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Combined Genomics and Experimental Analyses of Respiratory Characteristics of *Shewanella putrefaciens* W3-18-1

Dongru Qiu,[a,b] Hehong Wei,[b] Qichao Tu,[a] Yunfeng Yang,[c] Ming Xie,[a] Jingrong Chen,[a] Mark H. Pinkerton, Jr.,[a] Yili Liang,[a] Zhili He,[a] Jizhong Zhou[a,d]

Institute for Environmental Genomics and Department of Microbiology and Plant Biology, University of Oklahoma, Norman, Oklahoma, USA[a]; Institute of Hydrobiology and Graduate University, Chinese Academy of Sciences, Wuhan, China[b]; State Key Joint Laboratory of Environment Simulation and Pollution Control, School of Environment, Tsinghua University, Beijing, China[c]; Earth Science Division, Lawrence Berkeley National Laboratory, Berkeley, California, USA[d]

It has previously been shown that the *Shewanella putrefaciens* W3-18-1 strain produces remarkably high current in microbial fuel cells (MFCs) and can form magnetite at 0°C. To explore the underlying mechanisms, we developed a genetic manipulation method by deleting the restriction-modification system genes of the SGI1 (*Salmonella* genome island 1)-like prophage and analyzed the key genes involved in bacterial respiration. W3-18-1 has less respiratory flexibility than the well-characterized *S. oneidensis* MR-1 strain, as it possesses fewer cytochrome *c* genes and lacks the ability to oxidize sulfite or reduce dimethyl sulfoxide (DMSO) and timethylamine oxide (TMAO). W3-18-1 lacks the hydrogen-producing Fe-only hydrogenase, and the hydrogen-oxidizing Ni-Fe hydrogenase genes were split into two separate clusters. Two periplasmic nitrate reductases (NapDAGHB and NapDABC) were functionally redundant in anaerobic growth of W3-18-1 with nitrate as the electron acceptor, though *napDABC* was not regulated by Crp. Moreover, nitrate respiration started earlier in W3-18-1 than in MR-1 (with NapDAGHB only) under microoxic conditions. These results indicate that *Shewanella putrefaciens* W3-18-1 is well adapted to habitats with higher oxygen levels. Taken together, the results of this study provide valuable insights into bacterial genome evolution.

Shewanella strains, most renowned for their dissimilatory metal reduction and potential applications in the bioremediation of heavy metal contamination, have frequently been isolated from redox-stratified environments. Some *Shewanella* strains, including the best-characterized *S. oneidensis* MR-1 strain, have been isolated from freshwater environments (1, 2). However, most of the sequenced *Shewanella* strains were isolated from marine environments and this genus was believed to have a marine origin (3). The dissimilatory metal reduction of *Shewanella* species has been intensively studied for the potential applications in bioremediation of radioactive waste of groundwater.

Shewanella putrefaciens is a facultative anaerobic Gram-negative psychrophile with relevance to fish spoilage, oil pipeline corrosion, and human infections (4). The type strain of *S. putrefaciens*, ATCC 8071, was isolated from butter in England during the 1930s. Other strains, W3-18-1, 200, and CN-32, were isolated from marine sediment, a Canadian oil pipeline, and anaerobic shale sandstone, respectively (4). *S. putrefaciens* strains originally composed a highly heterogeneous phylogenetic group and have been subjected to frequent reclassification (1, 5, 6). A number of novel species, including *S. algae*, *S. baltica*, *S. frigidimarina*, *S. pealeana*, and *S. oneidensis* (ATCC 700550^T [MR-1]), were initially classified as *S. putrefaciens* but renamed later. *S. putrefaciens* and *S. algae* are also human pathogens associated with septicemia, cellulitis, ear infection, cerebellar abscesses, leg ulcers, osteomyelitis, and arthritis (7).

To date, many *Shewanella* species have been sequenced, providing a unique opportunity for exploring bacterial genome evolution and adaptation to their specific habitats (4). *S. putrefaciens* W3-18-1 is particularly interesting because it achieves high current production in microbial fuel cells (8) and reduces metals and forms magnetite at 0°C (9, 10). Here we report an integrated genetics and physiological analysis of the respiration of W3-18-1 in order to provide insights into the underlying mechanisms. Our results indicate that the respiratory flexibility of *S. putrefaciens* W3-18-1 is lower than that of *S. oneidensis* MR-1 and that the strain is well adapted to its habitats with higher oxygen levels.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Shewanella putrefaciens* W3-18-1 and *S. putrefaciens* CN-32 were previously isolated from the deep marine sediments underlying the depth of 670 m below the sea level off the Washington state coast (9, 10) and from anaerobic shale sandstone at the depth of 250 m in the Morrison formation of Cerro Negro, New Mexico (4), respectively. Bacterial strains were usually cultured in Luria-Bertani broth (or plates) (supplemented with 15 and 50 μg/ml of gentamicin [Gm] and kanamycin when necessary) and modified M1 minimal medium (1, 11).

Genome sequencing, annotation, and bioinformatics analysis. The DNA sequencing, assembly, and annotation of the W3-18-1 genome were conducted by Joint Genome Institute (http://genome.jgi-psf.org/she_w/she_w.home.html) (Fig. 1). The orthologs are identified by using bidirectional BLASTP (best hits) for comparisons of W3-18-1 and other *Shewanella* strains and also on the basis of genome synteny. The paralog(s) of polypeptides was searched by BLAST against the same genome (>70% sequence similarity).

Electron donor and acceptor utilization assays. Biolog dye was used to monitor sulfite and hydrogen utilization in modified M1 minimal medium. Electron acceptor utilization was assayed in the modified M1 me-

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Address correspondence to Jizhong Zhou, jzhou@ou.edu.
D.Q. and H.W. contributed equally to this article.
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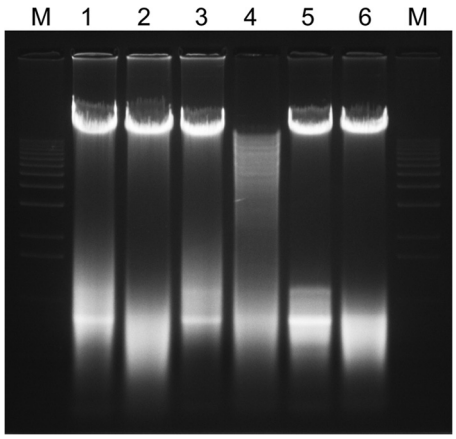

FIG 1 PtsI restriction digestion of the chromosomal DNAs of the wild-type (lane 6), Δ*pstI* (lane 2), and Δ*pstI pstM* (lane 4) strains. The DNAs were subjected to electrophoresis on the 1% (wt/vol) agarose gel. M, 1-kb DNA markers. Lanes 1, 3, and 5 are the undigested chromosomal DNAs as the controls.

dium supplemented with 50 mM sodium lactate as the electron donor and carbon source and the electron acceptors to be tested. The bacterial cultures were incubated under anaerobic or microoxic conditions (without shaking to limit aeration). Nitrate and the nitrite concentrations were measured by using a standard colorimetric method (12).

Genetic manipulation. The two-step protocol of selection (antibiotics resistance for the single crossover) and counterselection (sucrose sensitivity for the double crossover) was applied for in-frame deletion of a specific gene(s) by using suicide vector pDS3.0 (R6K replicon, *sacB*, Gm^r)-based constructs carrying a fusion of upstream and downstream sequences of target genes (see Tables S1 and S2 in the supplemental material) as previously described (13). The suicide vector was introduced into *Shewanella* by mating using *Escherichia coli* WM3064 as the donor strain.

RNA extraction and RT-PCR analysis of gene transcription. Total RNA was extracted by using RNAiso Plus (TaKaRa) and an RNAprep Pure Cell/Bacteria kit (Tiangen Biotech [Beijing] Co., Ltd.), and RNA was further purified using DNase I treatment. The integrity of RNA was evaluated by agarose (0.8%) gel electrophoresis. The RNA concentration and purity were measured on a spectrophotometer (Nanodrop Technologies, Wilmington, DE). To prepare cDNA, 2 μg of total RNA was reverse transcribed (RT) using a PrimeScript RT reagent kit with gDNA Eraser (TaKaRa) and a TIANscript RT kit (Tiangen Biotech [Beijing] Co., Ltd.) according to the manufacturer's protocol. The PCR thermal cycles were as follows: 5 min at 95°C for cDNA denaturation followed by 27 to 30 cycles of 40 s at 95°C, 40 s at 51°C, and 30 s at 72°C. A final elongation step was performed for 10 min at 72°C. RT-PCR products were separated on a 0.8% agarose gel containing ethidium bromide and visualized by UV light and Bio-Rad Image software. The data presented represent relative mRNA levels normalized to 16S rRNA transcript levels, and the value of the control was set to 1. All the experiments described were performed in triplicate to obtain means and standard deviations (SD). The PCR products were also sequenced to confirm amplification of target genes. The primers used are listed in Table S2 in the supplemental material.

RESULTS

Establishment of genetic manipulation in strain W3-18-1. *S. putrefaciens* W3-18-1 has a genome size of 4,708 kb and harbors a single circular chromosome with a predicted total of 4,237 open reading frames (ORFs). Among them, about 700 genes, many of which encode bacteriophages, lateral flagella, and bacterial microcompartments, are absent in the MR-1 genome (Table 1). Unlike MR-1 and CN-32, W3-18-1 is recalcitrant to genetic manipulations, which may be due to DNA restriction mediated by prophage-borne restriction-modification systems and CRISPR (clustered regularly interspaced short palindromic repeat) elements. Five prophage elements and a few degenerate phages are present in the W3-18-1 genome (Table 1). The phage (Sputw3181_4072-4090) shares many genes with SGI1 (*Salmonella* genome island 1) of pathogenic *Salmonella enterica* serovar Typhimurium DT120 (14). We noted that the SGI1 island of W3-18-1 encodes a PstI-like restriction-modification system. Consistently, its chromosomal DNA could not be digested by commercial PstI endonuclease (New England BioLabs), probably due to the methylation of recognition sites (Fig. 2). To facilitate genetic manipulation, we deleted the putative endonuclease (encoded by Sputw3181_4075) and DNA methylase (designated *pstM*; Sputw3181_4074) genes. The *pstM* deletion resulted in sucessful digestion of chromosomal DNA of strain W3-18-1ΔpstIΔpstM by PstI, confirming that PstM mediated methylation of DNA.

There are several transposons and insertion sequences in W3-18-1, most of which are present in multiple copies. The IS*4* family insertion sequences (sputw3181_1472, -3732, and -3852) and Tn*5* family transposons (sputw3181_1767, -1850, -2145, -2150, and -3364) are present in W3-18-1 but not other sequenced *Shewanella* strains. The transposon-disrupted genes include *ifcA* (sputw3181_3363), *rtxB* (sputw3181_3731), and *pilN* (sputw3181_3851). The *ifcA* gene encodes a Fe(III)-induced flavocytochrome c_3, while *rtxB* is part of an operon involved in biofilm and pellicle formation in MR-1 (SO_4317-4321) (15). The *pilN* gene encodes the pilus assembly protein for the type IV pili (*pilM* to -Q) involved in cell adhesion and twitching motility of bacteria (16). These genetic differences may have contributed to the phenotypic variations between W3-18-1 and CN-32 revealed by previous studies (8, 17).

Respiratory chains, *c*-type cytochromes, and respiratory flexibility. Based on the CXXCH motif and comparative genomic analysis, a total of 32 *c*-type cytochrome genes were identified in W3-18-1, while 42 and 55 are present in *S. oneidensis* MR-1 and *S. piezotolerans* WP3, respectively (18, 19, 20). W3-18-1 does not harbor unique *c*-type cytochrome genes that are absent in other sequenced *Shewanella* genomes. Both W3-18-1 and MR-1 could grow anaerobically with fumarate as the electron acceptor (21), but only the *S. putrefaciens* strains (W3-18-1 and CN-32) contain the four-subunit integral-membrane fumarate reductase complex genes *frdABCD* (Table 1 and Fig. 2) among the sequenced *Shewanella* strains. FrdABCD does not contain heme groups or contribute to the proton gradient (22). In addition, W3-18-1 lacks the gene clusters encoding sulfite hydrogenase SorAB, octaheme tetrathionate reductase Otr, timethylamine oxide (TMAO) reductase, dimethyl sulfoxide (DMSO) reductase, and the secondary metal reductase MtrDEF (see Tables S3 and S4 in the supplemental material). A number of in-frame deletion mutants have been generated in W3-18-1 and MR-1 for experimentally testing the cellular functions of *c*-type cytochromes and other respiratory genes as described below.

Nitrate reduction. Like several other *Shewanella* strains, W3-18-1 contains two periplasmic nitrate reductase operons, *nap*-α (*napDABC*) and *nap*-β (*napDAGHB*), and two nitrite reductase paralog (pentaheme cytochrome NrfA) genes (Table 1). In contrast, *S. oneidensis* MR-1 harbors only *napDAGHB* whereas the denitrifier *S. denitrificans* OS217 contains only *napDABC*. It has been proposed that *nap*-α is involved in denitrification and redox

TABLE 1 Specific genetic loci of *S. putrefaciens* W3-18-1 that are absent in *S. oneidensis* MR-1

Locus	Length (kb)	Gene(s)	Predicted function(s)
Sputw3181_0088-0096	10.4	Cytochrome *bo* oxidase genes *cyoABCDE*	Electron transfer and energy transduction
Sputw3181_0197-0204	9.1	Anion transporter, fumarase, and fumarate reductase genes *frdABCD*	Nutrient uptake and fumarate reduction
Sputw3181_0305-0330	27	Degenerate phage elements, type I restriction-modification system	
Sputw3181_0341-0367	27.8	Drugs efflux genes, metal resistance genes	Toxin and heavy metal resistance[a]
Sputw3181_0408-0445	82.4	Bacterial microcompartment operon, including vitamin B_{12}-independent diol dehydratase genes, and phosphotransferase system genes	1,2-Propanediol utilization and sugar uptake
Sputw3181_0454 −0493	35.8	Lateral flagellum operon	Cell motility and colonization
Sputw3181_0554-0559	8.6	Tannase/feruloyl esterase, outer member porin, and fumarate reductase-like flavoprotein genes	
Sputw3181_0862-0868	8.5	Potassium ion-transporting ATPase operon and two-component regulatory system genes	Osmotic stress response
Sputw3181_0872-0873	4.1	Nitric oxide reductase gene *norZ* and regulatory gene *norR*	Denitrification and detoxification of nitric oxide
Sputw3181_1077-1183	108.6	SXT/R391 ICE (integrating conjugative element)-like prophage inserted into *prfC* gene	Mobile efflux pumps[a]
Sputw3181_1380-1395	4.3	Acylneuraminate cytidylyltransferase and sugar nucleotidyltransferase genes	Utilization of amino and nucleotide sugars; lipopolysaccharide O antigen biosynthesis and flagellin glycosylation
Sputw3181_1667-1670	7.2	Cobaltochelatase (CobN), siderophore receptor, and *tolQ* genes	Porphyrin metabolism
Sputw3181_1944-1966	32.8	L-Arabinose and polymer utilization operon	Uptake and degradation of L-arabinose and arabinan
Sputw3181_2014-2044	31.3	Aldo/keto reductase and glutathione S-transferase genes	—[a]
Sputw3181_2102-2107	4.6	Periplasmic nitrate reductase operon *napDABC (nap*-α)	Dissimilatory nitrate reduction and anaerobic respiration
Sputw3181_2184-2212	37.7	CRISPR elements	Plasmid and phage restriction
Sputw3181_2399	2.5	Retron- and RNA-directed DNA polymerase gene	—[a]
Sputw3181_2877-2921	37.5	Phi phage element	
Sputw3181_2930-2954	24.1	Mu phage element and arsenate reductase genes	Arsenate resistance
Sputw3181_2974-2977	6.0	Aldehyde oxidoreductase, 4Fe-4S ferredoxin, and decaheme cytochrome genes	Carbon source utilization and respiration
Sputw3181_3133-3136	3.5	Sodium ion-translocating oxaloacetate decarboxylase genes	Energy transduction
Sputw3181_3204-3212	7.2	Mercury resistance operon	Mercury resistance
Sputw3181_3245-3248	3.6	Cyanide-insensitive terminal oxidase genes *cioAB*	Aerobic respiration
Sputw3181_3326-3332	4.9	Mlt-interacting MipA family proteins/OmpV, MipA, and two-component system	Osmotic stress response and envelope biogenesis
Sputw3181_3435-3437	3.0	Radical *S*-adenosylmethionine (SAM) domain protein genes	
Sputw3181_3472-3473	2.6	NnrS family protein and nitrite reductase NrfA-like cytochrome C_{552} genes	Nitrite reduction and detoxification
Sputw3181_3508-3513	9.7	Tetrathionate reductase and two-component sensory kinase-response regulator genes	Tetrathionate reduction
Sputw3181_3902-3909	11.8	*Pseudomonas* Cup type IV pilus operon	Biofilm formation
Sputw3181_3982-3996	20.9	Proline biosynthesis and sodium/proline symporter genes	Proline uptake and osmotic stress response
Sputw3181_4067-4090	20.7	Prophage genes, similar to SGI1 element from *Salmonella*, PstI restriction and modification genes	Modification and restriction[a]

[a] The indicated loci are absent in the *Shewanella putrefaciens* CN-32 strain.

balance and *nap*-β is involved in nitrate ammonifcation in *Shewanella* (23). To test this hypothesis, we generated the in-frame deletion mutants of *nap*-β and *nap*-α operons. Our results showed that both single mutants grew on nitrate at rates similar to that seen with the wild-type strain (Fig. 3). Nonetheless, nitrate reduction was abolished in the double mutant, suggesting that both *nap* operons were functional in the nitrate reduction in W3-18-1. W3-18-1 harbors the nitric oxide (NO) reductase gene *norZ* and regulatory gene *norR*, which may be involved in NO detoxification (21).

In MR-1, anaerobic respiration is mainly regulated by cyclic AMP (cAMP) and the cAMP receptor protein (Crp), and the *crp* mutant is deficient in anaerobic growth with, and reduction of, Fe(III), Mn(IV), nitrate, nitrite, fumarate, and DMSO (24). It has been previously noted that the consensus binding sequence of Crp is absent in the upstream region of the *nap*-α (*napDABC*) operon in some *Shewanella* species, though it is present upstream of *nap*-β (*napDAGHB*) and *nrfA* (encoding nitrite reductase) in those species as well as MR-1 (25). This is also the case for W3-18-1 (see Fig. 5). Thus, we monitored the time course of dissimilatory nitrate reduction (represented by the changes in nitrate and nitrite levels in the media) of W3-18-1 and MR-1 under micoaerobic conditions (cultivation without shaking to limit aeration [Fig. 4]). The results showed that nitrate respiration started earlier in W3-18-1 than in MR-1 (with NapDAGHB only). In addition, the level of transcription of *napA*-α, as indicated by RT-PCR, was still high in

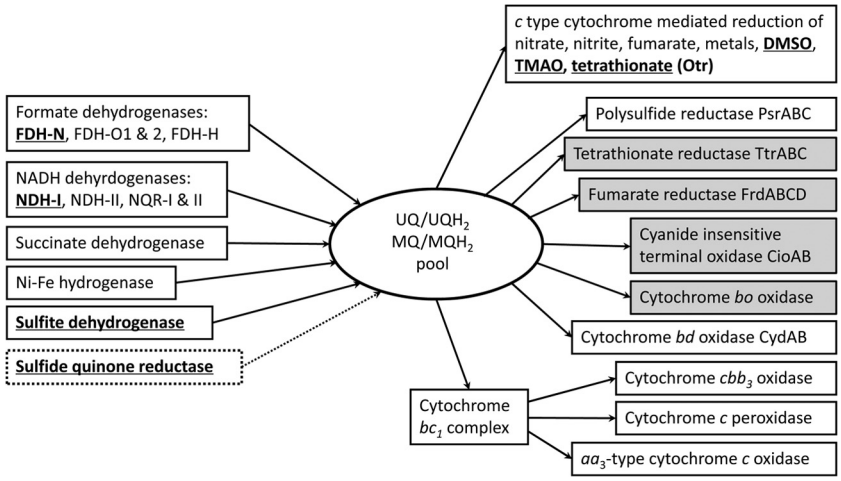

FIG 2 Comparison of respiratory chain components between W3-18-1 and MR-1. The highlighted items are present in MR-1 but absent in W3-18-1. The shaded boxes indicate items present in W3-18-1 but not in MR-1. The broken box indicates that the gene is mutated. UQ/UQH$_2$ and MQ/MQH$_2$ represent ubiquinone/ubiquinol and menaquinone/menaquinol, respectively. See Table S4 in the supplemental material for more details.

the *crp* null mutant *W3-18-1* Δ*crp* (Fig. 5). Furthermore, the anaerobic growth of the W3-18-1 Δ*crp* mutant with nitrate as the electron acceptor was not completely abolished, in contrast to what was shown in the MR-1 *crp* mutant (24), albeit the growth of the Δ*crp* Δ*nap*-α double mutant was totally abolished (see Fig. S1 in the supplemental material). Together, these results suggested that the expression of the *nap*-α operon was truly independent of the presence of Crp, allowing for an earlier transcription for nitrate respiration when oxygen supply is limited.

Hydrogen utilization. W3-18-1 lacks the Fe-only hydrogenase (HydAB) gene cluster that enables *S. oneidensis* MR-1 to use protons as electron acceptors in the absence of an external electron acceptor, resulting in hydrogen production (26). On the other hand, Ni-Fe hydrogenase is involved in the utilization of hydrogen as the electron donor in *S. decolorationis* S12 and MR-1 (27, 28). In most sequenced *Shewanella* strains, Ni-Fe hydrogenase is encoded by a single operon (e.g., SO_2089 to SO_2099 in MR-1), which is split into two separate gene clusters (Sputw3181_1919-1924 and Sputw3181_2173-2178) in W3-18-1. To examine utilization of hydrogen, Biolog dye and a previously described method (27) were used. Dye color changes were observed in both W3-18-1 and MR-1 cultures supplemented with hydrogen via a syringe compared to the results seen with the control experiments performed with nitrogen gas (Table 2) that resulted in only slight color changes, indicative of hydrogen utilization as an electron donor in both strains.

Deficiency in DMSO and TMAO reduction in W3-18-1. Most *Shewanella* strains, including MR-1, can use trimethylamine oxide (TMAO) and dimethyl sulfoxide (DMSO) as electron acceptors under anoxic conditions (29). These species harbor the TMAO reductase gene cluster (SO_1228 to SO_1233 in MR-1), but W3-18-1 lacks the pentaheme *c* TorC and MtrAD-like decaheme *c*

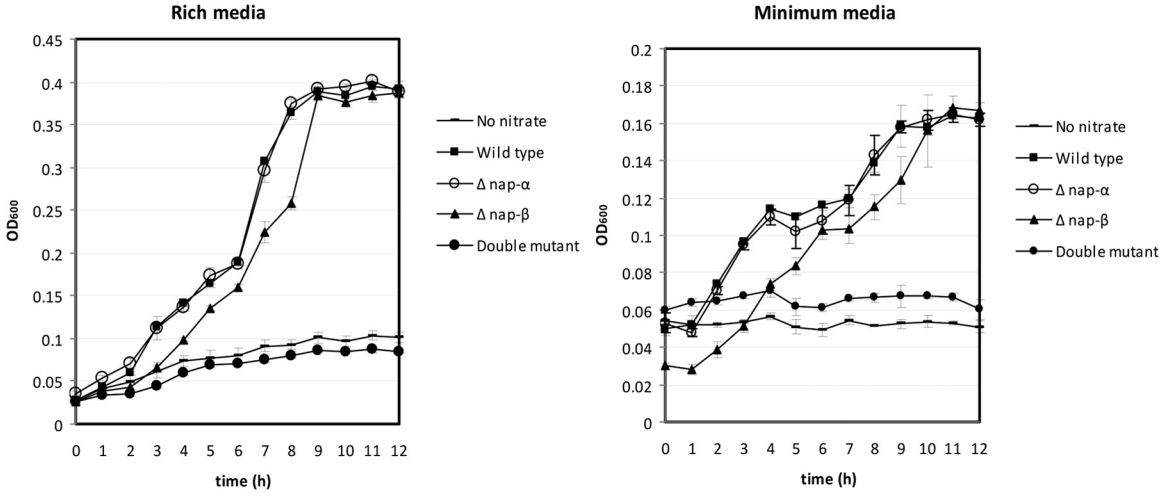

FIG 3 Bacterial growth (optical density at 600 nm [OD$_{600}$]) of the *S. putrefaciens* W3-18-1 wild-type strain, *nap*-α in-frame deletion mutant, *nap*-β mutant, and double mutant of *nap*-α and *nap*-β on nitrate (2 mM) in the rich (1% [wt/vol] tryptone and 0.5% [wt/vol] yeast extract) and modified M1 minimum media supplemented with 50 mM sodium lactate as the electron donor and carbon source. The wild-type strain grown in media without nitrate was used as the control. Error bars represent SD.

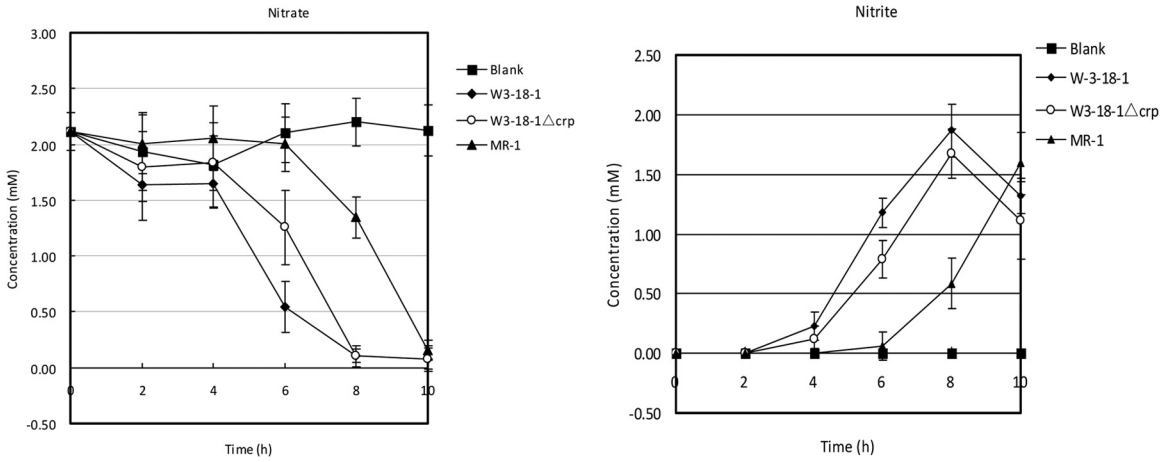

FIG 4 Nitrate reduction of *S. putrefaciens* W3-18-1, the *crp* deletion mutant (W3-18-1Δcrp), and *S. oneidenesis* MR-1 under microoxic conditions. Bacteria were cultivated in the modified M1 minimal media supplemented with 2 mM sodium nitrate (8 ml of bacterial culture in the 15-ml culture tubes incubated without shaking for enhanced aeration). The blank represents the used culture media without bacterial inoculation. Error bars represent SD.

proteins (SO_1427 and SO_4360, involved in reduction of DMSO). The *torF* gene (SO_4694) and *torECAD* are positively coregulated by the transcriptional regulator TorR (30). These genes are absent in W3-18-1 (see Tables S3 and S4 in the supplemental material). Accordingly, W3-18-1 respired neither TMAO nor DMSO in our assays (Table 2).

Oxidation and reduction of sulfur compounds. MR-1 harbors several *c*-type cytochrome genes that are probably involved in sulfur compound metabolisms, such as reduction of thiosulfate and/or other sulfur-containing molecules (11). We noted that two types of tetrathionate reductases, tetrathionate reductase (TTR) and octaheme tetrathionate reductase (OTR), exist in the *Shewanella* strains. W3-18-1 harbors the tetrathionate reductase structural genes *ttrA*, *ttrB*, and *ttrC* as well as *ttrS* and *ttrR* encoding the two-component regulatory system (Table 1; see also Table S4 in the supplemental material). The gene products are homologous to TtrRSBCA in *Salmonella* Typhimurium LT2, which are responsible for respiring tetrathionate (31). On the other hand, MR-1 harbors the octaheme tetrathionate reductase encoded by SO_4144 (32), which is also present in many other *Shewanella*

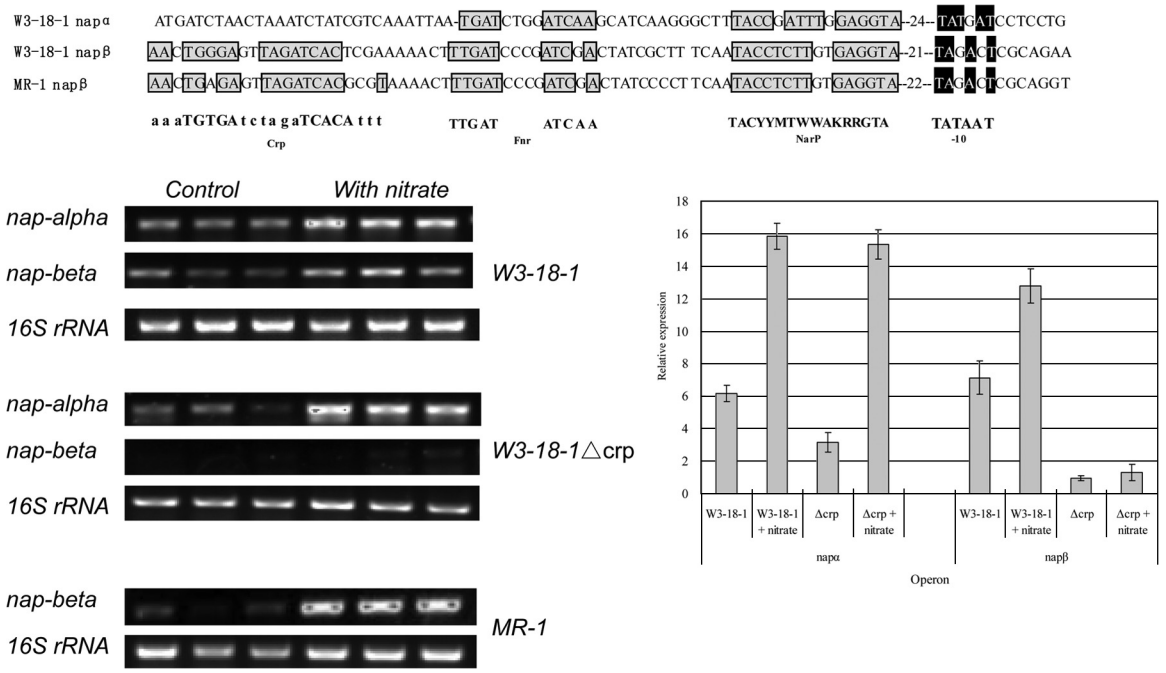

FIG 5 Expression of *napA*-α and *napA*-β of *S. putrefaciens* W3-18-1, the *crp* deletion mutant (W3-18-1Δcrp), and *S. oneidenesis* MR-1 (harboring only the nap-β operon) under microoxic conditions. Bacteria were cultivated in the modified M1 minimal media (8 ml of bacterial culture in the 15-ml culture tubes incubated without shaking for enhanced aeration). The blank represents the used culture media without bacterial inoculation. The presented data represent the samples collected at 6 h of incubation. The three lanes on the left represent the controls without nitrate, and the three on the right represent the treatments supplemented with 2 mM sodium nitrate. Error bars represent SD. (MR-1 sequence alignment adapted from reference 25.)

TABLE 2 Electron donors and acceptors utilized by *Shewanella putrefaciens* W3-18-1 and *S. oneidensis* MR-1 for respiration[a]

Chemical	Electron donor or acceptor	*S. putrefaciens* W3-18-1	*S. oneidensis* MR-1	Enzyme
Fumarate	Acceptor	+	+	Fumarate reductase
Nitrate	Acceptor	+	+	Nitrate reductase
Nitrite	Acceptor	+	+	Nitrite reductase
Ferric citrate	Acceptor	+	+	Metal reductase
Dimethyl sulfoxide (DMSO)	Acceptor	−	+	DMSO reductase
Timethylamine oxide (TMAO)	Acceptor	−	+	TMAO reductase
Thiosulfate	Acceptor	+	+	Polysulfide reductase
Tetrathionate	Acceptor	+	+	Tetrathionate reductase
Hydrogen ion (proton)	Acceptor	−	+	Fe-only hydrogenase
Hydrogen gas	Donor	+	+	Ni-Fe hydrogenase
Sulfite	Donor	−	+	Sulfite dehydrogenase

[a] + and − indicate that utilization of an electron donor or acceptor was and was not observed, respectively.

strains. OTR can also reduce nitrite and hydroxylamine (33). Several *Shewanella* strains, including *S. baltica* strains MR-4 and ANA-3, contain both tetrathionate reductases. Both W3-18-1 and MR-1 could grow on tetrathionate (20 mM) under anaerobic condition (Table 2). Our assays showed that the growth of MR-1 was better than that of W3-18-1 under thiosulfate-reducing conditions, probably due to the *psrABC* gene cluster (34).

W3-18-1 lacks the monoheme cytochrome *c* genes of SO_0714, SO_0716, and SO_0717 in MR-1. SO_0715 and SO_0716 encode the periplasmic sulfite dehydrogenase subunits SorA and SorB, respectively. SorAB could oxidize sulfite (SO_3^{2-}) and transfer the electrons to cytochrome c_{552}, which could be oxidized by the terminal cytochrome *c* oxidase. To experimentally test the oxidation of sulfite (2 to 4 mM), Biolog dye was used to monitor aerobic respiration by MR-1 and W3-18-1 in the minimal medium supplemented by sulfite. Respiration (dye color change) was observed in MR-1 but not in W3-18-1 (Table 2), which was indicative of active sulfite oxidation and/or detoxification by MR-1. In addition, W3-18-1 was more sensitive to higher (>16 mM) levels of sulfite than MR-1 in the modified M1 minimum medium (supplemented with 50 mM pyruvate as the carbon source) and LB broth. These results indicated that MR-1 could utilize sulfite as an electron donor under aerobic condition.

Dissimilatory metal reduction. Previous studies showed that W3-18-1 exhibited a lower capability of metal oxide reduction than several other *Shewanella* strains (8). The metal-reductase-containing locus is highly diverse among the sequenced *Shewanella* genomes (4). W3-18-1 lacks the *mtrFED* genes encoding the secondary metal reductase as shown in *S. oneidensis* MR-1. Furthermore, the allele of *omcA1* of MR-1 (SO_1779, decaheme *c*) encodes an 11-heme cytochrome (designated UndA) in W3-18-1 and *S. baltica* OS223 (4). In addition, W3-18-1 lacks the orthologs for cytochromes encoded by SO_1413 (split tetraheme flavocytochrome), SO_1427 (decaheme *c*), SO_2930 (diheme c), SO_2931 (diheme *c*), SO_3300 (split tetraheme flavocytochrome), SO_4360 (decaheme *c*), SO_3623 (split tetraheme flavocytochrome), SO_4570 (monoheme *c*), and SO_4572 (triheme *c*) in MR-1 (see Table S3 in the supplemental material). Moreover, W3-18-1 grew only with Fe(III), Mn(IV), and Se(IV) whereas *S. putrefaciens* CN-32 could grow with all of six metal ions and metalloids tested, including Co(III), Cr(VI), and As(V) (8). We noted that similar sets of the *c*-type cytochromes are present in both W3-18-1 and CN-32 strains whereas the sputw3181_3363 (*ifcA*) locus is disrupted by a transposon (sputw3181_3364) in W3-18-1 as aforementioned. The *ifcA* gene encodes the ferric ion-induced flavocytochrome c_3 (Ifc3), a soluble periplasmic low-potential heme *c* cytochrome involved in Fe(III) respiration and modulation of fumarate reduction rates in *S. frigidimarina* NCIMB 400 (35, 36). The Δ*mtrD*, Δ*mtrF*, and Δ*ifcA1* mutants of MR-1 had an even greater capacity to reduce the solid-phase iron, a combination of geothite, hematite, and nanoparticles of hydrous ferric oxide (37). Moreover, deletion of *mtrD*, *mtrF*, *ifcA*, SO_0714, *sorB*, SO_0717, SO_1427 (*dmsC*), SO_2930 (diheme *c*), SO_3300, and *otr* resulted in higher current density in microbial fuel cells (MFC) (37).

The NapC/NirT family tetraheme CymA is required for Fe(III), fumarate, nitrate, and arsenate respiration (38, 39) and is responsible for electron transfer from the quinol pool to periplasmic and outer-membrane-bound reductases. We generated an in-frame *cymA* deletion mutant in W3-18-1 and compared the reduction of ferric citrate of this mutant to that of the wild-type strain. The results showed that deletion of *cymA* led to a significant decrease in the rate of reduction of ferric citrate (Table 2), suggesting CymA plays a role similar to that of its ortholog in MR-1 and *Shewanella* sp. ANA-3 strains.

DISCUSSION

The respiratory chains of *Shewanella* strains are highly diversified and could utilize a series of electron acceptors for respiration (Fig. 2). *S. putrefaciens* W3-18-1 has less respiratory flexibility than *S. oneidensis* MR-1, as it possesses fewer cytochrome *c* genes, resulting in the inability to reduce DMSO and TMAO or oxidize sulfite. In most *Shewanella* strains, there are two functionally redundant dissimilatory periplasmic nitrate reductases encoded by *nap*-α and *nap*-β operons; OS217 harbors only the former and MR-1 encodes the latter (23). Our results demonstrated that these seemingly redundant respiratory-chain components may be differentially expressed and function under the different environmental conditions, particularly with respect to oxygen levels and availability of other electron acceptors. These subtly different gene clusters may have enhanced the ecological fitness of *Shewanella* strains (23).

Genomics analyses also revealed other characteristics of respiratory chains. W3-18-1 and MR-1 share three terminal oxidases, i.e., cbb_3-type cytochrome *c* oxidase (CcoNOQP, a proton-pumping heme-copper oxidase), cytochrome *c* oxidase COX (complex IV, proton pump), and cytochrome *d* ubiquinol oxidase CydAB (38, 40). There are two alternative quinol terminal oxidase operons, *cyoABCDE* and *cioAB* (41, 42), present in W3-18-1 but absent in MR-1 (Fig. 2; see also Table S4 in the supplemental material). W3-18-1 harbors more terminal oxidases and fewer *c*-type cytochromes and terminal reductases than MR-1. Moreover, W3-18-1 lacks the anaerobic nitrate-inducible formate dehydrogenase FDH-N, the hydrogen-producing Fe-only hydrogenase, and sulfite dehydrogenase, which are present in MR-1 and many marine *Shewanella* strains. W3-18-1 does not produce hydrogen in the absence of the Fe-only hydrogenase. These observations suggest that W3-18-1 may also have evolved in and is well adapted to environments with relatively high oxygen tensions. The lower anaerobic respiratory flexibility that resulted from the absence or mutation of respiratory genes, including *mtrDEF*, *ifcA1*, and Fe-

only hydrogenase genes, may confer the phenotypes of higher current production of W3-18-1 in MFC (17) and its inability to reduce Co(III), Cr(VI), and As(V) (8). *S. putrefaciens* W3-18-1 is not only suitable for MFC, which could be utilized for electricity generation and wastewater treatment, but also useful for the *in situ* bioremediation of marine and brackish water environments with higher oxygen tensions and at lower temperatures.

Formate dehydrogenase and NADH dehydrogenase catalyze oxidation of formate and NADH, donating electrons to respiratory chains. The formate dehydrogenase *fdh-O* and NADH dehydrogenase *nqr* gene clusters appear to have been duplicated in most *Shewanella* strains (see Table S4 in the supplemental material). The RNF electron transport complex was thought to be a sodium-translocating NADH dehydrogenase. Only the denitrifier *S. denitrificans* OS217 harbors a single NQR (NqrABCDEF-2) and lacks many other respiratory chain components such as formate dehydrogenase and hydrogenase. A notable feature of MR-1 is the acquisition of NADH dehydrogenase I operon (*ndh-I*), composed of *nuoA* to *-N*, which is unique to MR-1 among *Shewanella* strains. This proton-pumping NADH:ubiquinone oxidoreductase (a minimal form of mitochondrion respiratory complex I) may be energetically more efficient under certain conditions such as neutral pH. The *ndh-I* gene clusters obviously arose from the lateral gene transfer. Such genome divergence could provide valuable insights into bacterial genome evolution and adaptation to their specific niches.

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