and complemented mutant PSRA+ were tested for anaerobic respiration on a combination of two electron donors (lactate and formate) and a set of alternate electron acceptors. PSRA1 was severely impaired in its ability to respire anaerobically on $S_2O_3^{2-}$ and S^0 with lactate or formate (Fig. 3 and 4) yet retained the ability to grow on all other combinations of electron donors and electron acceptors, including O_2, DMSO, Fe(III)-citrate, hydrous ferric oxide, NO_3^-, fumarate, Mn(III)-pyrophosphate, and TMAO (see Fig. S2 in the supplemental material). Anaerobic growth of wild-type *S. oneidensis* and PSRA+ on S^0-containing solid growth medium was accompanied by the production of a clearing zone in the colony periphery. The clearing zone, indicative of S^0 reduction, was more pronounced with formate than with lactate as an electron donor (Fig. 4). Neither cell mass nor a clearing zone was observed in anaerobic incubations of PSRA1 on S^0-containing solid growth medium.

PSRA1 retained $S_4O_6^{2-}$-respiratory activity and consequently produced $S_2O_3^{2-}$, although not at wild-type rates (Fig. 5). With formate as an electron donor, the wild-type strain and PSRA+ depleted $S_4O_6^{2-}$ to low levels and switched to anaerobic respiration of the produced $S_2O_3^{2-}$ (noted by a depletion of $S_2O_3^{2-}$ after 24 h). With lactate as an electron donor, the wild-type strain and PSRA+ reduced $S_4O_6^{2-}$ to $S_2O_3^{2-}$; however, neither strain was able to respire the produced $S_2O_3^{2-}$. PSRA1, on the other hand, reduced $S_4O_6^{2-}$ and produced $S_2O_3^{2-}$ but was unable to respire the produced $S_2O_3^{2-}$ with either lactate or formate as an electron donor. As a result, PSRA1 displayed a marked decrease in growth rate and $S_4O_6^{2-}$ reduction activity (Fig. 5).

DISCUSSION

Metal-respiring members of the genus *Shewanella* provide attractive models for determining the molecular mechanism of anaerobic respiration of metals, metalloids, and radionuclides.

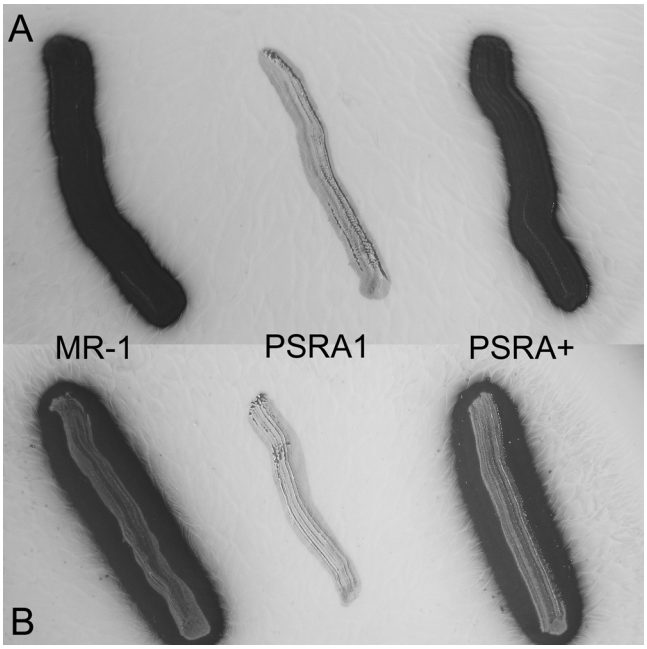

FIG. 4. S^0-respiratory phenotypes of the wild-type (MR-1), *psrA* deletion mutant (PSRA1), and "knock-in" complementation (PSRA+) strains. Anaerobic cell growth was measured on solid medium containing S^0 (40 mM) as the electron acceptor with either lactate (18 mM) (A) or formate (30 mM) (B) as the electron donor (90% N_2, 10% CO_2 atmosphere). Following a 48-h anaerobic incubation, plates were visualized against a black background to observe the clearing zone associated with S^0 reduction by MR-1 and PSRA+ (but not by PSRA1).

Genetic manipulations in *Shewanella* can be performed aerobically prior to subsequent phenotypic tests under anaerobic conditions. With the recent release of the genomic sequences of 17 *Shewanella* strains (Joint Genome Institute; http://www.jgi.doe.gov), targeted, in-frame gene deletion mutagenesis may now be carried out to examine the molecular mechanism of anaerobic respiration. Earlier genetic manipulation techniques, such as transposon and chemical mutagenesis, have been problematic for a variety of reasons, including polar effects on downstream genes, inadvertent genomic rearrangements and deletions, and altered growth rates due to the energetic requirements for maintaining antibiotic resistance markers (7, 13, 43). The suicide vector constructed in the present study (pKO2.0) contains features ideal for generating in-frame, markerless gene deletions in *Shewanella*: pKO2.0 replicates efficiently in *E. coli* donor strains but not in *S. oneidensis*, contains an appropriate broad-host-range origin of transfer (*oriT*), and encodes a functional counterselection marker (SacB) (16, 18, 19). In addition, pKO2.0 is ~5 kb smaller than previously constructed vectors (18) and contains a LacZ polylinker for more-efficient ligation of DNA constructs. *Shewanella* deletion mutants may therefore be constructed more efficiently due to increased transformation efficiency and elimination of problems with blunt-end cloning (19). pKO2.0 was used to construct in-frame deletions of the gene encoding the putative PsrA homolog identified in the *S. oneidensis* genome.

The majority of studies of anaerobic respiration by *S. oneidensis* have focused on the mechanism of electron transport to transition metals and radionuclides, while the mechanism of electron transport to metalloids such as inorganic sulfur compounds remains poorly understood (8, 11, 14, 46, 56). Previous studies demonstrated that several members of the *Shewanella* genus couple the oxidation of organic matter (or hydrogen) to the dissimilatory reduction of S^0, a process that does not require direct contact between the cell and the S^0 particle (41, 47). S^0 respiration in *S.* Typhimurium and *W. succinogenes* involves a PhsA (PsrA)-dependent intraspecies sulfur cycle. In *S.* Typhimurium, PhsA reduces $S_2O_3^{2-}$ to HS^- and SO_3^{2-}. The produced SO_3^{2-} diffuses from the cell and reacts chemically with S^0 to form additional $S_2O_3^{2-}$, which reenters the periplasm and is reduced by PhsA to HS^- and SO_3^{2-}, thereby sustaining an intraspecies sulfur cycle. In *W. succinogenes*, PsrA reduces S_n^{2-} stepwise to S_{n-1}^{2-} and HS^-. The HS^- diffuses from the cell and reacts with S^0 to form additional S_n^{2-}, which reenters the periplasm and is rereduced (24). In this manner, the terminal reduction step for S^0 respiration by *S.* Typhimurium and *W. succinogenes* is driven by the abiotic interaction of S^0 with either SO_3^{2-} or HS^- and not by direct enzymatic reduction of S^0.

In the present study, all predicted PsrA homologs identified in the recently sequenced *Shewanella* genomes contain Pfam domains indicative of the Phs (Psr) family of thiosulfate reductases, including 4Fe-4S cluster and MGD binding motifs. *psrA*-like genes are not found in the genomes of *S. denitrificans* or *S. woodyi*, two *Shewanella* species unable to respire S^0 or $S_2O_3^{2-}$ (57). Interestingly, *S. denitrificans* and *S. woodyi* are also unable to respire Fe(III) oxides as the terminal electron acceptor (57), a pattern of electron acceptor utilization reflecting the close association of the S^0 and Fe(III) reduction phenotypes in metal-respiring bacteria (57).

To determine if *S. oneidensis psrA* is required for S^0 and $S_2O_3^{2-}$ respiration, the *S.* Typhimurium *phsA* homolog identified in the *S. oneidensis* genome was deleted in frame with the new pKO2.0-based in-frame gene deletion system, and the resulting mutant was tested for anaerobic respiration on a combination of two electron donors and a set of 11 alternate electron acceptors, including S^0 and $S_2O_3^{2-}$. *S. oneidensis* deletion mutant PSRA1 is unable to respire anaerobically on $S_2O_3^{2-}$ or S^0 as an electron acceptor, yet it retains respiratory activity on nine other electron acceptors tested, including $S_4O_6^{2-}$. Following depletion of $S_4O_6^{2-}$, wild-type *S. oneidensis* switches to anaerobic respiration of the produced $S_2O_3^{2-}$. PSRA1, on the other hand, is unable to respire the produced $S_2O_3^{2-}$ and, as a result, displays decreased cell growth and $S_4O_6^{2-}$ depletion rates (Fig. 5). This finding indicates that $S_4O_6^{2-}$ is reduced to HS^- stepwise with $S_2O_3^{2-}$ as an intermediate. Cells respiring $S_4O_6^{2-}$ with lactate as an electron donor were unable to reduce the produced $S_2O_3^{2-}$ (Fig. 5A). The reasons for this finding are not clear, but it may be due to repression of the Psr system by residual (1 mM) $S_4O_6^{2-}$ remaining in the system. A similar growth pattern was recently reported for cytochrome *c* maturation (*ccmB*) mutants of *S. putrefaciens* 200 that are unable to reduce NO_2^- but retain NO_3^- reduction activity (11). The *S. putrefaciens* CCMB1 mutant (with an in-frame deletion of *ccmB*) stoichiometrically converts NO_3^- to NO_2^- but is unable to respire the produced NO_2^-, and growth halts.

$S_4O_6^{2-}$-respiring bacteria generally contain the three-gene cluster *ttrBAC*, in which *ttrA* encodes the major subunit (TtrA)

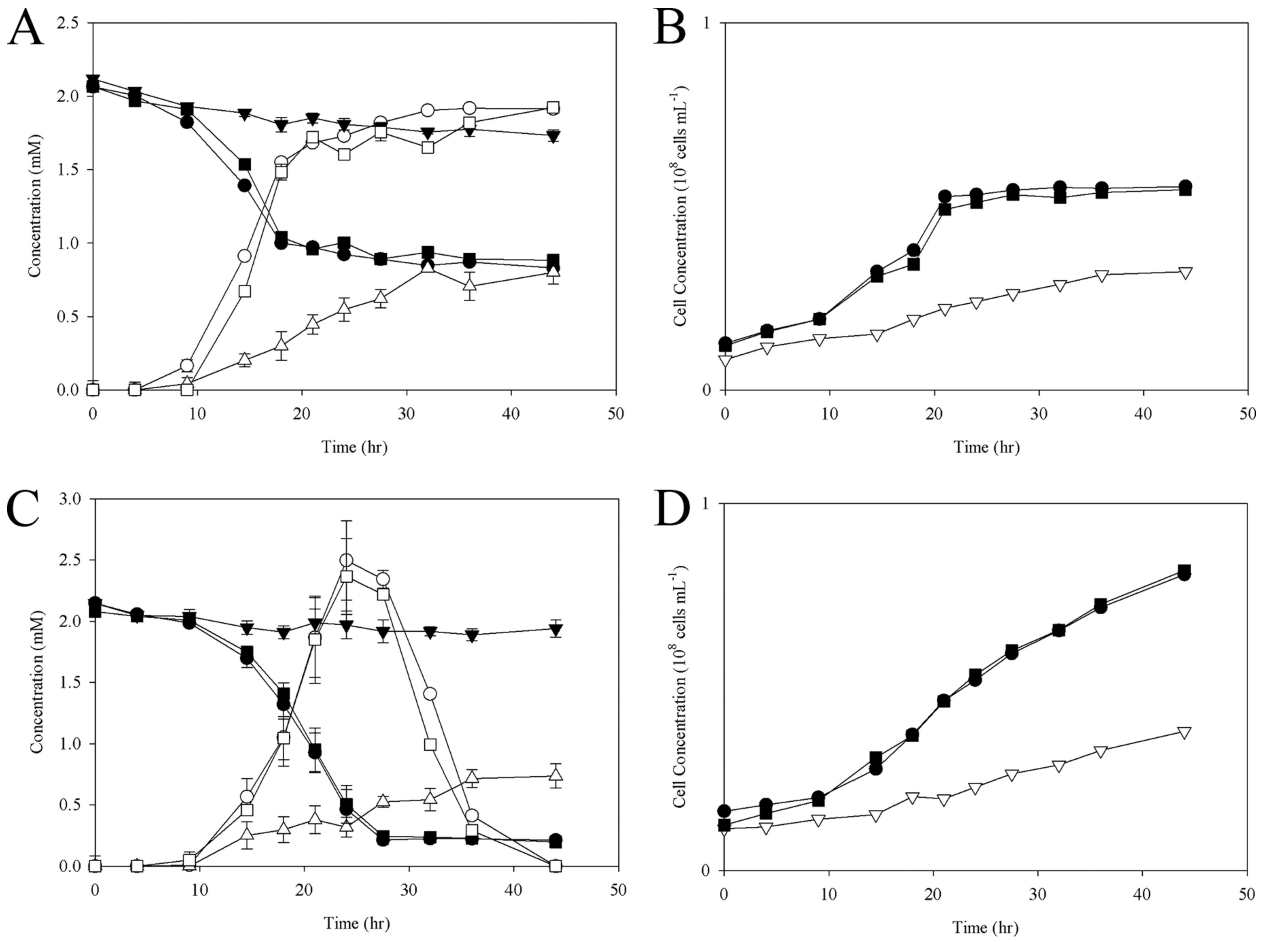

FIG. 5. $S_4O_6^{2-}$-respiratory phenotypes of the wild-type (MR-1), *psrA* deletion mutant (PSRA1), and "knock-in" complementation (PSRA+) strains. (A and C) $S_4O_6^{2-}$ reduction by the wild-type (filled circles), PSRA1 (filled triangles), or PSRA+ (filled squares) strain with lactate (18 mM) (A) or formate (30 mM) (C) as the electron donor. Respiration of $S_4O_6^{2-}$ results in an accumulation of $S_2O_3^{2-}$ by the wild-type (open circles), PSRA1 (open triangles), or PSRA+ (open squares) strain. (B and D) Cell growth as a function of time for the wild-type (filled circles), PSRA1 (open triangles), or PSRA+ (filled squares) strain with lactate (B) or formate (D) as the electron donor. In some cases, error bars are smaller than the symbol.

of typical $S_4O_6^{2-}$ terminal reductases. TtrA contains ferredoxin and MGD binding motifs, similarly to the polysulfide (thiosulfate) reductases PhsA and PsrA (23). The ability of PSRA1 to respire $S_4O_6^{2-}$ indicates that PsrA does not function as the $S_4O_6^{2-}$ reductase in *S. oneidensis*. Interestingly, the genomes of other $S_4O_6^{2-}$-respiring members of the genus *Shewanella* contain the traditional *ttrBAC* gene cluster, while *S. oneidensis* does not (21). A possible candidate for $S_4O_6^{2-}$ reductase in *S. oneidensis* is an atypical octaheme *c*-type cytochrome (SO4144) that displays $S_4O_6^{2-}$, NO_2^-, and NH_2OH reduction activities in vitro (4, 42). The physiological role of SO4144 in vivo remains to be examined.

The results of the present study indicate that pKO2.0 is an effective suicide vector for generating markerless, in-frame gene deletions in *S. oneidensis* MR-1. The vector is also suitable for generating "knock-in" complements of the deletion mutants, thereby avoiding problems associated with genetic complementation in *trans*. The results also indicate that PsrA is required for *S. oneidensis* to respire anaerobically on either S^0 or $S_2O_3^{2-}$ as an electron acceptor, most likely functioning as the $S_2O_3^{2-}$ terminal reductase. The large clearing zone observed in the periphery of wild-type *S. oneidensis* colonies incubated anaerobically on solid medium amended with S^0 corroborates previous findings that direct contact is not required for S^0 respiration by *S. oneidensis* (41). *S. oneidensis* may therefore respire S^0 via an intraspecies sulfur cycle catalyzed by extracellular, abiotic (purely chemical) interactions between S^0 and either HS^- or SO_3^{2-} produced during anaerobic S^0 respiration. The results of the present study are unable to differentiate between the HS^-- and SO_3^{2-}-catalyzed pathways. One potential strategy for differentiating between the HS^- and SO_3^{2-} cycling pathways is to construct SO_3^{2-} reductase deletion mutants. An increase in the diameters of clearing zones in the periphery of SO_3^{2-} reductase deletion mutants incubated anaerobically on S^0-amended solid growth medium is an indication that SO_3^{2-} contributes to S^0 mobilization in *S.* Typhimurium (24) (produced SO_3^{2-} is not reduced and is therefore more readily available to interact with extracellular S^0, resulting in a larger clearing zone). This experimental strategy is not currently possible for *S. oneidensis*, however, since a genome-

wide scan indicated that genes encoding traditional dissimilatory SO_3^{2-} terminal reductases (DsrA or AsrA) are missing from the *S. oneidensis* genome. Current work is focused on identification of the novel SO_3^{2-} reductase in *S. oneidensis* and examination of the S^0 reduction activity of the corresponding SO_3^{2-} reductase deletion mutants.

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