Respiratory Nitrate Ammonification by *Shewanella oneidensis* MR-1[▽]

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Anaerobic cultures of *Shewanella oneidensis* MR-1 grown with nitrate as the sole electron acceptor exhibited sequential reduction of nitrate to nitrite and then to ammonium. Little dinitrogen and nitrous oxide were detected, and no growth occurred on nitrous oxide. A mutant with the *napA* gene encoding periplasmic nitrate reductase deleted could not respire or assimilate nitrate and did not express nitrate reductase activity, confirming that the NapA enzyme is the sole nitrate reductase. Hence, *S. oneidensis* MR-1 conducts respiratory nitrate ammonification, also termed dissimilatory nitrate reduction to ammonium, but not respiratory denitrification.

Anaerobic respiration is a primary means by which bacteria participate directly in biogeochemical cycles. It generates a proton motive force with any of a variety of terminal electron acceptors, including inorganic compounds such as nitrate (NO_3^-) and thiosulfate ($\text{S}_2\text{O}_3^{2-}$), organic compounds such as fumarate and dimethyl sulfoxide, and metals such as Fe(III) and Mn(IV). *Shewanella oneidensis* MR-1 (32), a member of the class *Gammaproteobacteria*, serves as a model system for exploring environmental impacts of this metabolism (13, 17, 26, 48).

The nitrate/nitrite couple has a relatively high standard redox potential, and thus nitrate is a preferred electron acceptor (18, 39). The reduction product nitrite (NO_2^-) also is an effective acceptor, and nitrite respiration proceeds by either of two pathways. In respiratory denitrification, nitrite is reduced sequentially to nitric oxide (NO), nitrous oxide (N_2O), and dinitrogen (N_2) in three separate reactions, each of which contributes independently to the proton motive force (53). In respiratory nitrate ammonification, also termed dissimilatory nitrate reduction to ammonium (47), a proton motive force is generated by sequential reduction of nitrate via nitrite to ammonium (NH_4^+) (43). Bacteria exhibit substantial diversity, even within a species, with respect to the capacity to respire by denitrification or nitrate ammonification.

Nitrate respiration involves either of two distinct enzyme systems, both of which have a molybdenum cofactor in the catalytic subunit (18, 39). One system, the NapAB enzyme, is localized in the periplasm, whereas the second system, the NarGHI enzyme, is localized on the cytoplasmic face of the cytoplasmic membrane (18, 38). The *S. oneidensis* MR-1 ge-

nome contains a *napDAGHB* operon (locus tags SO0849 through SO0845) (21) but does not contain a *narGHJI* operon. Respiratory nitrite reduction is catalyzed by three distinct enzymes. The NrfA enzyme, localized in the periplasm, is a cytochrome *c* that reduces nitrite to ammonium (43), whereas the NirK and NirS enzymes reduce nitrite to nitric oxide, thereby initiating the denitrification pathway (53). The *S. oneidensis* MR-1 genome contains an *nrfA* gene (locus tag SO3980) (21) but does not contain a *nirK* or *nirS* gene.

Thus, genome sequence analysis predicts that *S. oneidensis* MR-1 conducts respiratory nitrate ammonification by means of the periplasmic NapAB and NrfA enzymes. Global analyses have demonstrated that the genes are expressed during anaerobic growth with nitrate (3, 5). However, well before the *S. oneidensis* MR-1 genome project was initiated, respiratory denitrification was reported for *S. oneidensis* MR-1 and *Shewanella* spp. strains MR-4 and MR-7 by Krause and Nealson (23). In that study, cultures were grown in nutrient broth with added nitrate in an atmosphere containing 10% hydrogen. Nitrous oxide was produced from the late exponential phase through the onset of the stationary phase and accounted for about 25% of the nitrate N. No ammonium accumulation was detected. In a separate study workers also detected nitrous oxide production by *S. oneidensis* MR-1 (16). Therefore, our goal in the present study was to establish the pathway(s) by which *S. oneidensis* MR-1 uses nitrate for anaerobic respiration.

Culture media and conditions. Bacterial strains are listed in Table 1. LML medium (4) contained 0.2 mg ml⁻¹ yeast extract, 0.1 mg ml⁻¹ peptone, 20 mM DL-lactate, and 10 mM HEPES (pH 7.4). HEPES medium, based on modified M1 medium (22), contained 4.35 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 1.34 mM KCl, 7.5 mM NaOH, 2 $\mu\text{g ml}^{-1}$ L-Arg, 2 $\mu\text{g ml}^{-1}$ L-Glu, 2 $\mu\text{g ml}^{-1}$ DL-Ser, 20 mM DL-lactate, and 50 mM HEPES (pH 7.4). LML and HEPES media both were supplemented with 0.1 mg ml⁻¹ vitamin-free acid-hydrolyzed casein, 10 ml liter⁻¹ of a trace mineral solution (52), and, added after autoclaving, 10 ml liter⁻¹ of a vitamin solution (52). MOPS medium was prepared as described previously (33), except that NaCl was added to a final concentration of 100 mM (25), Na_2MoO_4 and Na_2SeO_3

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TABLE 1. Bacterial strains, plasmids, and oligonucleotide primers

Strain, plasmid, or primer	Relevant genotype or sequence	Source
Strains		
<i>Enterobacter aerogenes</i> ENT-5-9	Prototroph	27
<i>Escherichia coli</i> K-12		
β2155	$\Delta dapA::erm\ pir::RP4$	14
VJS5433	<i>narGHJI</i> ⁺ $\Delta napF1::Km$	45
VJS5436	<i>narG205::Tn10 napFDAGHBC</i> ⁺	45
<i>Flavobacterium</i> sp. strain ATCC 33514	Prototroph	27
<i>Pseudomonas stutzeri</i> ZoBell	Prototroph	ATCC 14405
<i>Shewanella oneidensis</i>		
MR-1 (=ATCC 700550 ^T)	Prototroph	32
CCG01	As MR-1 but $\Delta napA::loxP$	This study
Plasmids		
pCM184	Ap ^r Tc ^r ; <i>loxP-kan-loxP</i>	29
pKNOCK-Gm	Gm ^r ; <i>ori</i> _{R6K} <i>oriT</i> _{RP4}	1
pCCG01	As pKNOCK-Gm but <i>napD-napA'-loxP-kan-loxP-napA-napG</i>	This study
Oligonucleotide primers		
napAN Fwd	5'-GCATATGGGCGGCTAATGCTCATAGTGTT	
napAN Rev	5'-CGAATTCTCTTGCCCCATTCCTCCCT	
napAC Fwd	5'-CGAGCTCCAGACATCGCAGCGTAATCCTT	
napAC Rev	5'-GCCGCGGAGTGCCCCGTAAGAGTGATGAA	
napAScreen Fwd	5'-AGACATCGCAGCGTAATCTC	
napAScreen Rev	5'-TCCCTCTCCAAAGGGATAGC	
napAScreenout Fwd	5'-GTGTCATGCTCTGCGGATT	
napAScreenout Rev	5'-AATGCGCCTGGGATTGAA	

were both added to a final concentration of 0.1 μ M (37), and 20 mM DL-lactate served as the principal carbon source and electron donor. A nitrogen source (NH₄Cl or NaNO₃) and acid-hydrolyzed casein were added as indicated below. For all media, the respiratory oxidants nitrate, nitrite, nitrous oxide, and fumarate were added as indicated below. The complex media used were LB medium (6) and nutrient broth.

Cultures were incubated at 30°C, and culture densities are reported below as mean optical densities at 600 nm (OD₆₀₀) for triplicate cultures for each growth condition examined. For anaerobic cultivation we employed the technique of Balch and Wolfe (2), whereas aerobic cultivation was performed in flasks filled to 10% of their capacity with medium and shaken at 150 to 200 rpm. Each experiment included two negative controls, uninoculated medium and medium with no added respiratory oxidant. Cultures initially were aerated overnight to saturation in LB medium. A 1:100 dilution from an LB medium culture was inoculated into test medium with no added respiratory oxidant, and the culture was aerated for 12 h. A 1:100 dilution from this adapted culture was inoculated into test medium with various respiratory oxidants, and the cultures were monitored to determine growth and other parameters as described below.

Nitrate and nitrite as anaerobic respiratory oxidants. We cultured *S. oneidensis* anaerobically in LML medium with added nitrate at concentrations ranging from 0.5 to 100 mM (Fig. 1). Cultures with 1 to 100 mM nitrate exhibited similar exponential-phase doubling times, ranging from about 1.5 to 2 h (Fig. 1 and data not shown). Cultures reached the stationary phase at relatively low densities, roughly 0.1 to 0.2 OD₆₀₀ unit (Fig. 1). Cultures with 3 to 4 mM nitrate attained the highest densities. Cultures with 0.5 mM nitrate exhibited a notably longer doubling time (>5 h) and lower stationary-phase density (OD₆₀₀, approximately 0.05).

Similarly, cultures with 1 to 2 mM added nitrite had exponential-phase doubling times of approximately 1.5 h and attained stationary-phase densities of at least 0.1 OD₆₀₀ unit (Fig. 1). Cultures with 0.5 mM nitrite had similar doubling times but lower densities (OD₆₀₀, approximately 0.06). Strikingly, cultures with 4 mM nitrite had pronounced lag periods and lower growth rates and attained final densities of only about 0.03 OD₆₀₀ unit. Thus, during growth in LML medium, the organism was inhibited by 4 mM nitrite yet was little affected by 100 mM nitrate.

Nitrate reduction to ammonium. We monitored the concentrations of nitrate, nitrite, and ammonium in anaerobic batch cultures (Fig. 2). Samples (2 ml) were sterilized by passage through a 0.22- μ m syringe filter, and then the ammonium content was analyzed by the salicylate colorimetric method (34) and the nitrate and nitrite contents were analyzed at the Michigan State University Soil Testing Laboratory using a Lachat QuickChem automated flow injection ion analyzer and the copperized cadmium reduction method of QuickChem method no. 10-107-04-1-A (Lachat Instruments, 1988).

Nitrate (initial concentration, 2 mM) was rapidly converted to nitrite, which accumulated to a concentration of 2 mM before it was converted to ammonium. The initial ammonium concentration (0.33 mM) did not change until nitrite began to be consumed, at which point ammonium accumulated to concentration of 2 mM at the onset of the stationary phase. Further accumulation of ammonium during the stationary phase (Fig. 2) presumably reflected N release from dead cells. Anaerobic cultures of *Escherichia coli* K-12 in medium with relatively low nitrate concentrations similarly accumulate nitrite quantitatively before reducing it to ammonium (12, 15). Therefore, during anaerobic growth, *S. oneidensis* MR-1 quantitatively reduced nitrate via nitrite to ammonium.

FIG. 1. Nitrate and nitrite respiration by *S. oneidensis* MR-1. Cultures in LML medium contained nitrate (A) or nitrite (B) at the following concentrations: 0.5 mM (○), 1.0 mM (●), 2.0 mM (□), 3.0 mM (△), 4.0 mM (■), and 40 mM (▲). The data are averages from three independent cultures. Error bars were omitted for clarity. Cultures with no added nitrate or nitrite did not grow (not shown).

No evidence for respiratory denitrification. We tested for anaerobic respiration with nitrous oxide as the sole electron acceptor and for production of gas (nitrous oxide plus dinitrogen) during anaerobic respiration with nitrate. Strains were cultured anaerobically for 12 h in LML medium with 4 mM nitrate. Samples (0.1 ml) from the cultures were used to inoculate sets of 10 Balch tubes, each containing 10 ml of LML medium along with a Durham tube to observe gas production. Nitrous oxide gas was added to one set of tubes, and nitrate (4 mM) was added to another. These cultures were incubated for 3 weeks.

No growth was observed in the *S. oneidensis* MR-1 cultures containing nitrous oxide, and no accumulated gas was observed in the cultures containing nitrate (data not shown). By contrast, the respiratory denitrifier *Pseudomonas stutzeri* ZoBell

grew well with nitrous oxide and accumulated substantial gas in cultures containing nitrate.

We also measured the amounts of nitrous oxide, dinitrogen, and ammonium that accumulated during nitrate respiration (Table 2). Production of nitrogen gases and ammonium was determined for cultures grown in nutrient broth as previously described (27), except that all cultures were grown at 30°C with 0.5, 1.25, or 2.0 mM nitrate. Dinitrogen and nitrous oxide were quantified by gas chromatography with thermal conductivity and ^{63}Ni electron capture detectors, respectively.

The amount of nitrous oxide plus dinitrogen recovered from cultures of *Flavobacterium* sp. strain ATCC 33514 (the positive control for denitrification) accounted for nearly 100% of the nitrate added at all concentrations tested, whereas the amount of nitrous oxide plus dinitrogen recovered from cultures of *Enterobacter aerogenes* ENT-5-9 (the negative control for denitrification) and *S. oneidensis* MR-1 was less than 10% of the nitrate added. Nitrous oxide accounted for less than 1% of this amount; the remainder represented dinitrogen contamination from the atmosphere, which also caused the variation seen at

FIG. 2. Production and consumption of nitrite and ammonium during nitrate respiration. Cultures in HEPES medium contained 2.0 mM nitrate, and the culture density (■) and the concentrations of nitrate (□), nitrite (△), and ammonium (○) were monitored. The data are averages from three independent cultures. Error bars were omitted for clarity.

TABLE 2. Nitrogen balances

Organism	Initial nitrate concn (mM) ^a	% Nitrogen recovered as ^b :	
		N ₂ O + N ₂	N ₂ O + N ₂ + NH ₄ ⁺
<i>S. oneidensis</i>	0.5	<0.1	85.9 ± 20.9
<i>S. oneidensis</i>	1.25	8.3 ± 11.7	88.1 ± 18.9
<i>S. oneidensis</i>	2.0	6.9 ± 11.1	87.2 ± 18.3
<i>Flavobacterium</i> sp.	0.5	91.4 ± 37.5	154 ± 37.7
<i>Flavobacterium</i> sp.	1.25	88.1 ± 6.3	145 ± 7.8
<i>Flavobacterium</i> sp.	2.0	88.9 ± 3.7	160 ± 6.5
<i>E. aerogenes</i>	0.5	0.3 ± 0.3	70.4 ± 1.4
<i>E. aerogenes</i>	1.25	8.4 ± 12.2	76.8 ± 19.7
<i>E. aerogenes</i>	2.0	11.3 ± 11.9	98.5 ± 13.0

^a Organisms were cultured anaerobically to the early stationary phase in nutrient broth containing the concentrations of nitrate indicated.

^b The values are averages ± standard deviations.

these low levels. By contrast, ammonium accounted for nearly 100% of the nitrate added for both *E. aerogenes* and *S. oneidensis* cultures (Table 2).

The percentage of recovery of nitrogen for *Flavobacterium* sp. strain ATCC 33514 was about 150% when the amount of ammonium was included (Table 2). Additional experiments revealed that in addition to performing denitrification, *Flavobacterium* sp. strain ATCC 33514 secreted ammonium when it was cultured in nutrient broth (data not shown).

Nitrous oxide is produced by a wide range of bacteria that do not conduct respiratory denitrification, including several species of enterobacteria (7). In the cases examined, nitrous oxide is produced from the late exponential phase through the stationary phase and accounts for 3 to 36% of the nitrate N. One criterion for establishing respiratory denitrification is that at least 80% of nitrate or nitrite N is recovered as nitrous oxide plus dinitrogen (27). By this criterion, the relatively low-level formation of nitrous oxide by *S. oneidensis* MR-1 (Table 2) (16, 23) is inconsistent with respiratory denitrification. The enzymes that contribute to the production of nitrous oxide by bacteria that do not conduct respiratory denitrification have not been established, although in *E. coli* K-12, which grows by respiratory nitrate ammonification, nitrous oxide production has been correlated with formation of nitrate reductase (44).

Construction of a chromosomal $\Delta napA::loxP$ allele. The routine procedures for cloning, analyzing, and engineering recombinant DNA molecules used were standard procedures (41). Primers for PCRs were designed with the aid of the Vector NTI software (InforMax, Inc.) and were synthesized at Integrated DNA Technologies (www.idtdna.com).

Deletion of the chromosomal $napA^+$ gene (locus tag SO0848) was performed by allelic replacement in three steps. The protocol was based on the *cre-lox* mutagenesis system developed by Marx and Lidstrom (29). First, two PCR-generated DNA fragments were cloned into the multiple cloning sites flanking the *kan* gene, encoding resistance to kanamycin (Km^r) ($25 \mu\text{g ml}^{-1}$), in plasmid pCM184 (Table 1). Primers *napAN* Rev (EcoRI) and *napAN* Fwd (NdeI) generated a 521-bp fragment containing the *nap* operon upstream control region, the entire *napD* gene, and the first five codons of the *napA* gene, which comprises 826 codons. Primers *napAC* Rev (SacII) and *napAC* Fwd (SacI) generated a 547-bp fragment containing the last nine codons of the *napA* gene and about two-thirds of the *napG* gene. The resulting *napD-napA'-loxP-kan-loxP-napA-napG'* assembly was cloned into the conditionally replicating plasmid pKNOCK-Gm (Table 1), which encodes resistance to gentamicin (Gm^r) ($7.5 \mu\text{g ml}^{-1}$), to generate plasmid pCCG01.

Second, plasmid pCCG01 was introduced into *S. oneidensis* MR-1 by conjugation from *E. coli* strain $\beta 2155$ (Table 1), which is auxotrophic for diaminopimelic acid, using a protocol provided by M. F. Romine (Pacific Northwest National Laboratory, Richland, WA). *S. oneidensis* Km^r Gm^s colonies were screened by PCR using primers *napAScreenout* Fwd and *napAScreenout* Rev, which anneal approximately 210 bp downstream and 90 bp upstream, respectively, from the boundaries of the *nap* fragment in plasmid pCCG01.

Third, the *kan* gene was excised by Cre recombinase-mediated site-specific recombination between the flanking *loxP* sites. Plasmid pCM157 (29), which encodes Cre recombinase and resistance to tetracycline (Tc^r) ($10 \mu\text{g ml}^{-1}$) (Table 1), was

FIG. 3. Nitrate respiration requires the $napA^+$ gene. Cultures in HEPES medium were aerated (circles) or cultured anaerobically with nitrate (squares) or nitrate plus fumarate (triangles). Closed symbols, $napA^+$ parent MR-1; open symbols, $\Delta napA::loxP$ mutant CCG01. The data are averages from three independent cultures. Error bars were omitted for clarity.

introduced into the $\Delta napA::loxP$ -*kan-loxP* strain by conjugation from *E. coli* strain $\beta 2155$. *S. oneidensis* Km^s Tc^r colonies were screened by PCR using the *napAScreenout* primers. Plasmid pCM157 was then segregated by culturing several generations in the absence of tetracycline. *S. oneidensis* Km^s Tc^s colonies were rescreened by PCR, and the amplified fragments were subjected to DNA sequence analysis to confirm the presence of the chromosomal $\Delta napA::loxP$ allele. The sequence confirmed that the deletion was in frame. DNA sequencing was performed at the Genomics Technical Support Facility at Michigan State University.

Nitrate respiration requires the $napA^+$ gene. We cultured both the $napA^+$ parent and the $\Delta napA::loxP$ mutant in HEPES medium with oxygen, 3 mM nitrate, or 3 mM nitrate plus 30 mM fumarate (19) as respiratory oxidants (Fig. 3). The two strains grew equally well with oxygen or fumarate, but the $\Delta napA::loxP$ mutant failed to grow with nitrate. Thus, the $napA^+$ gene was required for nitrate respiration.

In a separate experiment, we analyzed the nitrate, nitrite, and ammonium contents of cultures (data not shown). In $napA^+$ cultures containing either nitrate or nitrate plus fumarate, nitrate was quantitatively converted via nitrite to ammonium (Fig. 2), whereas in the $\Delta napA::loxP$ culture containing nitrate plus fumarate, the nitrate concentration remained at the original concentration and neither nitrite nor ammonium accumulated. Aerated cultures in HEPES medium containing 3 mM nitrate failed to reduce nitrate irrespective of the *napA* genotype.

Anaerobic nitrate assimilation. Regulation of respiratory nitrate ammonification responds to electron acceptor availability and is indifferent to the nitrogen source (11). Nevertheless, the resulting ammonium can be assimilated into biomass, and *E. coli* K-12 uses nitrate as a sole nitrogen source during anaerobic growth (11). We therefore examined growth of *S. oneidensis* MR-1 in MOPS medium with either ammonium or nitrate as the principal nitrogen source. The culture medium contained 40 mM fumarate as an electron acceptor, as well as

50 $\mu\text{g ml}^{-1}$ of acid-hydrolyzed casein, which provided a low level of fixed nitrogen (supporting growth to a density of about 0.03 OD₆₀₀ unit).

Ammonium and nitrate both increased the growth yield of the wild-type to the same extent, to densities of about 0.16 OD₆₀₀ unit with 0.5 mM N source and about 0.24 OD₆₀₀ unit with 1 mM N source (data not shown). This demonstrated that all added nitrate N was assimilated. The growth yield of the $\Delta napA::loxP$ mutant with ammonium was similar, but the biomass of this mutant did not increase with nitrate. No further increase in culture density was observed with higher N source concentrations. Thus, during anaerobic growth with fumarate as an electron acceptor, the biomasses of cultures were equivalent with equal amounts of N added as ammonium or nitrate, demonstrating that most of the nitrate was reduced to ammonium.

Nitrate reductase activity. We cultured both the $napA^+$ parent and the $\Delta napA::loxP$ mutant anaerobically in MOPS medium containing 40 mM fumarate or 4 mM nitrate plus 40 mM fumarate and monitored the nitrate reductase enzyme activity in intact cells by measuring the nitrite accumulation with reduced methyl viologen as an electron donor, as previously described (45, 46). Cultures of the $napA^+$ strain exhibited robust nitrate reductase activity (data not shown) that was induced about 100-fold during growth with nitrate. The activity was indifferent to 50 μM sodium azide (NaN_3), as expected for the NapA enzyme (18). Parallel assays with *E. coli* mutant strains ($\text{NarG}^+ \text{NapA}^-$ and $\text{NarG}^- \text{NapA}^+$) (Table 1) confirmed the efficacy of azide for inhibition of the NarG nitrate reductase. Nitrate reductase activity was undetectable in cultures of the $\Delta napA::loxP$ mutant.

Nitrate metabolism and the *S. oneidensis* MR-1 genome. For genome database searches we employed the BLAST programs (30) of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>). Draft genome sequence data for various *Shewanella* spp. were accessed through the Joint Genome Institute (http://genome.jgi-psf.org/mic_home.html).

Periplasmic nitrate reductase transfers electrons from quinol through the NapC and NapB cytochrome *c* subunits to the catalytic NapA subunit (38). The NapG and NapH proteins participate in electron transfer from ubiquinol (9), and the NapD and NapF proteins are involved in enzyme assembly (35, 49). A variety of *nap* clusters with different gene contents and organizations are present in diverse bacterial species (28). Most species belonging to the class *Gammaproteobacteria* that have been examined contain a *napFDAGHBC* (or *napFDABC*) operon (38), and therefore the *S. oneidensis* MR-1 *napFDAGHB* operon is unusual. Likewise, in most species examined, the *nrfA* gene is adjacent to either the *nrfH* gene or the *nrfBCD* genes encoding cytochromes *c* (43); hence, the *S. oneidensis* MR-1 single *nrfA* gene is also unusual.

The NapC/NirT/NrfH family of tetraheme cytochromes *c* transfer electrons from quinol to the corresponding periplasmic redox enzymes (38, 43). In *Shewanella* spp., the CymA tetraheme cytochrome *c*, a member of the NapC/NirT/NrfH family, is required for electron transport with nitrate, nitrite, and at least three other terminal electron acceptors (31, 42). Thus, the absence of *napC* and *nrfH* genes from the *S. oneidensis* MR-1 genome suggests that the CymA protein provides the requisite functions.

An unlinked gene annotated as *napF* (locus tag SO1663) (21) encodes a 161-residue protein in which 44% of the residues are identical to residues in the *E. coli* 164-residue NapF protein. The identical residues include all four groups of four Cys residues involved in iron-sulfur cluster formation, as well as the characteristic IRPPW motif (38) near the amino terminus. The NapF protein is hypothesized to catalyze formation of the NapA iron-sulfur cluster (35). The possible involvement of the *S. oneidensis* MR-1 NapF protein in nitrate respiration remains to be determined.

The Hcr and Hcp enzymes likely catalyze the NADH-dependent reduction of hydroxylamine (10, 50, 51), an intermediate of nitrite reduction to ammonium (43). The *S. oneidensis* MR-1 genome contains an *hcp-hcr* operon (locus tags SO1363 and SO1364) (21).

Many bacteria synthesize soluble, cytoplasmic assimilatory nitrate and nitrite reductases (24). Synthesis of these enzymes is regulated in response to available fixed nitrogen rather than electron acceptor availability. The *S. oneidensis* MR-1 genome does not contain genes for assimilatory nitrate or nitrite reductase. Nitrate assimilation during anaerobic growth with fumarate as the electron acceptor (see above) constitutes gratuitous use of ammonium resulting from respiratory nitrate ammonification (11).

The *S. oneidensis* MR-1 genome does not contain structural genes (36) for denitrifying nitrite reductase (nitric oxide forming; *nirK* or *nirS*), nitric oxide reductase (*norBC*), or nitrous oxide reductase (*nosZ*). By contrast, the genome sequence of *Shewanella denitrificans* OS217 (8) includes *napA*, *nirK*, *norBC*, and *nosZ* genes, and this strain has been shown experimentally to be a respiratory denitrifier. So far, however, this property has been found in only one strain of *Shewanella*.

The *S. oneidensis* MR-1 genome contains five genes originally annotated as *nos* (nitrous oxide reductase) genes: *nosLDFY* (locus tags SO0485 to SO0488) and *nosA* (locus tag SO0630) (21). Homologous genes in other species encode proteins required for assembly of the copper center in nitrous oxide reductase (*nosZ* gene product) (53). However, transcription of the *S. oneidensis* MR-1 "*nos*" genes, along with adjacent genes whose products likely are involved in cytochrome *c* maturation, is induced by thiosulfate during anaerobic growth (3). Therefore, these gene products probably are involved in formation of a copper-containing enzyme for thiosulfate respiration.

Nitrate in relation to anaerobic respiration. In *E. coli*, which also uses an array of electron acceptors, nitrate represses synthesis of enzymes for respiration with most of the other acceptors (18). Nitrate has been found also to influence aspects of anaerobic respiration in *Shewanella* spp. (20, 40). Thus, nitrate, which is present in a wide range of environments, probably affects the utilization of other acceptors in a variety of contexts. The studies reported here, which established the pathway for nitrate respiration, provide a platform for understanding the interactions between nitrate and other anaerobic respiratory pathways in *S. oneidensis* MR-1.

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