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Metal Reduction and Protein Secretion Genes Required for Iodate Reduction by *Shewanella oneidensis*

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ABSTRACT The metal-reducing gammaproteobacterium *Shewanella oneidensis* reduces iodate (IO_3^-) as an anaerobic terminal electron acceptor. Microbial IO_3^- electron transport pathways are postulated to terminate with nitrate (NO_3^-) reductase, which reduces IO_3^- as an alternative electron acceptor. Recent studies with *S. oneidensis*, however, have demonstrated that NO_3^- reductase is not involved in IO_3^- reduction. The main objective of the present study was to determine the metal reduction and protein secretion genes required for IO_3^- reduction by *Shewanella oneidensis* with lactate, formate, or H_2 as the electron donor. With all electron donors, the type I and type V protein secretion mutants retained wild-type IO_3^- reduction activity, while the type II protein secretion mutant lacking the outer membrane secretin GspD was impaired in IO_3^- reduction. Deletion mutants lacking the cyclic AMP receptor protein (CRP), cytochrome maturation permease CcmB, and inner membrane-tethered *c*-type cytochrome CymA were impaired in IO_3^- reduction with all electron donors, while deletion mutants lacking *c*-type cytochrome MtrA and outer membrane β-barrel protein MtrB of the outer membrane MtrAB module were impaired in IO_3^- reduction with only lactate as an electron donor. With all electron donors, mutants lacking the *c*-type cytochromes OmcA and MtrC of the metal-reducing extracellular electron conduit MtrCAB retained wild-type IO_3^- reduction activity. These findings indicate that IO_3^- reduction by *S. oneidensis* involves electron donor-dependent metal reduction and protein secretion pathway components, including the outer membrane MtrAB module and type II protein secretion of an unidentified IO_3^- reductase to the *S. oneidensis* outer membrane.

IMPORTANCE Microbial iodate (IO_3^-) reduction is a major component in the biogeochemical cycling of iodine and the bioremediation of iodine-contaminated environments; however, the molecular mechanism of microbial IO_3^- reduction is poorly understood. Results of the present study indicate that outer membrane (type II) protein secretion and metal reduction genes encoding the outer membrane MtrAB module of the extracellular electron conduit MtrCAB are required for IO_3^- reduction by *S. oneidensis*. On the other hand, the metal-reducing *c*-type cytochrome MtrC of the extracellular electron conduit is not required for IO_3^- reduction by *S. oneidensis*. These findings indicate that the IO_3^- electron transport pathway terminates with an as yet unidentified IO_3^- reductase that associates with the outer membrane MtrAB module to deliver electrons extracellularly to IO_3^-.

KEYWORDS *Shewanella oneidensis*, iodate, iodine, metals, reduction

Iodine is a biologically active element commonly found in freshwater and marine environments in the forms of iodide (I^-; −1 oxidation state) and iodate (IO_3^-; +5 oxidation state) (1). IO_3^- is more thermodynamically stable than I^-, yet I^- is the predominant form in the environment, potentially indicating that microbial IO_3^-

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reduction is a major component of the iodine biogeochemical reaction network (1–3). In marine environments, microbial IO_3^- reduction is the primary mechanism for IO_3^- reduction to I^- (4–8). Microbial IO_3^- reduction has also attracted interest as a component of alternative strategies for remediation of waters and sediments contaminated with radioactive iodine released to the environment (9). The nuclear waste product and radioactive isotope ^{129}I is produced during uranium and plutonium fission reactions and displays a half-life of 1.6×10^7 years (10). Following the 2011 Fukushima nuclear reactor catastrophe, westerly winds deposited a large portion of the radioactive iodine in the Pacific Ocean, where radioactive IO_3^- and I^- were the predominant ^{129}I forms (11–13). Radioactive iodine is also found in contaminated groundwater at the U.S. Department of Energy Savannah River and Hanford sites (9, 13, 14). Despite the human health concerns surrounding the fate and transport of radioactive iodine in the environment, the molecular mechanism of microbial IO_3^- reduction remains poorly understood (15).

IO_3^--reducing microorganisms include the facultative anaerobe *Shewanella oneidensis*, which reduces a wide range of terminal electron acceptors, including oxidized forms of iron, manganese, nitrogen, sulfur, uranium, plutonium, technetium, and iodine (16–18). *S. oneidensis* also transfers electrons to a variety of extracellular electron acceptors, including Fe(III), Mn(III), and Mn(IV) oxides (19, 20). To transfer electrons to external Fe(III) oxides, *S. oneidensis* employs a variety of novel respiratory strategies, including (i) direct enzymatic reduction via decaheme *c*-type cytochromes associated with the extracellular electron conduit (EEC) located on the surface or surface extensions of the *S. oneidensis* outer membrane (21–23), (ii) extracellular electron transfer via endogenous or exogenous electron shuttling compounds (24–26), and (iii) nonreductive Fe(III) solubilization by organic ligands to produce more readily reducible soluble organic Fe(III) complexes (27–29).

Previous findings for other IO_3^--reducing microorganisms indicated that nitrate (NO_3^-) reductase may catalyze the reduction of IO_3^- as an alternative electron acceptor (30–32). However, neither assimilatory nor dissimilatory NO_3^- reductases are required for IO_3^- reduction by *S. oneidensis* (33). The molecular mechanism of IO_3^- reduction by *S. oneidensis* has yet to be examined. The electron transport pathways of *S. oneidensis* consist of upstream dehydrogenases linked via the menaquinone pool and the inner membrane-tethered *c*-type cytochrome CymA to downstream terminal reductase complexes, including the metal-reducing EEC (19, 34, 35). The *S. oneidensis* EEC is comprised of outer membrane β-barrel protein MtrB (and essential cysteine residue C42) (36) and decaheme *c*-type cytochromes MtrA and MtrC (34, 37–40). MtrC is translocated to the outside face of the outer membrane through GspD, the outer membrane secretin of the type II protein secretion system (21, 41, 42). Other proteins essential for electron transport to external metal oxides include the *c*-type cytochrome maturation permease CcmB (43) and the cAMP receptor protein (CRP), required for anaerobic respiratory gene expression in *S. oneidensis* (44).

Although NO_3^- reductase is not required for IO_3^- reduction by *S. oneidensis*, identification of metal reduction and protein secretion genes involved in this process will aid in development of biomarkers to examine the potential for microbial IO_3^- reduction, a prominent process in iodine cycling in natural and contaminated environments such as the ^{129}I-contaminated Hanford and Savannah River sites. Likewise, such biomarkers could be used to track ^{129}I cycling in a pump-and-treat system currently treating contaminated groundwater at Hanford. Formation of I^- could lead to increased adsorption onto organic material, such as granular activated carbon (GAC) in a fluidized bed reactor (FBR) that is part of the pump-and-treat process, or by organic matter in environmental systems such as those found at Savannah River. The main objective of the present study was to test the hypothesis that the *S. oneidensis* metal reduction and protein secretion pathways required for Fe(III), Mn(III), and Mn(IV) oxide reduction are also involved in IO_3^- reduction. The experimental strategy to test the hypothesis included (i) construction of additional *S. oneidensis* gene deletion mutants lacking metal reduction and protein secretion pathway components and (ii) tests of the

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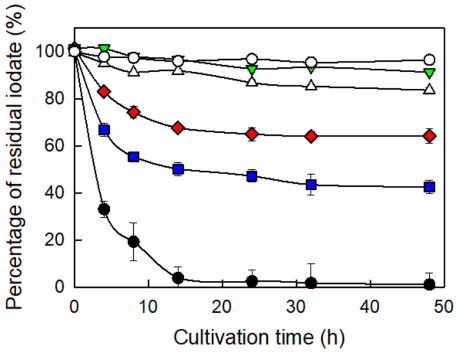

FIG 1 Effect of IO_3^- concentration on IO_3^- reduction activity of *S. oneidensis* MR-1. IO_3^- reduction was performed in M1 medium amended with 20 mM lactate and 250 μM IO_3^-, ranging from 0.1 to 2 mM iodate at room temperature and 300 rpm. Values are means of triplicate samples from anaerobic incubations. Error bars represent SDs. Some error bars cannot be seen due to small SDs. Symbols: ●, 0.10 mM; ■, 0.25 mM; ♦, 0.50 mM; ▲, 1.00 mM; ▼, 1.50 mM; ○, 2.00 mM.

battery of metal reduction and protein secretion pathway mutants for IO_3^- reduction activity.

RESULTS

Effect of electron donor on IO_3^- reduction activity by the *S. oneidensis* wild-type strain. A set of anaerobic incubations with batch cultures of the *S. oneidensis* wild-type strain was carried out to determine the optimum IO_3^- concentration that avoided IO_3^- or produced I^- toxicity and maximized the IO_3^- reduction activity of the *S. oneidensis* wild-type strain at cell densities of 10^8 ml^{-1}. IO_3^- concentrations of >500 μM inhibited IO_3^- reduction activity, while 250 μM was the optimum initial IO_3^- concentration (Fig. 1). The IO_3^- reduction activities of the *S. oneidensis* wild-type strain with lactate and formate as electron donors were similar (512 and 455 nmol h^{-1} mg of protein^{-1}, respectively), while the IO_3^- reduction activity with H_2 as the electron donor was approximately 4-fold lower (120 nmol h^{-1} mg of protein^{-1}) (Table 1). The extents of reaction (of the initial 250 μM IO_3^- starting concentration) for IO_3^- reduction by the *S. oneidensis* wild-type strain with lactate and formate as electron donors (60% and 55%, respectively) were approximately 4-fold greater than the extent of reaction with H_2 as the electron donor (16%) (Table 1).

IO_3^- reduction activity of *S. oneidensis* EEC mutant strains. The IO_3^- reduction activity of the *S. oneidensis* EEC mutant strains was determined with either lactate, formate, or H_2 as the electron donor. The Δ*mtrB* and *mtrB*-C42A site-directed mutant strains were severely impaired in IO_3^- reduction activity with lactate as the electron donor (3% and 7% of the wild-type rate, respectively, and 2% and 7% of the wild-type extent of reaction, respectively). In contrast, the Δ*mtrB* mutant strain retained wild-type IO_3^- reduction activity with formate or H_2 as the electron donor (107% and 145% of the wild-type rate, respectively) (Fig. 2; Table 1).

The IO_3^- reduction activity of the Δ*mtrB* mutant strain was restored to wild-type rates by providing a wild-type copy of *mtrB* in *trans*. With lactate as the electron donor, the Δ*mtrB*/*mtrB* transconjugant strain reduced IO_3^- at a rate almost 2-fold higher (179% of the wild-type rate) than that of the wild-type strain and displayed an extent of reaction approximately 143% of that of the wild-type strain (Fig. 2B; Table 1). In contrast, the *mtrB*-C45A site-directed mutant reduced IO_3^- at near wild-type rates (96% of the wild-type rate) and displayed a near wild-type extent of reaction (102% of the wild-type extent of reaction). The Δ*mtrA* mutant was also severely impaired in IO_3^- reduction activity with lactate as the electron donor (20% of the wild-type rate) and displayed a significantly lower extent of reaction (16% of the wild-type extent of reaction) (Fig. 2A; Table 1). However, with formate as the electron donor, the Δ*mtrA* mutant reduced IO_3^- at near wild-type rates (82% of the wild-type rate) and displayed a near wild-type extent of reaction (79% of the

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TABLE 1 IO_3^- reduction activity of wild-type and mutant strains of *S. oneidensis* with lactate, formate, and H_2 as electron donors[a]

Condition or strain	Lactate		Formate		H_2	
	IO_3^- reduction rate[b] (nmol h^{-1} mg of protein^{-1})[d]	Extent of reaction[c] (% of IO_3^- reduced to I^-)[d]	IO_3^- reduction rate[b] (nmol h^{-1} mg of protein^{-1})[d]	Extent of reaction[c] (% of IO_3^- reduced to I^-)[d]	IO_3^- reduction rate[b] (nmol h^{-1} mg of protein^{-1})[d]	Extent of reaction[c] (% of IO_3^- reduced to I^-)[d]
Abiotic	0 ± 0 (0)	0 ± 0 (0)	0 ± 0 (0)	0 ± 0 (0)	0 ± 0 (0)	0 ± 0 (0)
MR-1	512.0 ± 19.9 (100)	59 ± 1 (100)	454.7 ± 25.7 (10)	55 ± 3 (100)	119.9 ± 48.3 (100)	16 ± 3 (100)
Δ*crp* mutant	77.5 ± 9.4 (15)	7 ± 0 (11)	122.1 ± 10.3 (27)	15 ± 1 (28)	45.2 ± 14.4 (38)	7 ± 4 (46)
Δ*crp/crp* mutant	274.9 (54)	50 (85)	ND	ND	ND	ND
Δ*mtrA* mutant	100.7 ± 14.1 (20)	9 ± 1 (16)	373 ± 9.2 (82)	43 ± 0 (79)	254.3 ± 41.1 (212)	32 ± 3 (197)
Δ*mtrA/mtrA* mutant	839.8 (164)	83 (141)	ND	ND	ND	ND
Δ*mtrB* mutant	14.6 ± 11 (3)	1 ± 0 (2)	488.1 ± 16.4 (107)	59 ± 3 (108)	174.4 ± 16.4 (145)	22 ± 0 (134)
Δ*mtrB/mtrB* mutant	916.2 (179)	85 (143)	ND	ND	ND	ND
Δ*mtrC* mutant	389.7 ± 29.3 (76)	49 ± 2 (82)	566.5 ± 20.5 (125)	62 ± 2 (113)	170.8 ± 5.1 (142)	24 ± 3 (147)
Δ*omcA* mutant	351.6 ± 19.9 (69)	44 ± 4 (75)	552.3 ± 0.5 (121)	63 ± 3 (116)	104.0 ± 7.2 (87)	16 ± 1 (98)
Δ*mtrC* Δ*omcA* mutant	565.3 ± 97.2 (110)	66 ± 15 (112)	644.9 ± 85.2 (142)	71 ± 3 (130)	171.1 ± 12.3 (139)	18 ± 3 (112)
Δ*cymA* mutant	52.7 ± 9.4 (10)	2 ± 7 (3)	114.1 ± 7.2 (25)	14 ± 1 (25)	67.7 ± 25.7 (56)	5 ± 0 (28)
Δ*cymA/cymA* mutant	407.1 (80)	58 (98)	ND	ND	ND	ND
Δ*ccmB* mutant	51.9 ± 10.5 (10)	5 ± 2 (8)	102.2 ± 20 (22)	11 ± 2 (20)	65.5 ± 4.1 (55)	3 ± 3 (16)
Δ*ccmB/ccmB* mutant	435.5 (85)	60 (101)	ND	ND	ND	ND
Δ*tolC* mutant	494.1 ± 22.3 (97)	62 ± 2 (104)	435.1 ± 21.6 (96)	50 ± 0 (92)	140.3 ± 27.7 (117)	16 ± 0 (101)
Δ*gspD* mutant	155.2 ± 68.5 (30)	16 ± 0 (27)	203.4 ± 4.1 (45)	22 ± 2 (40)	66.9 ± 37 (56)	9 ± 2 (55)
Δ*SO3800* mutant	581.0 ± 9.4 (113)	64 ± 2 (109)	586.8 ± 26.7 (129)	65 ± 1 (118)	166.4 ± 42.1 (139)	20 ± 3 (124)
mtrB-C42A mutant	37 ± 71.4 (7)	4 ± 7 (7)	ND	ND	ND	ND
mtrB-C45A mutant	489.9 ± 23.4 (96)	60 ± 3 (102)	ND	ND	ND	ND

[a]Values represent means of triplicate samples; errors represent 1 SD. ND, not determined.
[b]Reaction rate was calculated from the first 4-h anaerobic incubation (lactate and formate) or 8-h incubation (H_2) values.
[c]Extent of reaction is reported as the percentage of IO_3^- reduced to I^- upon completion of the 24-h incubation period, after which further IO_3^- reduction was minimal.
[d]The values in parentheses are in comparison with the wild-type rates (percent) within each set of lactate, formate, or H_2 values.

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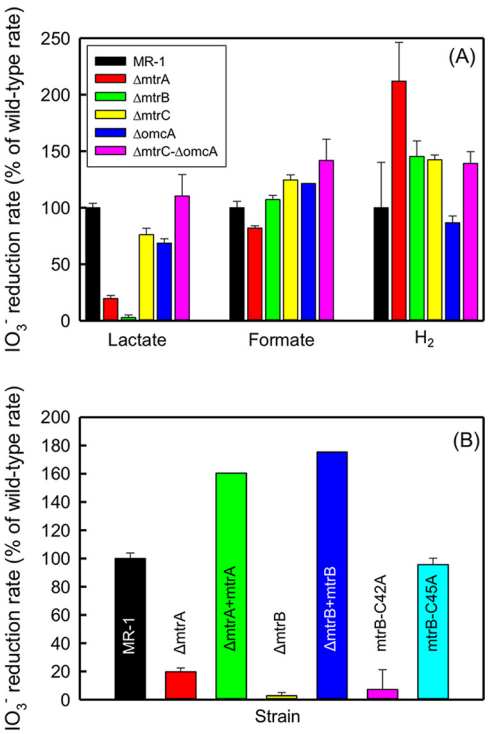

FIG 2 IO_3^- reduction activity of *S. oneidensis* wild-type (MR-1) and EEC mutant strains with IO_3^- as the electron acceptor and lactate, formate, or H_2 as the electron donor and *mtrB*-CxxC motif mutants and complemented strains of Δ*mtrA* and Δ*mtrB* with pBBR*mtrA* and pBBR*mtrB*, respectively (A), and with IO_3^- as the electron acceptor and lactate as the electron donor (mutant strains normalized to wild-type levels) (B). Values are means from triplicate samples from anaerobic incubations. Error bars represent SDs. Some error bars cannot be seen due to small SDs.

wild-type extent of reaction). With H_2 as the electron donor, the Δ*mtrA* mutant reduced IO_3^- at rates over 2-fold higher that of the wild-type strain (212% of the wild-type rate) and displayed an extent of reaction almost 2-fold higher than that of the wild-type strain (197% of the wild-type extent of reaction). With lactate as the electron donor, the Δ*mtrA*/*mtrA* transconjugant strain reduced IO_3^- at a rate nearly 2-fold greater than that of the wild-type strain (164% of the wild-type rate) and displayed a higher extent of reaction (141% of the wild-type extent of reaction) (Fig. 2B; Table 1).

Conversely, with lactate as the electron donor, the Δ*mtrC*, Δ*omcA*, and Δ*mtrC* Δ*omcA* EEC mutant strains reduced IO_3^- at near wild-type rates with lactate (76%, 69%, and 110% of the wild-type rate, respectively) and displayed near wild-type extents of reaction (82%, 75%, and 112% of the wild-type extent of reaction) (Fig. 2A; Table 1). In a similar fashion, with formate as the electron donor, the Δ*mtrC*, Δ*omcA*, and Δ*mtrC* Δ*omcA* mutant strains reduced IO_3^- at near wild-type rates (125%, 121%, and 142% of the wild-type rate, respectively) and displayed near wild-type extents of reaction (113%, 116%, and 130% of the wild-type extent of reaction, respectively). With H_2 as the electron donor, the Δ*mtrC*, Δ*omcA*, and Δ*mtrC* Δ*omcA* mutant strains also reduced IO_3^- at near wild-type rates (142%, 87%, and 139% of the wild-type rate, respectively) and displayed near wild-type extents of reaction (147%, 98%, and 112% of the wild-type extent of reaction, respectively) (Fig. 2A; Table 1).

IO_3^- reduction activity of additional *S. oneidensis* *c*-type cytochrome and *crp* mutants. With lactate as the electron donor, Δ*cymA* and Δ*ccmB* *c*-type cytochrome mutant strains were also severely impaired in IO_3^- reduction activity (both 10% of the wild-type rate) and displayed significantly lower extents of reaction (3% and 8% of the wild-type extent of reaction, respectively) (Fig. 3A; Table 1). With formate as the electron donor, the mutant strains were also impaired in IO_3^- reduction activity (25%

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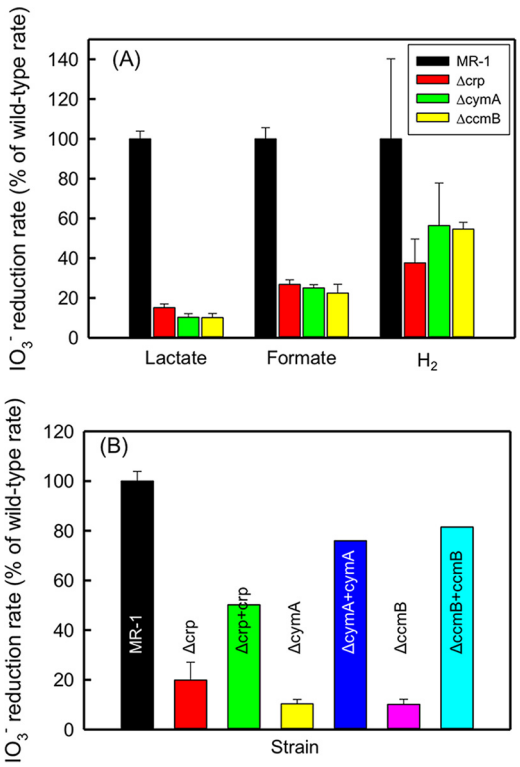

FIG 3 IO_3^- reduction activity of *S. oneidensis* wild-type (MR-1) and *c*-type cytochrome and *crp* mutants with IO_3^- as the electron acceptor and lactate, formate, or H_2 as the electron donor and their complemented strains with pBBR*cymA*, pBBR*ccmB*, and pBBR*crp*, respectively (A), and with IO_3^- as the electron acceptor and lactate as the electron donor (mutant strains normalized to wild-type levels) (B). Values are means of triplicate samples from anaerobic incubations. Error bars represent SDs. Some error bars cannot be seen due to small SDs.

and 22% of the wild-type rate, respectively) and displayed significantly lower extents of reaction (25% and 20% of the wild-type extent of reaction, respectively). With H_2 as the electron donor, the Δ*cymA* and Δ*ccmB* mutant strains were also impaired in IO_3^- reduction activity (56% and 55% of the wild-type rate, respectively) and displayed significantly lower extents of reaction (28% and 16% of the wild-type extent of reaction, respectively). The Δ*cymA/cymA* and Δ*ccmB/ccmB* transconjugant strains recovered near wild-type rates of IO_3^- reduction (80% and 85% of the wild-type rate, respectively), and displayed near wild-type extents of reaction (98% and 101% of the wild-type extent of reaction, respectively) with lactate as the electron donor (Fig. 3B; Table 1). With lactate, formate, and H_2 as electron donors, the Δ*crp* mutant strain was severely impaired in IO_3^- reduction activities (15%, 27%, and 38% of the wild-type rate, respectively) and displayed significantly lower extents of reaction (11%, 28%, and 46% of wild-type extent of reaction, respectively) (Fig. 3A; Table 1), while the IO_3^- reduction activity of the Δ*crp/crp* transconjugant strain was partially restored to wild-type rates with lactate as the electron donor (54% of the wild-type rate and 85% of wild-type extent of reaction) (Fig. 3B; Table 1). The IO_3^- reduction activities of the transconjugant strains with formate or H_2 as the electron donor were not determined.

IO_3^- reduction activity of *S. oneidensis* type I, II, and V protein secretion mutants. With lactate as the electron donor, the type I (Δ*tolC*) and the type V (Δ*SO3800*) protein secretion mutants reduced IO_3^- at near wild-type rates (97% and 113% of the wild-type rate, respectively) and displayed near wild-type extents of reaction (104% and 109% of the wild-type extent of reaction, respectively) (Fig. 4; Table 1). With formate as the electron donor, the mutants similarly reduced IO_3^- at near wild-type rates (96% and 129% of the wild-type rate, respectively) and displayed near wild-type extents of reaction (118% and 92% of the wild-type extent of reaction, respectively). With H_2 as

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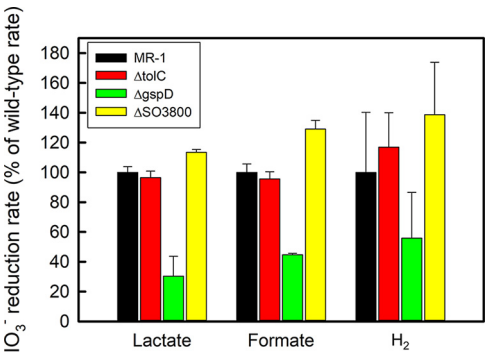

FIG 4 IO_3^- reduction activity of *S. oneidensis* wild-type (MR-1) and Δ*tolC*, Δ*gspD*, and Δ*SO3800* protein secretion mutants with IO_3^- as the electron acceptor and lactate, formate, or H_2 as the electron donor (mutant strains normalized to wild-type levels). Values are means of triplicate samples from anaerobic incubations. Error bars represent SDs.

the electron donor, the type I and type V protein secretion mutants reduced IO_3^- at near wild-type rates (117% and 139% of the wild-type rate, respectively) (Fig. 4; Table 1) and displayed near wild-type extents of reaction (101% and 124% of the wild-type extent of reaction, respectively). However, with lactate, formate, and H_2 as electron donors, the Δ*gspD* type II protein secretion mutant was severely to partially impaired in IO_3^- reduction activity (30%, 45%, and 56% of the wild-type rate, respectively) and displayed significantly lower extents of reaction with all three electron donors (27%, 40%, and 55% of the wild-type extent of reaction, respectively).

DISCUSSION

The molecular mechanism of microbial IO_3^- reduction is poorly understood. Under NO_3^--reducing anaerobic conditions, microorganisms, including the phytoplankton *Navicula* and the bacteria *Pseudomonas* sp. strain SCT, *Agrobacterium*-related strain DVZ35, and *Escherichia coli*, reduce IO_3^- to I^-, which led to the hypothesis that NO_3^- reductase reduces IO_3^- as an alternative terminal electron acceptor (6, 7, 30, 32, 45, 46). The IO_3^--reducing NO_3^- reductase hypothesis was recently brought into question, however, by findings with *S. oneidensis*, which demonstrated that NO_3^- and IO_3^- reduction activities were not inhibited by the presence of saturating levels of the competing electron acceptor and that NO_3^- reductase-deficient deletion mutants retained wild-type IO_3^- reduction activity (33).

S. oneidensis also reduces external metal oxides via EEC-mediated electron transfer either at the outside face of the outer membrane or via outer membrane extensions (i.e., nanowires) (19, 35, 47). The *S. oneidensis* EEC (MtrCAB) is composed of outer membrane β-barrel protein MtrB, which forms a ternary complex with decaheme *c*-type cytochromes MtrC and MtrA (48, 49). *S. oneidensis* mutants lacking MtrCAB display Fe(III), Mn(III), and Mn(IV) reduction-deficient phenotypes (39, 47, 50, 51). In addition, methyl viologen-reduced proteoliposomes containing only the *S. oneidensis* MtrCAB complex transfer electrons to external soluble and solid Fe(III) substrates (20, 52, 53). In the present study, Δ*mtrA* and Δ*mtrB* deletion mutants were severely impaired in IO_3^- reduction activity with lactate as the electron donor, indicating that MtrA and MtrB also function as critical components of the lactate-dependent IO_3^- reduction pathway. However, IO_3^- was reduced at wild-type rates with formate or H_2 as the electron donor, indicating that the electron transport pathway components required for IO_3^- reduction are electron donor dependent.

In contrast to the lactate-dependent, IO_3^- reduction-deficient phenotypes displayed by the Δ*mtrA* and Δ*mtrB* mutant strains, the Δ*mtrC* mutant strain retained wild-type IO_3^- reduction activity regardless of electron donor, indicating that MtrC is not required for IO_3^- reduction by *S. oneidensis*. These findings differed from those of previous studies which demonstrated that MtrC is required for Fe(III), Mn(IV), Mn(III),

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Tc(VII), U(VI), and flavin reduction by *S. oneidensis* (28, 39, 54–57). In addition, previous studies demonstrated that OmcA was required for Fe(III), Mn(IV), and Mn(III) reduction (39). In the present study, the Δ*omcA* and Δ*omcA* Δ*mtrC* double mutant strains retained wild-type IO_3^- reduction activity with each electron donor, thus indicating that neither OmcA nor MtrC is required for electron transport to IO_3^-.

Previous studies with the *mtrB*-C42A and *mtrB*-C45A site-directed mutants demonstrated that cysteine at MtrB amino acid position 42 (but not at position 45) was required for Fe(III), Mn(IV), and Mn(III) reduction by *S. oneidensis* (36, 39). In a similar fashion, results of the present study demonstrated that the*mtrB*-C42A mutant was severely impaired in IO_3^- reduction activity, while the *mtrB*-C45A mutant reduced IO_3^- at wild-type rates. Residues C42 and C45 comprise a conserved CXXC motif in MtrB homologs of metal-reducing gammaproteobacteria (36). The biochemical function of the CXXC motif of *S. oneidensis* MtrB is currently unknown but may involve MtrB maturation via disulfide bond formation or metal cofactor binding (36, 58, 59). The detection of a CXXC motif in MtrB homologs of gammaproteobacteria is diagnostic for microbial Fe(III) reduction (36). Future IO_3^- reduction activity assays will be required to determine if the CXXC motif of MtrB homologs in gammaproteobacteria is also diagnostic for microbial IO_3^- reduction. Such information will guide interpretation of *in situ* meta(omic) signals indicative of microbial IO_3^- reduction in natural and contaminated environments such as the ^{129}I-contaminated Hanford and Savannah River sites. These types of molecular signatures will be important for monitoring the ^{129}I cycling in sites like Hanford, which may affect overall mobility of ^{129}I in the oligotrophic aquifer. Diagnostic markers may also be used to monitor conversion of $^{129}IO_3^-$ in FBRs currently treating contaminated groundwater at Hanford. Conversion of $^{129}IO_3^-$ to $^{129}I^-$ facilitates uptake of ^{129}I on biofilm or GAC in FBRs.

S. oneidensis CcmB functions as the integral membrane component of the cytochrome *c* maturation complex (43). The Δ*ccmB* mutant strain was severely impaired in IO_3^- reduction activity regardless of electron donor, which indicates that the *c*-type cytochrome pool is involved in anaerobic electron transport to IO_3^-. Correspondingly, the Δ*cymA* mutant strain was also severely impaired in IO_3^- reduction activity regardless of electron donor. Inner membrane-tethered tetraheme *c*-type cytochrome CymA functions as the central branch point in anaerobic electron transport by *S. oneidensis* (60, 61). CymA accepts electrons from the menaquinone pool for subsequent delivery to a variety of periplasmic or outer membrane localized terminal reductase complexes, including MtrA of the *S. oneidensis* EEC (61, 62). The IO_3^- reduction-deficient phenotype of the Δ*cymA* strain indicates that the electron transport chain to IO_3^- also includes CymA. The Δ*crp* mutant strain (lacking the cyclic AMP receptor protein) was also severely impaired in IO_3^- reduction activity. CRP regulates expression of genes required for anaerobic respiration by *S. oneidensis* (44). The IO_3^- reduction-deficient phenotype displayed by Δ*crp* indicates that IO_3^- reduction gene expression is also regulated by CRP/cAMP levels in *S. oneidensis*.

The *S. oneidensis* genome encodes type I, II, and V protein secretion systems (39, 41, 42, 63). Δ*tolC* and Δ*SO3800* deletion mutants retained wild-type IO_3^- reduction activity regardless of electron donor, thus indicating that IO_3^- reduction requires neither TolC-mediated type I protein secretion (for efflux of antibiotics, heavy metals, or toxic proteins) (64) nor SO3800-mediated type V protein secretion (an autotransporter-like serine protease involved in *S. oneidensis* adhesion to Fe(III) oxide surfaces [63]). In contrast, the IO_3^- reduction-deficient phenotype displayed by the mutant with a deletion of *gspD* (encoding GspD, the outer membrane secretin of type II protein secretion) indicates that IO_3^- reduction is linked to type II protein secretion in a manner similar to that of Fe(III), Mn(IV), and Mn(III) reduction. The type II protein secretion system is required for outer membrane localization of MtrC and OmcA (21, 65), and impairment of type II protein secretion results in mislocalization of MtrC and OmcA, with corresponding Fe(III), Mn(IV), and Mn(III) reduction-deficient phenotypes. The Δ*mtrC*, Δ*omcA*, and Δ*mtrC* Δ*omcA* mutant strains retained wild-type IO_3^- reduction activity. These findings demonstrate that IO_3^- reduction by *S. oneidensis* does not

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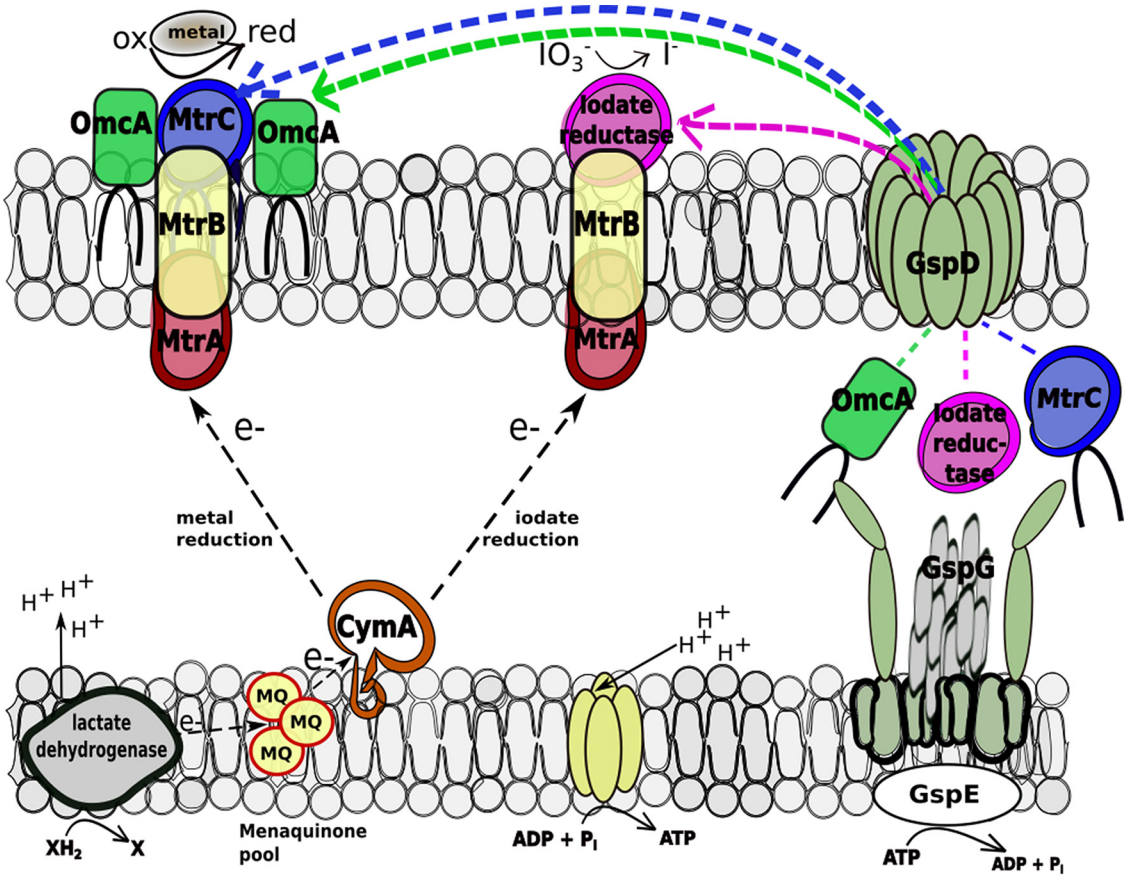

FIG 5 Working model of the lactate (MtrAB)-dependent IO_3^- reduction electron transport pathway in *S. oneidensis*, including a comparison with the MtrAB-dependent metal reduction pathway. In both the metal and IO_3^- reduction pathways, electrons originating from lactate dehydrogenase located at the head end of the electron transport chain are transferred to the inner membrane-localized menaquinone pool and subsequently to CymA, which facilitates electron transfer across the periplasmic space to decaheme cytochrome MtrA. At this location in the electron transport chain, the metal and IO_3^- reduction pathways diverge and terminate with either metal-reducing *c*-type cytochrome MtrC or an unknown terminal IO_3^- reductase, both of which associate with MtrA and β-barrel protein MtrB. MtrC and the unknown IO_3^- reductase are both secreted extracellularly by the type II protein secretion system to form a ternary complex with the MtrAB module on the outside face of the outer membrane. The formate- and H_2-dependent IO_3^- reduction pathways are MtrAB-independent and thus are not depicted in this working model.

require either of the EEC cytochromes MtrC and OmcA but does require type II protein secretion of an as-yet-unidentified IO_3^- reductase to the outside face of the outer membrane. In the current model of the lactate (MtrAB)-dependent *S. oneidensis* IO_3^- reduction system (Fig. 5), electrons originating from lactate dehydrogenase are transported via the menaquinone pool, CymA, and MtrAB to the terminal IO_3^- reductase that is translocated to the outside face of the outer membrane via type II protein secretion. Current work is focused on identification of the *S. oneidensis* IO_3^- reductase via comparison of the IO_3^--reducing protein fractions harvested from the outside face of the outer membrane of *S. oneidensis* wild-type and Δ*gspD* mutant strains.

MATERIALS AND METHODS

Growth and cultivation conditions. *S. oneidensis* strains were routinely cultured aerobically at 30°C in lysogeny broth (LB) (10 g liter^{-1} of NaCl, 10 g liter^{-1} of tryptone, 5 g liter^{-1} of yeast extract). IO_3^- reduction rate experiments were conducted under anaerobic conditions in M1 minimal medium (66) amended with 20 mM lactate, 10 mM formate, or 2% H_2 gas as the electron donor and 250 μM IO_3^- as the anaerobic electron acceptor. When required for selection, gentamicin (20 μg ml^{-1}) was amended to the appropriate growth medium.

In-frame deletion mutagenesis of *S. oneidensis* genes. The genes *crp* and *ccmB* were deleted in frame from the *S. oneidensis* MR-1 genome following previously described procedures (67). Regions corresponding to ~750 bp upstream and downstream of *crp* and *ccmB* were PCR amplified with iProof ultrahigh-fidelity polymerase (Bio-Rad, Hercules, CA) (primers D1/D2 and D3/D4 [Table 2]) and subse-

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TABLE 2 Primers used in this study

Primer	Sequence (5′–3′)	Remark
Δ*crp*		
D1	CTGATAGGATCCTCTTTATACCAACGTTCGGCC	BamHI (underlined)
D2	GGCTTAAATCAAGCTGAAGTCTAACTGTCGATGTTCCTCGATTGATTAA	
D3	TTAATCAATCGAGGAACATCGACAGTTAGACTTCAGCTTGATTTAAGCC	
D4	TCGATCGTCGACAGTGCCTGAATTCGCGCTA	SalI (underlined)
TF	GCGTAAATAAAACCTAAACGGAACT	
TR	TAGCTAAGTTGCTTGTTGGGATT	
Δ*ccmB*		
D1	CTGATAACTAGTACATCTAGTCCTAAGCAATTGTAAACC	SpeI (underlined)
D2	GGGTGTAACCATTTCCACATTT TATGCCAAACCTTATACGAAGC	
D3	GCTTCGTATAAGGTTTGGCATA AAATGTGGAAATGGTTACACCC	
D4	TCGATCGTCGACTTGCATTATTTGACCTCCTCAG	SalI (underlined)
TF	TCGATTTGTACGAGAAATATTGC	
TR	TAAAAGGCATAGCCACCCAT	

quently joined using overlap extension PCR (primers D1/D4 [Table 2]). The resulting fragment was cloned into suicide vector pKO2.0, which does not replicate in *S. oneidensis*, and mobilized into wild-type *S. oneidensis* MR-1 via conjugation with *E. coli* donor strain β2155 λ *pir* (Table 3) (68). *S. oneidensis* strains with the integrated plasmid were selected on LB agar containing gentamicin (15 μg ml^{-1}). Single-crossover integrations were verified using PCR with primers flanking the recombination region (TF/TR) and were resolved from the genomes by plating on LB agar lacking NaCl and containing sucrose (10% [wt/vol]). The in-frame deletion strains (Δ*crp* and Δ*ccmB*) were verified by PCR with primers TF/TR (Table 2). Genetic complementation analysis of Δ*mtrA*, Δ*mtrB*, Δ*crp*, Δ*cymA*, and Δ*ccmB* strains was carried out by cloning the wild-type gene into broad-host-range cloning vector pBBR1MCS (69) and conjugally transferring the recombinant vector into the respective mutant strains via biparental mating procedures (67).

Anaerobic incubation conditions. Mutant strains were initially inoculated in liquid LB growth medium and incubated at 30°C for 24 h. Ten-milliliter subcultures at an initial optical density at 600 nm (OD_{600}) of 0.02 were incubated at 30°C for 24 h. Subcultures were centrifuged at 4,000 rpm for 30 min,

TABLE 3 Strains used in this study

Strain or plasmid	Features	Source or reference
S. oneidensis		
MR-1	Wild-type strain	ATCC
Δ*crp* mutant	In-frame *crp* deletion mutant	This study
Δ*ccmB* mutant	In-frame *ccmB* deletion mutant	This study
Δ*cymA* mutant	In-frame *cymA* deletion mutant	39
Δ*mtrA* mutant	In-frame *mtrA* deletion mutant	39
Δ*mtrB* mutant	In-frame *mtrB* deletion mutant	36
Δ*mtrC* mutant	In-frame *mtrC* deletion mutant	39
Δ*omcA* mutant	In-frame *omcA* deletion mutant	39
Δ*mtrC* Δ*omcA* mutant	In-frame *mtrC* and *omcA* double deletion mutant	39
Δ*tolC* mutant	In-frame *tolC* deletion mutant	39
Δ*gspD* mutant	In-frame *gspD* deletion mutant	39
Δ*SO3800* mutant	In-frame *SO3800* deletion mutant	63
mtrB-C42A mutant	Site-directed mutant with cysteine at amino acid position 42 replaced with alanine	36
mtrB-C45A mutant	Site-directed mutant with cysteine at amino acid position 45 replaced with alanine	36
E. coli		
E100D *pir-116*	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80d*lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara, leu*)7697 *galU galK* λ^- *rpsL* (Str^r) *nupG pir-116*(DHFR)	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	71
Plasmids		
pKO2.0	In-frame gene deletion vector; 4.5 kb γR6K, *mobRP4 sacB* Gm^r *lacZ*	63
pBBR1MCS	Broad-host-range cloning vector; Cm^r *lacZ*	69
pBBRmtrA	pBBR1MCS containing wild-type *mtrA*	39
pBBRmtrB	pBBR1MCS containing wild-type *mtrB*	39
pBBRcymA	pBBR1MCS containing wild-type *cymA*	This study
pBBRccmB	pBBR1MCS containing wild-type *ccmB*	This study
pBBRcrp	pBBR1MCS containing wild-type *crp*	This study

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resuspended in 10 ml of M1 growth medium amended with 20 mM lactate, and incubated aerobically at room temperature for 8 h. The preconditioned cells were inoculated in 30-ml serum bottles at an initial OD_{600} of 0.1 in M1 growth medium amended with 250 μM IO_3^- and either 20 mM lactate or 10 mM formate and incubated anaerobically via continuous sparging with 100% high-purity (hydrated) N_2 gas. For IO_3^- reduction activity assays with H_2 as the electron donor, the preconditioned cells were incubated anaerobically via continuous sparging with high-purity (hydrated) anaerobic gas mix consisting of 2% H_2 and 98% N_2. Cultures were incubated at room temperature with gentle stirring under anaerobic conditions maintained by continuous sparging with high-purity hydrated N_2 gas. At preselected time points, OD_{600} was measured and IO_3^- concentrations were determined using the IO_3^--triiodide formation method described below.

Determination of IO_3^- concentrations via IO_3^--triiodide formation with I^- at acidic pH. The extent of IO_3^- reduction was determined using the IO_3^--triiodide method (33, 70). Culture samples were added to 96-well 500-μl microtiter plates. Sodium citrate buffer (0.1 M; pH 3.3) and potassium iodide solution (75 mM) were added to each well to initiate triiodide formation ($IO_3^- + 5I^- + 6H^+ \rightarrow 3H_2O + 3I_2$). Absorbance at 352 nm was measured with a UV spectrophotometer (Multiskan Go; Thermo Scientific) after a 4-min reaction time. IO_3^- concentrations were determined from a previously generated calibration curve.

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Y.J.T., J.K.M., and H.D.S. performed part of the experiments, developed part of the protocol, and cowrote the manuscript. M.H.L., B.D.L., and T.J.D. developed the concept and part of the protocol, coanalyzed all data, and cowrote the manuscript.

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