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Iodate Reduction by *Shewanella oneidensis* Does Not Involve Nitrate Reductase

Jung Kee Mok[a,†], Yael J. Toporek[a], Hyun-Dong Shin[a,†], Brady D. Lee[b], M. Hope Lee[c], and Thomas J. DiChristina[a]

[a]School of Biological Sciences, Georgia Institute of Technology, Atlanta, GA, USA; [b]Energy and Environment Directorate, Pacific Northwest National Laboratory, Richland, WA, USA; [c]Environmental Stewardship Directorate, Savannah River National Laboratory, Aiken, SC, USA

ABSTRACT

Microbial iodate (IO_3^-) reduction is a major component of iodine biogeochemical cycling and is the basis of alternative strategies for remediation of iodine-contaminated environments. The molecular mechanism of microbial IO_3^- reduction, however, is not well understood. In several microorganisms displaying IO_3^- and nitrate (NO_3^-) reduction activities, NO_3^- reductase is postulated to reduce IO_3^- as alternate electron acceptor. In the present study, whole genome analyses of 25 NO_3^--reducing *Shewanella* strains identified various combinations of genes encoding one assimilatory (cytoplasmic Nas) and three dissimilatory (membrane-associated Nar and periplasmic Napα and Napβ) NO_3^- reductases. *Shewanella oneidensis* was the only *Shewanella* strain whose genome encoded a single NO_3^- reductase (Napβ). Terminal electron acceptor competition experiments in *S. oneidensis* batch cultures amended with both NO_3^- and IO_3^- demonstrated that neither NO_3^- nor IO_3^- reduction activities were competitively inhibited by the presence of the competing electron acceptor. The lack of involvement of *S. oneidensis* Napβ in IO_3^- reduction was confirmed via phenotypic analysis of an in-frame gene deletion mutant lacking *nap*β*A* (encoding the NO_3^--reducing NapβA catalytic subunit). *S. oneidensis* Δ*nap*β*A* was unable to reduce NO_3^-, yet reduced IO_3^- at rates higher than the wild-type strain. Thus, NapβA is required for dissimilatory NO_3^- reduction by *S. oneidensis*, while neither the assimilatory (Nas) nor dissimilatory (Napα, Napβ, and Nar) NO_3^- reductases are required for IO_3^- reduction. These findings provide the first genetic evidence that IO_3^- reduction by *S. oneidensis* does not involve nitrate reductase and indicate that *S. oneidensis* reduces IO_3^- via an as yet undiscovered enzymatic mechanism.

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Introduction

Isotopes of iodine released during nuclear weapons testing and nuclear fuel reprocessing at facilities such as the Hanford Site (WA) have recently received heightened attention due to long half-lives and human health concerns (Buraglio et al. 2001; Hou et al. 2000; Kaplan et al. 2014; Moran et al. 1999; Muramatsu and Ohmomo 1986; Raisbeck and Yiou 1999). ^{129}I, for example, is present in multiple plumes at the Hanford Site and displays a half-life of 1.6×10^7 years and presents serious long-term radiological threats to human health (Chapman and McKinley 1987). Iodine is accumulated by brown algae, bacteria, and the thyroid glands of vertebrates (Amachi et al. 2008; De La Vieja et al. 2000; Eskandari et al. 1997; Küpper et al. 1998; Smyth and Dwyer 2002). Iodine is found in appreciable concentrations in contaminated soils, with iodine concentrations reported up to 5 mg kg^{-1} (Bowen 1979), and in anoxic marine basins, where iodine concentrations approach 1 mM (Nakayama et al. 1989). Iodate (IO_3^-, +5 oxidation state) and iodide (I^-, −1 oxidation state) represent the dominant iodine redox species in the environment (Whitehead 1984; Wong 1991). The iodine biogeochemical reaction network consists of coupled abiotic (purely chemical) and biotic (enzymatic) reactions (Amachi 2008). In marine environments, for example, IO_3^- is reduced to I^- by IO_3^--reducing microorganisms (Amachi 2008). The produced I^- is subsequently volatilized from marine surface waters via transformation to a variety of volatile organic iodine compounds, including methyl iodide (CH_3I), iodomethane (CH_2I_2), iodoethane (C_2H_5I), and iodopropane (C_3H_7I) (Carpenter et al. 1999; Rasmussen et al. 1982). I^- methylation activity is displayed by algae, phytoplankton, and bacteria (Lovelock 1975; Lovelock et al. 1973; Moore and Tokarcyak 1993; Rasmussen et al. 1982; Seigo et al. 2001). I^- oxidation to IO_3^- occurs step-wise via conversion of I^- to iodine (I_2) by I^--oxidizing microorganisms (Amachi et al. 2008; Gozlan and Margalith 1973; Ruse et al. 2003). I_2 is then rapidly hydrolyzed to HOI (+1 oxidation state), which is subsequently disproportionated to IO_3^-, completing the iodine biogeochemical cycle (Wong 1982, 1991).

Iodide (I^-) concentrations reach 0.3 μM in marine surface waters (Campos et al. 1996; Tian and Nicholas 1995; Tian et al. 1996) and approach 1 mM in deeper waters of anoxic marine basins (Chapman 1983; Farrenkopf and Luther III 2002; Farrenkopf et al. 1997b; Luther and Campbell 1991; Nakayama et al. 1989; Ullman et al. 1990; Wong et al. 1985). High I^- concentrations in marine environments are generally attributed to IO_3^- reduction by nitrate (NO_3^-)-reducing bacteria and

CONTACT Thomas J. DiChristina thomas.dichristina@biology.gatech.edu School of Biological Sciences, Georgia Institute of Technology, 311 Ferst Dr., NW, Atlanta, GA 30332.
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†These authors contributed equally to this work.

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phytoplankton (Councell et al. 1997; Farrenkopf et al. 1997a; Tsunogai and Sase 1969). NO_3^--reducing *Pseudomonas* sp. strain SCT, for example, reduces IO_3^- to I^- under anaerobic conditions (Amachi et al. 2007), while *Escherichia coli* cell-free extracts reduce IO_3^- to I^- under NO_3^--reducing conditions (Tsunogai and Sase 1969). These findings have led to speculation that microbial IO_3^- reduction is catalyzed by NO_3^- reductase (Amachi et al. 2007; Councell et al. 1997; Farrenkopf et al. 1997a; Tsunogai and Sase 1969).

IO_3^--reducing bacteria also include members of the genus *Shewanella*, which respire both aerobically and anaerobically with a myriad of compounds as terminal electron acceptor, including IO_3^- and NO_3^- (Borloo et al. 2007; Cooper et al. 2016; Cruz-Garcia et al. 2007; DiChristina et al. 2002; Farrenkopf et al. 1997a; Gao et al. 2009; Richter et al. 2012; Simpson et al. 2010; Szeinbaum et al. 2014; Venkateswaran et al. 1999). The dissimilatory NO_3^- reduction pathways of 23 *Shewanella* species have been identified via previously reported whole genome sequence analyses (Chen and Wang 2015; Simpson et al. 2010). The *Shewanella denitrificans* and *S. amazonensis* genomes encode classical denitrification pathways that reduce NO_3^- to N_2, while the remaining 21 *Shewanella* species encode dissimilatory NO_3^- reduction to ammonia (DNRA) pathways that reduce NO_3^- to NH_4^+ (Chen and Wang 2015). The 23 *Shewanella* genomes contain various combinations of genes predicted to encode membrane-bound (Nar) and periplasmic (Napα and Napβ) NO_3^- reductases (Chen and Wang 2015). Prior to the present study, the genes encoding the cytoplasmic assimilatory (Nas) NO_3^- reductases had not been analyzed in the 23 *Shewanella* genomes.

While a range of mechanisms have been proposed for microbial IO_3^- reduction, identification of specific genes or proteins involved in the process is a first step in developing biomarkers to probe for activity in the environment. This type of biomarker may then be used to determine the potential for attenuation of ^{129}I in contaminant plumes at locations such as the Hanford Site. The main objective of the present study was to test the hypothesis that microbial NO_3^- reductase is required for IO_3^- reduction under anaerobic conditions. The experimental strategy to test the main hypothesis included (i) phenotypic and genomic analyses to identify NO_3^-- and IO_3^--reducing *Shewanella* strains whose genomes encode a single (assimilatory or dissimilatory) NO_3^- reductase (i.e., *S. oneidensis*), (ii) terminal electron acceptor competition experiments to determine the IO_3^- and NO_3^- reduction activities of *S. oneidensis* batch cultures amended simultaneously with IO_3^- and NO_3^-, (iii) generation of *S. oneidensis* in-frame gene deletion mutants Δ*nap*β*A* (lacking the catalytic subunit of the single NO_3^- reductase, Napβ), and (iv) comparison of the IO_3^- and NO_3^- reduction activities of the *S. oneidensis* wild-type and Δ*nap*β*A* mutant strains.

Materials and methods

Bacterial strains and plasmids for genetic manipulations. Bacterial strains and plasmids used for the genetic manipulations in this study are listed in Table 1. For genetic manipulations, *S. oneidensis* and *E. coli* overnight cultures were grown aerobically in Luria-Broth (LB) (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, and 10 g l^{-1} NaCl) at 30°C and 37°C, respectively. When required for strain selection, LB medium was amended with chloramphenicol (25 μg ml^{-1}), ampicillin (100 μg ml^{-1}), and gentamicin (15 μg ml^{-1}) at the noted concentrations. All chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Features	Source
Shewanella oneidensis		
MR-1	Wild-type strain	56
Δ*nap*β*A*	In-frame *nap*β*A* deletion mutant	This study
Δ*crp*	In-frame *crp* deletion mutant	This study
Escherichia coli		
EC100D *pir*-116	*F- mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80d*lacZ*Δ*M15* Δ*lacX*74 *recA*1 *endA*1*araD*139 Δ(*ara, leu*)7697 *galU galK* λ^- *rpsL nupG pir*-116(DHFR)	Epicentre
B2155 λ *pir*	*thrB1004 pro thi strA hsds lacZ*ΔM15 F *lacZ*ΔM15 *lacI^q traD36 proA1 proB1) dapA::erm pir::*RP4 Km^r	58
Plasmids		
pKO2.0	4.5 kb γR6 K, *mob*RP4 *sacB* Gm^R *lacZ promoter*	58

Identification of the assimilatory NO_3^- reductase (*nas*) gene in 25 *Shewanella* genomes. The 25 *Shewanella* genomes included those from *S. putrefaciens* CN32 and recently sequenced *S. algae* BrY along with the 23 *Shewanella* genomes previously analyzed for the presence of the *nap*α, *nap*β, and *nar* gene clusters (Chen and Wang 2015): *S. oneidensis* MR-1, *S. denitrificans*, *S. frigidimarina*, *S. amazonensis*, *S. baltica* OS155, *S. baltica* OS185, *S. baltica* OS195, *S. baltica* OS223, *S. baltica* OS678, *S. baltica* OS117, *S. baltica* BA175, *S. loihica* PV-4, *S. algae* BrY, *S. putrefaciens* CN-32, *S. putrefaciens* strain 200, *S. sediminis*, *S. pealeana*, *Shewanella* sp. MR-4, *Shewanella* sp. MR-7, *Shewanella* sp. ANA-3, *Shewanella* sp. W3–18-1, *S. halifaxensis*, *S. woodyi*, *S. piezotolerans*, and *S. violacea*. The KEGG Prokaryotes and NCBI Reference/ Representative Genome databases (http://www.genome.jp/dbget-bin/www_bfind_sub?mode=bfind&max_hit=1000&dbkey=genome&keywords=shewanella) and BLASTP (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) were used for identification of the *nas* gene in the 25 *Shewanella* genomes via manual searches (Myers and Nealson 1988; Simpson et al. 2010) with the *S. sediminis* Nas amino acid sequence (Ssed_2799) as search query. Analogous genome analyses were used to identify the *nap*α, *nap*β, and *nar* gene clusters in the newly sequenced genomes of *S. alage* BrY and *S. putrefaciens* CN-32 gene cluster with *nap*α (*S. denitrificans*), *nap*β (*S. oneidensis* MR-1), and *nar* (*S. halifaxensis*) as search queries, respectively.

IO_3^- and NO_3^- reduction activity assays. The 25 sequenced *Shewanella* strains included in the genomic analyses of genes encoding the assimilatory and dissimilatory NO_3^- reductases were pared down to 10 strains for phenotypic analyses by selecting NO_3^--reducing *Shewanella* strains whose genomes contained various combinations of the *nas*, *nap*α, *nap*β, and *nar* gene clusters (bolded strain names in Figure 1). The 10 selected *Shewanella* strains were tested for IO_3^- reduction activity under previously determined optimal growth conditions (Garcia-Descalzo et al. 2014; Satomi 2014), which consisted of anaerobic incubation at room temperature in half-strength 2216 marine broth (ICN Biomedicals, Aurora, OH) amended with 20 mM lactate as carbon and energy source.

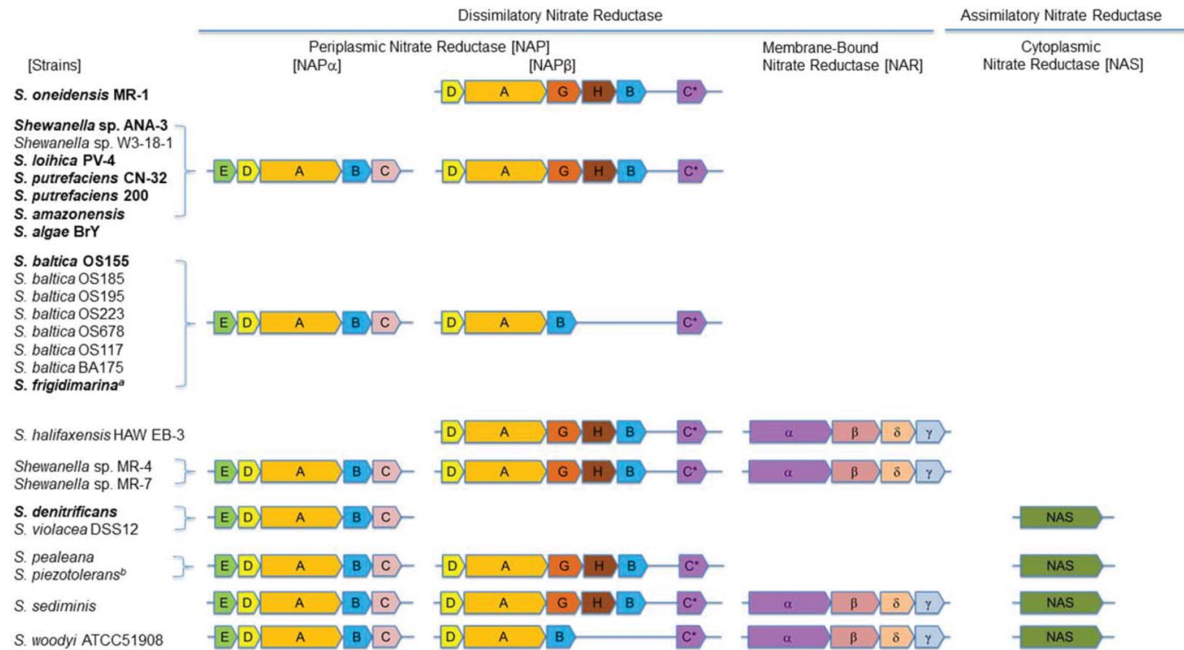

Figure 1. Organization of genes clusters encoding the assimilatory (Nas) and dissimilatory (Napα, Napβ, and Nar) NO_3^- reductases in the 25 *Shewanella* genomes. C* represents noncontiguous NapC-like homolog(s) identified in the *nap*β-containing genomes. The 10 bolded *Shewanella* strains were selected for determination of IO_3^- reduction activity (Table 4).

Shewanella overnight cultures were washed twice, and resuspended to an OD_{600} of 0.10 in 50 ml batch cultures of identical growth medium. IO_3^- was added at a previously determined optimal concentration of 250 μM (data not shown). *S. oneidensis* batch cultures were also tested for IO_3^- reduction activity at room temperature in M1 growth medium at the optimal concentration of 250 μM IO_3^-. To maintain anaerobic conditions, the batch cultures were continuously sparged with high purity (hydrated) N_2 gas. Total protein concentration was measured with a Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Rockford, IL).

IO_3^- reduction rates and extents of reaction (defined as % initial IO_3^- reduced) were determined by measuring IO_3^- concentration via the IO_3^--triiodide method (Afkhmi et al. 2001). Culture subsamples were transferred to 96-well microtiter plates and 0.1 M sodium-citrate buffer (pH 3.3) and 75 mM potassium-iodide solution were added to each well to initiate triiodide formation ($IO_3^- + 8I^- + 6H^+ \rightarrow 3I_3^- + 3H_2O$). After 4 min of reaction time, absorbance at 352 nm was measured with a UV-visible spectrophotometer. NO_3^- reduction rates were determined by measuring NO_2^- production spectrophotometrically after quenching samples in sulfanilic acid-N-1-naphthyl-ethylene-diamine dihydrochloride solution (Montgomery and Dymock 1961). IO_3^- and NO_2^- concentrations were determined from previously generated calibration curves.

IO_3^- and NO_3^- terminal electron acceptor competition experiments. *S. oneidensis* wild-type and select mutant strains were tested for simultaneous IO_3^- and NO_3^- reduction activities in a series of terminal electron acceptor competition experiments carried out anaerobically in M1 growth medium amended with 20 mM lactate, 250 μM IO_3^-, and either equimolar (250 μM) or 10X molar excess (2.5 mM) NO_3^-. Control experiments included heat-killed controls and incubations with either bacterial cells (abiotic controls), IO_3^-, or NO_3^- omitted. Anaerobic (abiotic) incubations with 250 μM NO_2^- and either 250 μM I^- or 250 μM IO_3^- were carried out to determine background chemical interactions that may otherwise mask microbial IO_3^- and NO_3^- reduction activities. IO_3^- and NO_3^- reduction activities were measured via the IO_3^--triiodide and sulfanilic acid-N-1-naphthyl-ethylene-diamine dihydrochloride methods described above. To determine the abiotic reactivity of NO_2^- on IO_3^- and I^- under anaerobic condition, NO_2^- and I^- or IO_3^- concentrations were monitored in M1 minimal growth medium amended with 20 mM lactate, 2.5 mM NO_2^-, and either 250 μM I^- or IO_3^-. IO_3^- and NO_3^- concentrations were measured via the IO_3^--triiodide and sulfanilic acid-N-1-naphthyl-ethylene-diamine dihydrochloride methods described above. I^- concentration was measured by spectrophotometric determination method which is based on complex formation of iodide ion with Bindschedler's Green Leuco Base after extraction of iodide ion with CCl_4 from the aqueous reaction mixture (Utsuni et al. 1987).

In-frame gene deletion mutagenesis. The *S. oneidensis* Δ*crp* mutant strain was constructed to provide a cAMP receptor protein-deficient *S. oneidensis* negative control strain unable to grow anaerobically on any terminal electron acceptor, including NO_3^- and (potentially) IO_3^- (57%). *nap*β*A* and *crp* were deleted in-frame from the *S. oneidensis* genome following previously described methods (Burns et al. 2010; Burns and DiChristina 2009). The primers used for construction of Δ*nap*β*A* and Δ*crp* are listed in Table 2. Regions corresponding to ~750 bp upstream and downstream of each open reading frame (ORF) were PCR-amplified with iProof ultrahigh-fidelity polymerase (Bio-Rad, Hercules, CA), generating fragments F1 and F2, which were fused by overlap extension PCR to generate fragment F3. Fragment F3 was cloned into pKO2.0 with *Bam*HI

Table 2. Primers used to construct Δ*nap*β*A* and Δ*crp* gene deletion mutants.

Primer	Sequence (5′ to 3′)	Remarks
Δ*nap*β*A deletion*		
NapA-TF	CAATCGTATTAAATATCTGTTCATTCA	
NapA-D1	GACTGGATCCCAACGCGCTTTAGACAAGG	BamH1 (underlined)
NapA-D2	*CATCGCTATCAAATGAAGGC*AGTGTTTCCTCACTCATTTTTTCTAAC	Reverse complementary sequence of NapA-D3 (*italic*)
NapA-D3	*GTTAGAAAAAATGAGTGAGGAAACACT*GCCTTCATTTGATAGCGATG	Reverse complementary sequence of NapA-D2 (*italic*)
NapA-D4	GACTGTCGACGGTTTCCTCAGTGTTGAGATAAGTG	SalI (underlined)
NapA-TR	AACGTCAGCCCCTTATTCAA	
Δ*crp deletion*		
Crp-TF	GCGTAAATAAAACCTAAACGGAACT	
Crp-D1	CTGATAGGATCC TCTTTATACCAACGTTCGGCC	BamH1 (underlined)
Crp-D2	*GGCTTAAATCAAGCTGAAGTCTAACT*GTCGATGTTCCTCGATTGATTAA	Reverse complementary sequence of Crp-D3 (*italic*)
Crp-D3	*TTAATCAATCGAGGAACATCGAC*AGTTAGACTTCAGCTTGATTTAAGCC	Reverse complementary sequence of Crp-D2 (italic)
Crp-D4	TCGATCGTCGACAGTGCCTGAATTCGCGCTA	SalI (underlined)
Crp-TR	TAGCTAAGTTGCTTGTTGGGATT	

and *Sal*I restriction endonucleases and electroporated into *E. coli* strain *β*2155 *λ pir*. pKO2.0-F3 was mobilized into the recipient *S. oneidensis* wild type via biparental mating procedures. A plasmid integrant was identified via PCR analysis, and the mutation was resolved on LB agar containing sucrose [10% (wt vol^{-1})]. Following counter selection, the corresponding *S. oneidensis* gene deletion mutant strains Δ*nap*β*A* and Δ*crp* were isolated and confirmed via PCR and DNA sequence analyses.

Results

Identification of various combinations of the assimilatory and dissimilatory NO_3^- reductase gene clusters in the 25 *Shewanella* genomes. Genes encoding assimilatory NO_3^- reductase (Nas) homologs were identified in 6 of the 25 *Shewanella* genomes (*S. sediminis*, *S. woodyi* ATCC51908, *S. violacea*, *S. denitrificans*, *S. pealeana*, *S. piezotolerans*; Figure 1 and Table 3). The six Nas homologs displayed moderate-to-high amino acid sequence similarity (50–96%), identity (31–91%), and E-values (5e^{-133}-0.0) to each other, and to the NO_3^- reductase catalytic subunit of the most similar Nas homolog outside of the genus *Shewanella* (*Thalassomonas actiniarum* WP_044831609.1; Table 3). The *nas* gene content reported in the present study was combined with the presence of genes encoding the dissimilatory NO_3^- reductase (Nap*α*, Nap*β*, and Nar) homologs reported in previous studies (Chen and Wang 2015; Simpson et al. 2010) to provide a genome-wide view of the assimilatory and dissimilatory NO_3^- reductase gene content of the 25 *Shewanella* genomes (Figure 1). As previously described (Chen and Wang 2015; Chen et al. 2011; Cruz-Garcia et al. 2007; Gao et al. 2009), the *Shewanella nap*α gene clusters included *napC* (encoding NapC/NirT cytochrome *c* family proteins), while the *Shewanella nap*β gene clusters lacked *napC* and harbored noncontiguous *napC* homologs (Nap C* in Figure 1) elsewhere in the *nap*β-containing *Shewanella* genomes. Only two genomes (*S. sediminis* and *S. woodyi* ATCC51908) contained the entire suite of *nas*, *nap*α, *nap*β, and *nar* gene clusters (Figure 1). The genomes of *Shewanella* spp. MR-4 and MR-7 contained the *nap*α, *nap*β, and *nar* gene clusters (but lacked the *nas* gene cluster) (Figure 1), while the genomes of *S. piezotolerans* and *S. pealeana* contained the *nap*α, *nap*β, and *nas* gene clusters (but lacked the *nar* gene cluster) (Figure 1 and Table 3). The genomes of *Shewanella* spp. ANA-3 and W3–18-1, *S. loihica*, *S. putrefaciens* CN-32, *S. putrefaciens* 200, *S. amazonensis*, *S. frigidimarina*, and *S. baltica* spp. (OS1155, OS185, OS195, OS223, OS678, OS117, and BA175) contained the *nap*α and *nap*β gene clusters (but lacked the *nas* and *nar* gene clusters) (Figure 1). The genomes of *S. denitrificans* and *S. violacea* DSS12 contained the *nas* and *nap*α gene clusters (but lacked the *nap*β and *nar* gene clusters) (Figure 1 and Table 3), while the genome of *S. halifaxensis* HAWEB-3 contained the *nap*β and *nar* gene clusters (but lacked the *nas* and *nap*α gene clusters) (Figure 1). *S. oneidensis* was the only *Shewanella* strain whose genome contained a single NO_3^- reductase gene cluster (*nap*β) (Figure 1). The presence of Nap*β* as the sole NO_3^- reductase in *S. oneidensis* facilitates interpretation of results from NO_3^- and IO_3^- terminal electron acceptor competition experiments and IO_3^- reduction activity assays with the *S. oneidensis* Δ*nap*β and Δ*crp* deletion mutants.

IO_3^- reduction activities of *Shewanella* wild-type strains. The IO_3^- reduction activities of the *Shewanella* strains differed over a 50-fold range, ranging from 2,295 nmol·h^{-1}·mg protein^{-1} by *S. putrefaciens* strain 200 to 45 nmol·h^{-1}·mg

Table 3. Amino acid sequence homology of assimilatory NO_3^- reductase (cytoplasmic Nas) homologs in the 25 *Shewanella* genomes.

	Within *Shewanella* genus[a]			Outside *Shewanella* genus[b]				
ORF	Sim %	ID %	E-value	Best hit	Sim %	ID %	E-value	Annotated function
Nas Gene Homolog (Reference: *S. sediminis*)								
Ssed_2799 (Nas)	50∼96	31∼91	5e^{-133}∼0.0	*Thalassomonas actiniarum* *WP_044831609.1	93	85	0.0	NO_3^- reductase catalytic subunit
Shewanella spp.	*S. sediminis*, *S. woodyi* ATCC51908, *S. violacea*, *S. denitrificans*, *S. pealeana*, *S. piezotolerans*							

[a]Percent sequence similarity (Sim), percent identity (ID), and E-value compared to reference gene obtained from BLASTp analysis. Ranges were determined by pair-wise comparison with translated sequence data from genome sequences of 6 strains of *Shewanella* in KEGG.
[b]Organisms outside of the genus *Shewanella* with homologs of the highest similarity (best hit) as determined by BLASTp analysis of the GenBank nonredundant database as shown.
*NCBI accession number.

Table 4. IO_3^- reduction activities and extents of reaction of 10 selected *Shewanella* strains.

Strain	IO_3^- Reduction Rate* (nmol·h^{-1}·mg protein^{-1})	Extent of reaction** (% of IO_3^- reduced to I^-)
Abiotic control	0.0	0.0
S. putrefaciens 200	2,295 ± 12	95 ± 0
S. algae BrY	2,099 ± 31	93 ± 0
S. putrefaciens CN-32	367 ± 6	79 ± 1
Shewanella sp. ANA-3	350 ± 7	36 ± 1
S. amazonensis	34 ± 3	31 ± 4
S. oneidensis MR-1	261 ± 7	64 ± 2
S. baltica OS155	150 ± 8	6 ± 4
S. frigidimarina	125 ± 2	5 ± 2
S. lihoica PV-4	65 ± 10	7 ± 3
S. denitrificans	45 ± 11	5 ± 5

All strains were incubated anaerobically in half strength 2,216 marine broth amended with 20 mM lactate as electron donor and 250 μM IO_3^- as electron acceptor. Values represent means of triplicate samples; error represents one standard deviation.
*IO_3^- reduction rate calculated from the first 2-h anaerobic incubation period (reported in nmol·h^{-1}·mg protein^{-1}).
**Extent of reaction is reported as the percentage of IO_3^- reduced to I^- at completion of 24-h incubation period.

protein^{-1} by *S. denitrificans* (Table 4). *S. algae* BrY also displayed high IO_3^- reduction activity nearly identical to *S. putrefaciens* strain 200. A group of four strains (*S. putrefaciens* CN-32, *Shewanella* sp. ANA-3, *S. amazonensis*, *S. oneidensis* MR-1) displayed IO_3^- reduction activities that were six to eightfold less than *S. putrefaciens* strain 200, while a group of four strains (*S. baltica* OS155, *S. fridigimarina*, *S. lihoica* PV-4 and *S. denitrificans*) displayed IO_3^- reduction activities that were up to 50-fold less than *S. putrefaciens* strain 200. IO_3^- reduction activity was below detection levels in all heat-killed control incubations.

NO_3^- and IO_3^- terminal electron acceptor competition experiments. Initial abiotic (purely chemical) control experiments indicated that neither I^- nor IO_3^- interacted chemically with NO_2^- to potentially mask the microbial IO_3^- and NO_3^- reduction activities of wild-type strain *S. oneidensis* MR-1 batch cultures (data not shown). The IO_3^- reduction activity of the *S. oneidensis* MR-1 was not competitively inhibited by the presence of equimolar NO_3^-. In the absence of NO_3^-, the *S. oneidensis* MR-1 reduced 250 μM IO_3^- at a rate of 258 nmol·h^{-1}·mg protein^{-1}, with a corresponding extent of reaction of 56% (Figure 2; Table 5(A)). By comparison, in the presence of 250 μM NO_3^-, the *S. oneidensis* MR-1 reduced 250 μM IO_3^- at a rate approximately 20% greater than the rate measured in the absence of NO_3^- [with an extent of reaction (59%) nearly identical to the absence of NO_3^-]. In the presence of NO_3^- amended at 10X molar excess (2.5 mM), the *S. oneidensis* MR-1 reduced 250 μM IO_3^- at a rate approximately 60% greater than the rate measured in the absence of NO_3^- [with an extent of reaction (57%) nearly identical to the absence of NO_3^-].

In an analogous fashion, the NO_3^- reduction activity of the *S. oneidensis* MR-1 was not competitively inhibited by the presence of equimolar IO_3^-. In the absence of IO_3^-, the *S. oneidensis* MR-1 reduced 250 μM NO_3^- at a rate of 2,496 nmol·h^{-1}·mg protein^{-1} with a corresponding extent of reaction of 102% (Figure 3; Table 5(B)). By comparison, in the presence of 250 μM IO_3^-, the *S. oneidensis* MR-1 reduced 250 μM NO_3^- at a rate approximately 92% of the rate measured in the absence of IO_3^- [with an extent of reaction (102%) nearly identical to the absence of IO_3^-]. A similar pattern was observed with NO_3^- amended at 2.5 mM levels. In the absence of IO_3^-, the *S. oneidensis* MR-1 reduced 2.5 mM NO_3^- at a rate of 5,248 nmol·h^{-1}·mg protein^{-1} with a corresponding extent of reaction of 86%. In the presence of 250 μM IO_3^-, the *S. oneidensis* MR-1 reduced 2.5 mM NO_3^- at a rate approximately 99% of the rate measured in the absence of IO_3^- [with an extent of reaction (80%) similar to the absence of IO_3^-]. NO_3^- reduction activity was below detection levels in heat-killed and abiotic control incubations.

NO_3^- and IO_3^- reduction activities of the *S. oneidensis* Δ*napA* and Δ*crp* mutant strains. Wild-type *S. oneidensis* MR-1 reduced 2.5 mM NO_3^- at a rate of 5,260 nmol·h^{-1}·mg protein^{-1} with a corresponding extent of reaction of 86% (Figure 4 and Table 6). The *S. oneidensis* Δ*napA* mutant strain, on the other hand, reduced 2.5 mM NO_3^- at a rate only 5% of the *S. oneidensis* wild-type strain (with a corresponding extent of reaction of 1%; Figure 4 and Table 6). As previously reported (Saffrarini et al. 2003), the *S. oneidensis* Δ*crp* mutant strain reduced 2.5 mM NO_3^- at a rate only 8% of the *S. oneidensis* wild-type strain with a corresponding extent of reaction of 2% (Figure 3). The *S. oneidensis* wild-type strain reduced 250 μM IO_3^- at a rate of 257 nmol·h^{-1}·mg protein^{-1} with a corresponding extent of reaction of 56% (Figure 4 and Table 6). The *S. oneidensis* Δ*napA* mutant strain, on the other hand, reduced

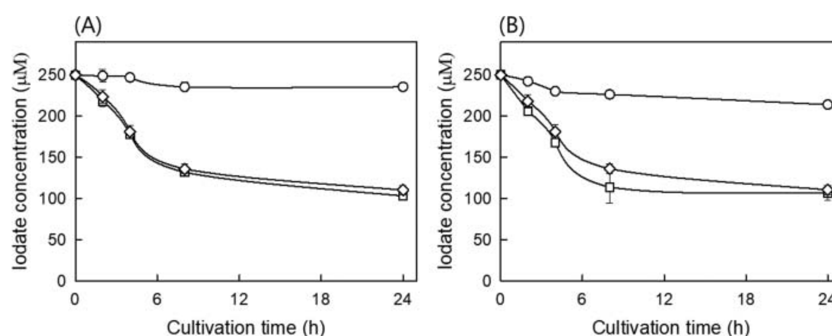

Figure 2. Effects of NO_3^- on IO_3^- reduction by *S. oneidensis* wild-type strain. IO_3^- reduction was monitored in M1 growth medium amended with 20 mM lactate, 250 μM IO_3^-, and either 250 μM (A) or 2.5 mM (B) NO_3^-. Initial cell density was 1×10^8 cells/ml. Symbols: ○, Δ*crp* mutant strain; ◇, *S. oneidensis* wild-type strain with NO_3^- omitted; □, *S. oneidensis* wild-type strain. Values represent means of triplicate samples; error bars represent the estimated standard deviations for triplicate samples. Error bars represent standard deviations, not shown if less than size of the symbol.

Table 5. Rates associated with terminal electron acceptor competition experiments: (A) IO_3^- reduction activities and extents of reaction of the *S. oneidensis* wild-type strain in the presence and absence of NO_3^-, and (B) NO_3^- reduction activities and extents of reaction of the *S. oneidensis* wild-type strain in the presence and absence of IO_3^-.

(A)

	NO_3^- omitted		250 μM NO_3^-		2.5 mM NO_3^-	
Strain	Rate* (% of WT)	Extent** (% of WT)	Rate (% of WT)	Extent (%) (% of WT)	Rate (% of WT)	Extent (%) (% of WT)
Heat-killed MR-1	11 ± 8 (4%)	5 ± 3 (9%)	ND***	ND	ND	ND
Wild-type MR-1	258 ± 10 (100%)	56 ± 4 (100%)	320 ± 14 (128%)	59 ± 3 (105%)	415 ± 24 (161%)	57 ± 4 (102%)

(B)

	250 μM NO_3^-				2.5 mM NO_3^-			
	IO_3^- omitted		250 μM IO_3^-		IO_3^- omitted		250 μM IO_3^-	
Strain	Rate* (% WT)	Extent** (% WT)	Rate (% WT)	Extent (% WT)	Rate (% WT)	Extent (% WT)	Rate (% WT)	Extent (% WT)
Heat-killed MR-1	ND	ND	ND	ND	75 ± 6 (1%)	1 ± 0 (1%)	ND	ND
Wild-type (MR-1)	2,496 ± 132 (100%)	102 ± 13 (100%)	2,306 ± 231 (92%)	102 ± 23 (100%)	5,248 ± 226 (100%)	86 ± 8 (100%)	5,183 ± 129 (99%)	80 ± 9 (96%)

*IO_3^- reduction rate calculated from the first 2-h anaerobic incubation period (reported in nmol·h^{-1}·mg $protein^{-1}$) (A), and NO_3^- reduction rate was calculated from the NO_2^- production rate for first 2-h reaction period (reported as nmol·h^{-1}·mg $protein^{-1}$) (B).
**Extent of reaction is reported as the percentage of IO_3^- reduced to I^- at completion of 24-h incubation period (A) and percentage of NO_2^- produced (at the highest NO_2^- concentration) from the initial NO_3^- concentration (B).
***ND, not determined. Values represent means of triplicate samples; error represents one standard deviation.

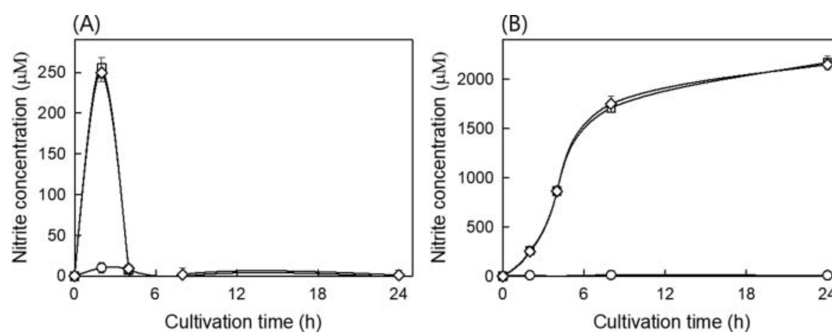

Figure 3. Effects of IO_3^- on NO_3^- reduction by *S. oneidensis* wild-type strain. NO_3^- reduction was monitored in M1 growth medium amended with 20 mM lactate, 250 μM IO_3^-, and 250 μM (A) or 2.5 mM (B) NO_3^-. Initial cell density was 1×10^8 cells/ml. Symbols: ○, Δ*crp* mutant strain; ◇, *S. oneidensis* wild-type strain with IO_3^- omitted; □, *S. oneidensis* wild-type strain. Values represent means of triplicate samples; error bars represent the estimated standard deviations for triplicate samples. Error bars represent standard deviations, not shown if less than size of the symbol.

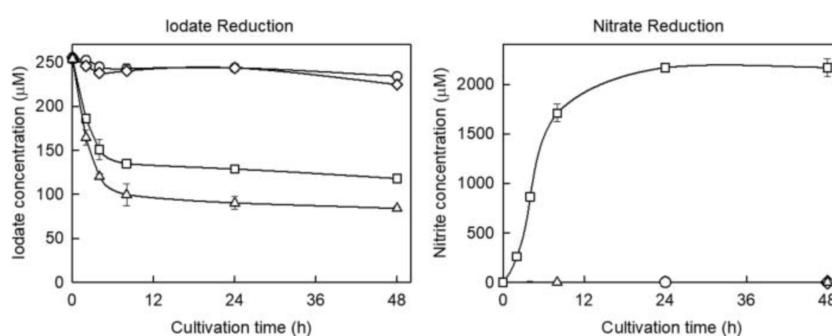

Figure 4. IO_3^- and NO_3^- reduction activities of the *S. oneidensis* wild-type and Δ*crp* and Δ*nap*β*A* mutant strains. IO_3^- and NO_3^- reduction activities were monitored in M1 minimal growth medium amended with 20 mM lactate and either 250 μM IO_3^- or 2.5 mM NO_3^- at room temperature. Initial cell density was 1×10^8 cells/ml. Symbols: ○, Cells omitted (abiotic control); ◇, Δ*crp* mutant strain; □;, *S. oneidensis* wild-type strain; Δ, Δ*napA* mutant strain. Values represent means of triplicate samples; error bars represent the estimated standard deviations for triplicate samples. Error bars represent standard deviations, not shown if less than size of the symbol.

250 μM IO_3^- at a rate approximately 2.6-fold greater than the *S. oneidensis* wild-type strain (with a corresponding extent of reaction of 65%; Figure 4 and Table 6), while the *S. oneidensis* Δ*crp* mutant strain reduced 250 μM IO_3^- at a rate only 13% of the *S. oneidensis* wild-type strain (with a corresponding extent of reaction of 11%; Figure 4 and Table 6).

Discussion

The iodine biogeochemical cycle consists of a coupled abiotic and biotic reaction network driven by the major microbially-catalyzed reactions IO_3^- reduction (Amachi 2008), I^- methylation (Carpenter et al. 1999; Rasmussen et al. 1982; Seigo et al. 2001), and I^- oxidation (Amachi et al. 2008, Gozlan and Margalith 1973, Ruse et al. 2003). Microbial IO_3^- reduction to I^- and subsequent I^- methylation to volatile iodocarbon compounds forms the basis of alternative strategies for bioremediation of iodine-contaminated environments (Amachi 2008; Kaplan et al. 2014; Moran et al. 1999). Microbial IO_3^- reduction is catalyzed by a variety of NO_3^--reducing microorganisms, including *Pseudomonas* sp. strain SCT, *E. coli*, and *S. putrefaciens* MR-4 (Amachi et al. 2007; Councell et al. 1997; Farrenkopf et al. 1997a; Tsunogai and Sase 1969). The highly oxidizing standard redox potentials of the NO_3^- and IO_3^- reduction half reactions (Amachi et al. 2007; Councell et al. 1997) and the correlation between microbial NO_3^- and IO_3^- reduction have led to the hypothesis that NO_3^- reductase reduces IO_3^- as alternate electron acceptor (Amachi et al. 2007; Councell et al. 1997; Farrenkopf et al. 1997a; Tsunogai and Sase 1969). The molecular mechanism of microbial IO_3^- reduction, however, remains poorly understood.

All members of the γ-proteobacterial genus *Shewanella* reduce NO_3^- as terminal electron acceptor (Cooper et al. 2016; Richter et al. 2012; Venkateswaran et al. 1999). Prior to the present study, *S. putrefaciens* MR-4 was the only member of the *Shewanella* genus tested for IO_3^- reduction activity (Farrenkopf et al. 1997a). All 10 *Shewanella* strains tested in the present study were capable of reducing both NO_3^- (as

Table 6. IO_3^- and NO_3^- reduction activities and extents of reaction of the *S. oneidensis* wild-type and △*nap*β*A* mutant strains.

	IO_3^- reduction (250 μM IO_3^-)		NO_3^- reduction (2.5 mM NO_3^-)	
Strain	Reduction Rate* (% of WT)	Extent of reaction** (% of WT)	Reduction Rate (% of WT)	Extent of reaction (% of WT)
Wild-type	258 ± 10 (100%)	56 ± 4 (100%)	5,248 ± 226 (100%)	86 ± 8 (100%)
Δ*nap*β*A*	669 ± 47 (260%)	65 ± 5 (116%)	242 ± 8 (5%)	1 ± 0 (1%)

*IO_3^- and NO_3^- reduction rates were calculated from the first 2-h anaerobic incubation period and reported as nmol·h^{-1}·mg $protein^{-1}$. Values represent means of triplicate samples; error represents one standard deviation.

**Extent of reaction is represented percentage IO_3^- reduced or NO_2^- produced after completion of the 24-h anaerobic incubation period.

previously described by Chen and Wang 2015; Chen et al. 2011; Cruz-Garcia et al. 2007; Gao et al. 2009) and IO_3^- as terminal electron acceptors. Although microbial IO_3^- reduction is well-documented in marine isolates, *Shewanella* strains isolated from both marine and freshwater environments displayed robust IO_3^- reduction activity. *S. putrefaciens* strain 200 [isolated from the crown of a corroding oil pipeline and selected for high Fe(III) reduction activity (Arnold et al. 1990; DiChristina 1992)] and *S. algae* strain BrY [enriched from estuarine sediments with acetate as carbon source and NO_3^- as electron acceptor (Caccavo et al. 1992; Satomi 2014)] reduced IO_3^- at the highest rates, while *S. denitrificans* [isolated from the oxic-anoxic interface of a marine basin (Brettar et al. 2002; Satomi 2014)] reduced IO_3^- at the lowest rates, approximately 50-fold lower than *S. putrefaciens* strain 200. The extent of the IO_3^- reduction reactions catalyzed by the wild-type *Shewanella* strains varied in a manner nearly identical to the IO_3^- reduction reaction rates, with extents of reaction ranging from 95% (*S. putrefaciens* strain 200) to 5% (*S. denitrificans*). Reasons for the correlation between IO_3^- reduction reaction rates and extents of reaction are unclear, but may correspond to the ability of the *Shewanella* strains displaying high rate IO_3^- reduction activity to withstand toxicity effects associated with the resulting high I^- concentrations.

Whole genome analyses indicated that the 25 *Shewanella* genomes encoded various combinations of gene clusters encoding the assimilatory (Nas) and dissimilatory (Napα, Napβ, and Nar) NO_3^- reductases (Figure 1). IO_3^- reduction activity did not correlate with the number or types of assimilatory and dissimilatory NO_3^- reductases encoded in the *Shewanella* genomes. For example, the *S. putrefaciens* strain 200 and *S. lihoica* PV-4 genomes encoded an identical pair of dissimilatory NO_3^- reductases (*nap*α and *nap*β gene clusters), yet the corresponding IO_3^- reduction activities differed by 35-fold (Table 4). Only two genomes (*S. sediminis* and *S. woodyi* ATCC51908) harbored the entire suite of NO_3^- reductase gene clusters encoding Nas, Napα, Napβ, and Nar, while *S. oneidensis* was the only *Shewanella* strain whose genome encoded a single NO_3^- reductase gene cluster (Napβ) (Figure 1). The presence of Napβ as the sole NO_3^- reductase in *S. oneidensis* facilitated interpretation of results from IO_3^- and NO_3^- terminal electron acceptor competition experiments with the *S. oneidensis* wild-type strain and IO_3^- reduction activity assays with the *S. oneidensis* Δ*nap*β deletion mutant. *S. oneidensis* was thus selected for further genetic and phenotypic analyses of the potential overlap between the IO_3^- and NO_3^- reduction systems.

Terminal electron acceptor competition experiments provide valuable insight into the electron transport chain physiology of anaerobically-respiring *Shewanella* strains (Arnold at al. 1990; DiChristina 1992). Fe(III) and NO_3^- terminal electron acceptor competition experiments with *S. putrefaciens* strain 200 (DiChristina 1992), for example, indicated that Fe(III) and NO_3^- were reduced simultaneously by separate terminal reductases and that the apparent inhibitory effect of NO_3^- on Fe(III) reduction activity was due to the abiotic (purely chemical) oxidation of Fe(II) (the product of microbial Fe(III) reduction) by NO_2^- (the product of microbial NO_3^- reduction) (Coby and Picardal 2005; DiChristina 1992). In the present study, the competitive inhibition of NO_3^- reductase activity by IO_3^- and, conversely, the competitive inhibition of IO_3^- reductase activity by NO_3^- were examined in *S. oneidensis* batch cultures amended with IO_3^- and NO_3^- as competing electron acceptors. Initial abiotic (purely chemical) control experiments indicated that I^- and IO_3^- did not interact chemically with NO_2^- and potentially mask the microbial IO_3^- and NO_3^- reduction activities of *S. oneidensis* batch cultures. In addition, the *S. oneidensis* genome encoded only a single NO_3^- reductase (Napβ; see below), which avoids the potential confounding effects of multiple NO_3^- reductases with varying IO_3^- reductase activities. The IO_3^- reduction activity of *S. oneidensis* was not competitively inhibited by the presence of equimolar NO_3^-, and conversely, the (Napβ-catalyzed) NO_3^- reduction activity of *S. oneidensis* was not competitively inhibited by the presence of equimolar IO_3^-. These findings may reflect the similarities between the standard redox potentials of the IO_3^- reduction to I^- (E_0 = +1.09 V) and NO_3^- reduction to NO_2^- (E_0 = +0.93 V) half reactions (Amachi et al. 2007). The IO_3^- reduction activity of wild-type *S. oneidensis* MR-1 in the presence of 10X molar excess NO_3^- was unexpectedly enhanced 60% higher than the IO_3^- reduction activity of *S. oneidensis* MR-1 in the absence of NO_3^-, potentially due to changes in NO_3^--responsive control elements regulating the activity of the *S. oneidensis* electron transport chain (Chen and Wang 2015). Results of the NO_3^- and IO_3^- electron acceptor competition experiments indicate that *S. oneidensis* does not preferentially channel electrons to IO_3^- or NO_3^- and suggest that NO_3^- and IO_3^- are reduced by separate terminal reductases (i.e., by Napβ and an as yet unidentified IO_3^- reductase, respectively).

The *Shewanella* Napβ gene clusters (*napDAGHB*) encode the NO_3^--reducing catalytic subunit NapβA, but do not encode the quinol dehydrogenase NapC, which is found at the terminus of the Napα gene cluster (*napEDABC*) (Chen and Wang 2015; Simpson et al. 2010). In the present study, genome-wide analyses of all *nap*β-containing *Shewanella* genomes identified a noncontiguous gene encoding a NapC-like quinol dehydrogenase (designated NapC*; Figure 1) that may transport electrons from the quinol pool to the NapβAB terminal reductase complex. To confirm that *S. oneidensis* NapβA was not required for IO_3^- reduction, an in-frame gene deletion mutant lacking *nap*β*A* (Δ*nap*β*A*) was constructed and tested for IO_3^- and NO_3^- reduction activities under anaerobic growth conditions. *S. oneidensis* Δ*nap*β*A* was unable to reduce NO_3^-, while Δ*nap*β*A* reduced IO_3^- at rates 2.6-fold greater than the wild-type strain. The enhanced IO_3^- reduction activity displayed by Δ*nap*β*A* was unexpected and is currently being investigated via complementary transcriptomic and proteomic analyses.

Napβ is the only NO_3^- reductase encoded in the *S. oneidensis* wild-type genome, thus the results of the IO_3^- and NO_3^- reduction activity assays with Δ*nap*β*A* demonstrate that NapβA is required for dissimilatory NO_3^- reduction by *S. oneidensis*, but neither the assimilatory (Nas) nor dissimilatory (Napα, Napβ, or Nar) NO_3^- reductases are required for IO_3^- reduction. These findings provide the first genetic evidence that iodate reduction by *S. oneidensis* does not involve nitrate reductase and indicate that *S. oneidensis* reduces IO_3^- via an as yet undiscovered enzymatic mechanism. Current work is focused on identification of the genes required for IO_3^- reduction by *S.*

oneidensis. Identification of IO_3^- reduction-specific genes will provide molecular information important for interpretation of the *in situ* (meta)omic signals obtained from iodine-contaminated environments undergoing remediation via monitored natural attenuation or biostimulation. Identification of these types of biomarkers will be important for monitoring attenuation in ^{129}I plumes such as those found at the Hanford Site.

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