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ORIGINAL ARTICLE

Reduction of nitrate in *Shewanella oneidensis* depends on atypical NAP and NRF systems with NapB as a preferred electron transport protein from CymA to NapA

Haichun Gao[1,2,3], Zamin K Yang[3], Soumitra Barua[2,3], Samantha B Reed[4], Margaret F Romine[4], Kenneth H Nealson[5], James K Fredrickson[4], James M Tiedje[6] and Jizhong Zhou[2,3]

[1]*College of Life Sciences and Institute of Microbiology, Zhejiang University, Hangzhou, Zhejiang, China;* [2]*Department of Botany and Microbiology, Institute for Environmental Genomics, University of Oklahoma, Norman, OK, USA;* [3]*Oak Ridge National Laboratory, Environmental Sciences Division, Oak Ridge, TN, USA;* [4]*Pacific Northwest National Laboratory, Richland, WA, USA;* [5]*Department of Earth Sciences, University of Southern California, Los Angeles, CA, USA and* [6]*Center for Microbial Ecology, Michigan State University, East Lansing, MI, USA*

In the genome of *Shewanella oneidensis*, a *napDAGHB* gene cluster encoding periplasmic nitrate reductase (NapA) and accessory proteins and an *nrfA* gene encoding periplasmic nitrite reductase (NrfA) have been identified. These two systems seem to be atypical because the genome lacks genes encoding cytoplasmic membrane electron transport proteins, NapC for NAP and NrfBCD/NrfH for NRF, respectively. Here, we present evidence that reduction of nitrate to ammonium in *S. oneidensis* is carried out by these atypical systems in a two-step manner. Transcriptional and mutational analyses suggest that CymA, a cytoplasmic membrane electron transport protein, is likely to be the functional replacement of both NapC and NrfH in *S. oneidensis*. Surprisingly, a strain devoid of *napB* encoding the small subunit of nitrate reductase exhibited the maximum cell density sooner than the wild type. Further characterization of this strain showed that nitrite was not detected as a free intermediate in its culture and NapB provides a fitness gain for *S. oneidensis* to compete for nitrate in the environments. On the basis results from mutational analyses of *napA*, *napB*, *nrfA* and *napBnrfA* in-frame deletion mutants, we propose that NapB is able to favor nitrate reduction by routing electrons to NapA exclusively.

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Introduction

Microbial reduction of nitrate, a complicated and extensively studied process, plays a predominant role in the global biogeochemical nitrogen cycle (N-cycle) (Richardson and Watmough, 1999). Nitrate reduction can fulfill various functions in cell metabolism ranging from providing ammonium for biosynthesis (assimilatory reduction), proton motive force for metabolic energy generation (nitrate respiration) to dissipating the excess of reducing power (dissimilatory reduction) (Moreno-Vivian and Ferguson, 1998; Moreno-Vivian *et al.*, 1999; Richardson, 2000; Richardson *et al.*, 2001).

The periplasmic nitrate reduction system (NAP) functions to reduce nitrate to nitrite, a process that can be coupled to the reduction of nitrite to either ammonium (ammonification) or nitrogen gas (denitrification) (Richardson *et al.*, 2001; Jepson *et al.*, 2006, 2007). NAP has been identified in the genomes of many Gram-negative bacteria but varies significantly in the complement and arrangement of associated genes detected (Marietou *et al.*, 2005).

Correspondence: H Gao or J Zhou, College of Life Sciences and Institute of Microbiology, Zhejiang University, 388 Yuhangtang Road, Hangzhou, Zhejiang 310058, China.
E-mails: haichung@zju.edu.cn or jzhou@ou.edu
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Until recently, the NAP system has been believed to require at least four components: NapA, B, C and D (Potter and Cole, 1999; Marietou *et al.*, 2005). Nitrate reductase (NapA), a molybdenum-containing protein, is the large subunit of the terminal nitrate reductase (Richardson, 2000). NapB, a diheme *c*-type cytochrome as the small subunit of the terminal nitrate reductase, functions to transfer electrons to NapA without a catalytic activity (Richardson, 2000). Both subunits are located in the periplasm. NapC, a membrane-anchored tetraheme *c*-type cytochrome of the NapC/NirT family, delivers electrons from the quinol pool through NapB to NapA. NapD, a cytoplasmic protein, is involved in NapA relocation by binding to the NapA twin-arginine signal peptide (Maillard *et al.*, 2007).

Nitrite can be further reduced to ammonium (NH_4^+) without the release of intermediate products by a periplasmic nitrite reduction system (NRF) (Simon, 2002). Two types of NRF have been described in organisms possessing this system: NrfAH type and NrfABCD type. Although the NrfAH type is found predominantly in the ε- and δ-*proteobacteria*, the NrfABCD system is most common in γ-*proteobacteria*. In both types, nitrite reductase (NrfA) functions as the terminal reductase. The NrfH protein is a tetraheme *c*-type cytochrome of the NapC/NirT family that is responsible for passing electrons from menaquinol to NrfA and functionally equivalent to NrfBCD of the NrfABCD type system (Simon *et al.*, 2000, 2001; Simon, 2002).

Shewanella oneidensis MR-1, a facultatively anaerobic member of the γ-*proteobacteria*, is renowned for its respiration versatility. Several lines of evidence suggest that nitrate reduction through the stepwise reduction of nitrate to nitrite and nitrite to ammonium (respiratory nitrate ammonification) is the dominant pathway, if not exclusive (Myers and Myers, 1997; Schwalb *et al.*, 2002; Cruz-Garcia *et al.*, 2007). The first step of the respiratory nitrate ammonification is carried out by a NAP system, which lacks NapC and the resulting nitrite, in the second step, can be further reduced to ammonium (Cruz-Garcia *et al.*, 2007). The second step is presumably catalyzed by an NRF system based on the presence of a periplasmic nitrite reductase encoded by *nrfA* (SO3980) without NrfBCD/NrfH. However, the experimental validation is lacking. It has been suggested that CymA, a *c*-type cytochrome belonging to the NapC/NirT family, is in the place of both NapC and NrfH for electron transport to the terminal reductases (Myers and Myers, 2000; Schwalb *et al.*, 2003).

Given these novel features of nitrate and nitrite reduction in *S. oneidensis*, a more comprehensive understanding of the process in this organism is worth acquiring. In this study, we systematically examined components of nitrate and nitrite reduction pathways by means of bioinformatics, microarrays and mutational analyses. Results presented led to the establishments that the NRF system is responsible for nitrite reduction of *S. oneidensis* and CymA functions to transport electrons to NapA and NrfA as a functional replacement for both NapC and NrfH. NapB, which is unexpectedly non-essential in the nitrate reduction of *S. oneidensis*, has been found to be a preferred electron-accepting protein from CymA. In addition, a competition assay showed that NapB provides a fitness gain for the bacterium living in the environment where nitrate is present.

Materials and methods

Bacterial strains, plasmids and culture conditions

A list of all bacterial strains and plasmids used in this study is given in Table 1. *Escherichia coli* and *S. oneidensis* strains were grown in Luria-Bertani (LB, Difco, Detroit, MI, USA) medium at 37 °C and room temperature for genetic manipulation, respectively. Where needed, antibiotics were added at the following concentrations: ampicillin at 50 μg ml^{-1}, kanamycin at 50 μg ml^{-1} and gentamycin at 15 μg ml^{-1}.

Mutagenesis and complementation of mutation

Two methods were used for mutant construction in this study. Genes *napA*, *nrfA* and *cymA* were deleted in-frame using a fusion PCR method with plasmid pDS3.0 as described earlier (Gao *et al.*, 2006a). Primers used for generating PCR products for mutagenesis are listed in Supplementary Table S1. To construct a *napA* in-frame deletion mutant, two fragments flanking *napA* were amplified by PCR with primers SO0848-5-F and SO0848-5-R, primers SO0848-3-F and SO0848-3-R, respectively. Fusion PCR products were generated by using the amplified fragments as templates with primers SO0848-5-F and SO0848-3-R, and ligated into the *Sac*I site of plasmid pDS3.0, resulting in the mutagenesis vector (pDS-NAPA). The vector was first introduced into *E. coli* WM3064 and then into MR-1 by conjugation. Integration of the mutagenesis construct into the chromosome was selected by gentamycin resistance and confirmed by PCR. Verified transconjugants were grown in LB broth in the absence of NaCl and plated on LB medium supplemented with 10% of sucrose. Gentamycin-sensitive and sucrose-resistant colonies were screened by PCR for the deletion of *napA*. The deletion mutation was then verified by sequencing of the mutated region, and the deletion strain was designated as JZ0848 (*ΔnapA*).

The same strategy was used for constructing *nrfA* and *cymA* in-frame deletion mutants with primers listed in Supplementary Table S1. The *napB* in-frame deletion mutants were generated using the *cre-lox* system as described elsewhere with primers listed in Supplementary Table S1, resulting in *ΔnapB* (Gao *et al.*, 2006b). Double mutants *ΔnapAΔnapB* and *ΔnapBΔnrfA* were constructed by introducing pDS-NAPA and pDS-NRFA into the *ΔnapB* mutant using the fusion PCR method, respectively.

Table 1 Strains and plasmids used in this study

Strain or plasmid	*Description*	*Reference or source*
E. coli strain		
WM3064	Host for *pir*-dependent plasmids and donor strain for conjugation; *ΔdapA*	Lab stock
S. oneidensis strains		
MR-1	Wild-type	Lab stock
JZ0845	*napB* deletion mutant derived from MR-1; *ΔnapB*::*loxP*	This study
JZ0848	*napA* deletion mutant derived from MR-1; *ΔnapA*	This study
JZ0845-0848	*napAnapB* double deletion mutant derived from MR-1; *ΔnapA ΔnapB*::*loxP*	This study
JZ0845-3980	*napBnrfA* double deletion mutant derived from MR-1; *ΔnapB*::*loxP ΔnrfA*	This study
JZ3980	*nrfA* deletion mutant derived from MR-1; *ΔnrfA*	This study
MR4591	*cymA* deletion mutant derived from MR-1; *ΔcymA*	This study
JZ0845-COM	JZ0845 with pBBR-NAP	This study
JZ0848-COM	JZ0848 with pBBR-NAP	This study
JZ0845-0848-COM	JZ0845-0848 with pBBR-NAP	This study
JZ0845-3980-COM0845	JZ0845-3980 with pBBR-NAP	This study
JZ0845-3980-COM3980	JZ0845-3980 with pBBR-NRFA	This study
JZ3980-COM	JZ3980 with pBBR-NRFA	This study
Plasmids		
pDS3.0	Ap^r, Gm^r, derivative from suicide vector pCVD442	Lab stock
pJK100	Allelic exchange vector	Lab stock
pCM157	*cre* expression vector	Lab stock
pDS-NAPA	pDS3.0 containing the PCR fragment for deleting *napA*	This study
pDS-NRFA	pDS3.0 containing the PCR fragment for deleting *nrfA*	This study
pDS-CYMA	pDS3.0 containing the PCR fragment for deleting *cymA*	This study
pJK-NAPB	pJK100 containing the PCR fragment for deleting *napB*	This study
pBBR1MCS-5	Gm^r vector used for complementation	Lab stock
pBBR-NAP	pBBR1MCS-5 containing *nap* and upstream promoter region from MR-1	This study
pBBR-NRFA	pBBR1MCS-5 containing *nrfA* and upstream promoter region from MR-1	This study

Complementing plasmids were constructed and used as a control to ensure that the phenotypes observed was because of the gene deletion, as described earlier (Gao *et al.*, 2008). All plasmids and primers used for PCR amplification were listed in Supplementary Table S1.

Physiological characterization of the mutant strains under anaerobic conditions

In this study, anaerobic growth was assayed in LB-1 (tryptone 10 g l^{-1}, yeast extract 5 g l^{-1}, NaCl 0.5 g l^{-1}, lactate 20 mM, pH = 7.0), derived from LB, supplemented with one of following electron acceptors: $NaNO_3$ (2 mM), $NaNO_2$ (2 mM), fumarate (20 mM), TMAO (20 mM), dimethyl sulfoxide (20 mM), MnO_2 (5 mM) and ferric citrate (10 mM). Although LB-l media support a faster growth rate and higher biomass than defined media M1, HEPES and MOPS as described earlier (Cruz-Garcia *et al.*, 2007), no contradictory results have ever been observed in this study. Furthermore, LB supplemented with lactate and electron acceptors have been used for physiological characterization of *S. oneidensis* under anaerobic conditions earlier (Gralnick *et al.*, 2005, 2006). To avoid interference by Cl^- in ion chromatography (IC) analysis, the final concentration of NaCl was reduced to 0.5%. Growth of *S. oneidensis* strains under anaerobic conditions was determined by monitoring an increase in OD_{600} in triplicate samples within a Bioscreen C microbiology reader (Labsystems, Helsinki, Finland).

Microarray analysis of MR-1 grown on nitrate vs fumarate

A total of 50 ml of LB-l, supplemented with either 2 mM sodium nitrate or 10 mM fumarate (control) as electron acceptors, was inoculated under anaerobic conditions to an OD_{600} of 0.01 and grown in an anaerobic chamber until mid-log phase ($OD \approx 0.15$ at 600 nm). Three cultures per electron acceptor, prepared independently as biological replicates, were centrifuged at 8000 rpm in a Sorvall RC5C plus for 3 min at the room temperature and the pellet was frozen immediately in liquid nitrogen and stored at −80 °C. Total RNA extraction, cDNA labeling, hybridization and slide scanning were conducted according to the standard procedure used in our laboratory (Gao *et al.*, 2004, 2008).

LOWESS was used to normalize the data set, which subsequently was applied to statistical analysis by analysis of variance (ANOVA) with Benjamini and Hochberg False Discovery Rate as multiple testing correction. Raw microarray data were deposited to gene expression omnibus (GEO) with the accession number GSE11198. In addition to the conventional two-color microarray analysis, which shows expression differences between two samples, the absolute expression value (signal

intensity) was calculated to determine absolute RNA levels in each sample. In this case, the signal intensity of each gene from all replicates was statistically analyzed as the data were obtained from the single dye microarray hybridization.

Determination of nitrate, nitrite and ammonium concentrations by Ion chromatography
At various time points, culture samples were collected and filtered with 0.2 μm filter and applied to IC. Nitrate and nitrite concentration in cultures was assayed using ICS-3000 with IonPac AS19 for nitrate and nitrite and ICS-1000 with IonPac CS12 for ammonium (Dionex, Sunnyvale, CA, USA). The eluents used were Na_2SO_4 at a concentration of 100 mM with a flow rate of 0.6 ml min^{-1} for ICS-3000 and methanesulfonic acid at a concentration of 20 mM with a flow rate of 0.6 ml min^{-1} for ICS-1000, respectively.

Reduction rates of nitrate to nitrite and nitrite to ammonium in whole cells
Cells grown under anaerobic conditions in LB-1 with 20 mM fumarate to the mid-log phase (OD ≈ 0.25 at 600 nm) were collected by centrifugation, washed twice with fresh LB-1 medium, and resuspended in LB-1 at the level of ~0.2 (OD_{600}). An aliquot of 200 μl was removed for determination of protein concentration with a bicinchoninic acid assay kit with bovine serum albumin as a standard according to the manufacturer's instruction (Pierce Chemical, Rockford, IL, USA). The nitrate/nitrite reduction reaction was initiated by the addition of 2 mM $NaNO_3$ or $NaNO_2$ to the assay medium. Aliquots of 200 μl were taken every 15 min up to 2 h, filtered with 0.2 μm filter and applied to IC for nitrate, nitrite and/or ammonium measurement as described above. The converting rates of nitrate to nitrite and nitrite to ammonium by whole cells were calculated by comparing the rate of disappearance of supplemented substrates and/or appearance of the corresponding products.

Competition assays in liquid media under anaerobic conditions
To prepare inocula for competition assays between the wild type and *ΔnapB* strains, anaerobic cultures of each strain were grown independently to stationary phase in LB-1 supplemented with 2 mM sodium nitrate to ~0.2 of OD_{600}. A total of 5 ml of each culture was mixed and taken as the sample of T0 and 100 μl of the same mixture was inoculated into 9.9 ml fresh LB-1 supplemented with 2 mM nitrate. After an incubation of 24 h, 100 μl of the competing cells was inoculated to fresh 9.9 ml of the same medium and the rest was taken as the sample of T1. The experiment was repeated the next day and the sample was collected as T2. In total, the procedure was repeated for 5 consecutive days. All samples were serially diluted with fresh LB and aliquots of 100 μl appropriate diluted samples were plated onto LB plates. A total of 100 colonies from plates containing 150–300 colonies were randomly picked and applied to colony PCR with primers listed in the Supplementary Table S1. Relative fitness, *W*, was calculated according to the method described earlier (Lenski *et al.*, 1991).

Results

Microarray analysis of S. oneidensis nap *and* nrf *genes*
The annotation of protein-coding genes in the *S. oneidensis* genome has changed over time (Heidelberg *et al.*, 2002; Daraselia *et al.*, 2003; Romine *et al.*, 2008). According to the original annotation, *S. oneidensis* possesses NAP encoded by operon *napDAGHB* (SO0845-9) and gene *napF* (SO1663) for nitrate reduction (Heidelberg *et al.*, 2002). In the case of nitrite reduction, the annotation shows a number of *NRF* genes, including *nrfA* (SO3980), *nrfF* (SO0477), *nrfGCD* (SO0482-4) and *nrfD-2* (SO4568). In the latest annotation, significant changes in the *nrf* genes have been made whereas the *nap* genes remain the same. On the basis of new transcriptional profiling data, the latest annotation related *nrfF* and *nrfGCD* to *c*-type cytochrome biogenesis and thiosulfate reduction, respectively (Beliaev *et al.*, 2005; Romine *et al.*, 2008). In addition to renaming *nrfD-2* (SO4568) *nrfD*, this annotation designates SO4570 and SO4569 next to *nrfD* (SO4568) on the chromosome as *nrfB* and *nrfC*. However, both of them are proposed to be pseudogenes because of truncation (Romine *et al.*, 2008).

To gain insights into the genes in nitrate reduction, transcriptional profiling was carried out using the *S. oneidensis* whole-genome cDNA microarray as described earlier (Gao *et al.*, 2004, 2008). A preliminary experiment was carried out to evaluate the toxicity of nitrate and nitrite on growth of *S. oneidensis*. In the presence of 2 mM nitrate, growth was not noticeably altered (data not shown). Although 2 mM nitrite delayed the initiation of growth ~2 h, it is impracticable to utilize a lower concentration which was unable to support detectable growth (data not shown). Therefore, for all experiments throughout the study we supplied nitrate and nitrite as the electron acceptors to the media at the level of 2 mM. Cells of MR-1 grown on 2 mM nitrate or 10 mM fumarate under anaerobic conditions were sampled at the exponential phase for the analysis. At the point of sampling, the concentrations of nitrate and nitrite were ~0.3 and 1.7 mM, respectively. The quality of the array data was statistically assessed using the method reported earlier (Gao *et al.*, 2004). Among the six annotated *nap* genes (*napDAGHB* and *napF*), the increased transcription of all members of operon *napDAGHB* was observed, whereas the transcription of *napF*

was unaffected (Figure 1). In the case of *nrf* genes, significantly increased transcription of *nrfA* was observed in our analysis but *nrfD*, along with its truncated partners *nrfC* and *nrfB*, was not affected, suggesting that NrfD may not be active in the nitrite reduction process.

In addition, the presence of nitrate had little influence on transcription of *nrfF* (SO0477) and *nrfGCD* (SO0482-4), predicted to be involved with nitrate/nitrite reduction in the original annotation but not in the latest annotation. All of these results suggest that the latest annotation is more likely to be correct with respect to genes in nitrate reduction.

NapA, but not NapB, is essential for nitrate respiration in S. oneidensis

A requirement for the *napA* gene in reduction of nitrate to nitrite of *S. oneidensis* has been reported recently (Cruz-Garcia *et al.*, 2007). In this study, we first examined whether the *napB* gene is essential for nitrate reduction. Our result showed that a *napB::loxP* strain was able to grow on nitrate (Figure 2a), indicating that the protein is not indispensable for the biological process. This is surprising given that the protein has long been regarded to be essential for the Nap system (Arnoux *et al.*, 2003; Tabata *et al.*, 2005). Interestingly, the *ΔnapB* strain grew to the maximum cell density earlier than the wild type although the maximum growth rates of both strains were not significantly different (Figure 2a). To confirm this, cultures of both strains were serially diluted and plated onto LB agar for colony counting (Figure 3). Compared with the wild type, the cultures of the *ΔnapB* strain started to be more populous in the window of ~7 h, from 5 to 12 h after inoculation. Eventually, the wild type reached the same level of cell densities. When nitrite was supplemented directly as the sole electron acceptor, the *ΔnapA* and *ΔnapB* strains exhibited a growth curve similar to that of the wild type (Figure 2b, data not shown), suggesting that

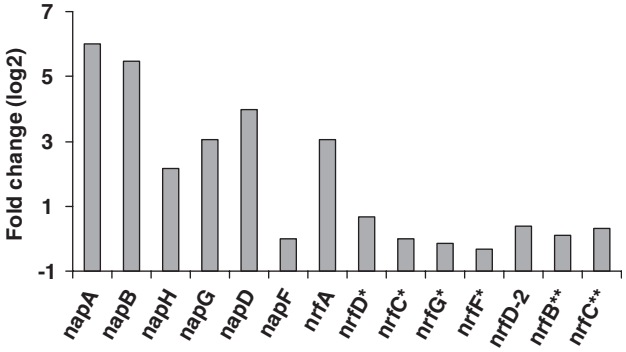

Figure 1 Transcription levels of predicted *nap* and *nrf* genes showed by the microarray analysis on MR-1 cells grown nitrate vs fumarate. Gene names used are from the TIGR annotation. Genes with a single asterisk marker have been renamed in the latest annotation. Genes with a double asterisk marker are degenerated.

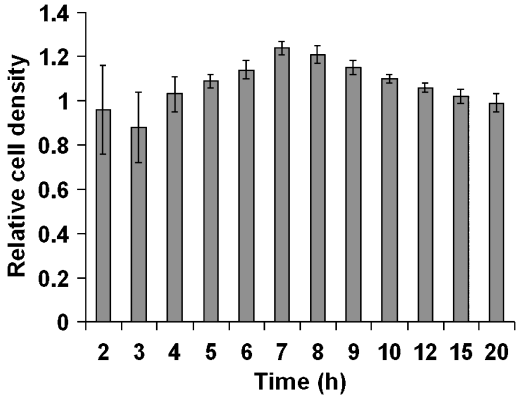

Figure 3 The cell densities of the wild type and *ΔnapB* strains grown on nitrate under anaerobic conditions. At each time point, cultures of each strain were taken, serially diluted and plated on LB agar for colony counting. The relative cell densities were calculated as the ratio of the number of the *ΔnapB* colonies to the number of the wild-type colonies.

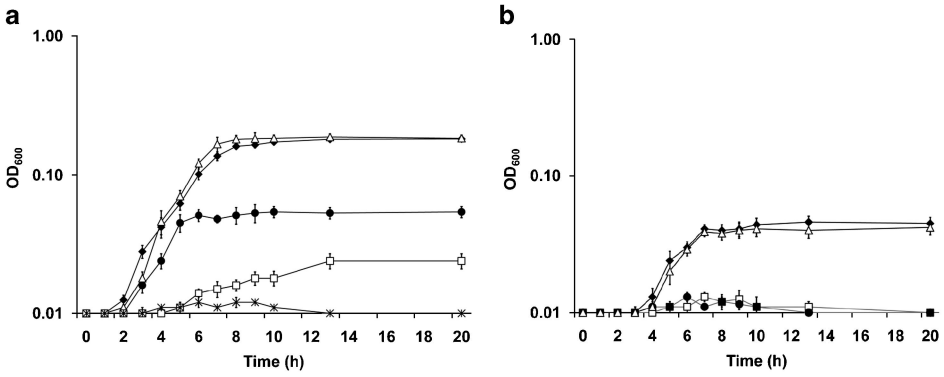

Figure 2 Growth of the wild type and mutant strains on nitrate and nitrite under anaerobic conditions. In both panels, MR-1 (♦), *ΔnapB* (△), *ΔnrfA* (●) and *ΔcymA* (□) are common. (**a**) Growth on 2 mM nitrate represented by OD_{600} readings. In addition to the common, *ΔnapA* (*) is shown. Like *ΔnapA*, *ΔnapAΔnapB* could not grow (not shown). (**b**) Growth on 2 mM nitrite represented by OD_{600} readings. In this panel, *ΔnapA* = *ΔnapAΔnapB* = *ΔnapB*, therefore *ΔnapA* and *ΔnapAΔnapB* were omitted for clarity. Experiments were performed in triplicate, and error bars indicate the standard deviation from the mean.

neither NapA nor NapB are required for nitrite reduction.

The unexpected result from the *ΔnapB* strain raises a possibility that *S. oneidensis* may possess another nitrate reduction pathway, which can only be functional in the absence of NapB. To test this hypothesis, we constructed a double mutant *ΔnapAΔnapB*. This strain, like the *ΔnapA* strain, failed to grow on nitrate or reduce nitrate to nitrite under anaerobic conditions (Figure 2a), suggesting that alternative nitrate reduction pathways may not exist in MR-1. To confirm this observation, the *napA* gene and the nap operon were independently cloned into plasmid pBBRMCS-5 for complementation. The ability of the *ΔnapAΔnapB* double mutant strain to grow on nitrate was restored by either of cloned DNA fragments (data not shown), indicating that NapA is the only nitrate reductase for converting nitrate to nitrite in *S. oneidensis*. In the following sections, results from all analyses on the *ΔnapA* strain and the *ΔnapAΔnapB* double mutant strain were practically the same and therefore the *ΔnapA* strain is used to represent both mutation strains unless otherwise noted.

NrfA is required for reduction of nitrite to ammonium in S. oneidensis

Although it is clear that the NAP system carries out reduction of nitrate to nitrite, whether the atypical NRF system of *S. oneidensis* is functional remains undefined experimentally. To this end, an *ΔnrfA* strain was constructed. Physiological characterization of this strain showed that the mutation in *nrfA* resulted in a severe defect in growth on nitrate compared with the wild type and completely failed to grow on nitrite (Figure 2b). For complementation, the *nrfA* gene on a plasmid restored the ability of the *ΔnrfA* strain to grow on nitrite (data not shown). These results indicate that NrfA is essential for reduction of nitrite. Interestingly, *S. oneidensis* showed substantially impaired growth on either nitrate alone (showed by the *ΔnrfA* strain on nitrate) or nitrite alone (showed by MR-1 on nitrite) compared with growth on both, suggesting that both steps of nitrate to ammonium reduction contribute to proton motive force for metabolic energy, resulting in higher biomass indicated by higher OD_{600} readings.

CymA is in place of the missing NapC and NrfH

Shewanella oneidensis genome lacks genes encoding proteins analogous to NapC and NrfH that specifically deliver electrons to the terminal reductases NapA and NrfA. Based on the fact that both NapC and NrfH known so far are membrane-bound *c*-type cytochrome proteins, it is most likely that the missing protein(s) is the same type. Earlier, CymA, a *c*-type cytochrome of 20.8 kDa, has been suggested to be the protein, playing both roles (Myers and Myers, 1997, 2000; Schwalb *et al.*, 2002, 2003). However, it is premature to assume that no other *c*-type cytochromes may be functionally in lieu of either NapC or NrfH given that *S. oneidensis* contains more than 40 *c*-type cytochromes and nine out of them are cytoplasmic membrane bound (Meyer *et al.*, 2004; Romine *et al.*, 2008). To gain insights into other candidate genes and/or verification of CymA in the process, we re-examined transcriptional profiles of MR-1 grown on nitrate *vs* fumarate (Table 2). Interestingly, none of the genes for the cytoplasmic membrane-bound proteins was induced significantly by nitrate over fumarate. However, *cymA* was transcribed at a level about five times higher than the average of all *c*-type cytochrome genes when either nitrate (31797/6520, signal intensity of *cymA*/average signal intensity) or fumarate (28154/5521) was used as the electron acceptor. The constitutive expression of *cymA* at the high level has been observed earlier when a variety of chemicals including oxygen were used as the sole electron acceptor, which has been suggested to be related to its pivotal role in electron transport (Myers and Myers, 2000; Beliaev *et al.*, 2005).

Although CymA is one of the most intensively investigated proteins by mutational analyses in *S. oneidensis*, its physiological function needs to be re-examined given its particular importance in anaerobic respiration. An in-frame deletion *cymA* mutation strain was constructed and this *ΔcymA* strain was unable to grow on fumarate, dimethyl sulfoxide, Fe(III), Mn(IV) or nitrite (Figure 2b), in agreement with earlier findings (Myers and Myers, 1997, 2000; Schwalb *et al.*, 2002, 2003). However, although the *ΔcymA* strain displayed a severe defect in growth on nitrate compared with the wild type, a small but noticeable increase in the OD_{600} reading was observed when compared with the *ΔnapA* strain (Figure 2a). Taken together, CymA is most likely the major and only proteins transferring electrons to NapA either through NapB or directly and to NrfA, respectively.

NapB is the preferred electron carrier from CymA to NapA but not to NrfA

The significant increase in the cell density of the *ΔnapB* strain at the early stages of growth was intriguing. To explore what occurred in the *ΔnapB* strain and the characteristics of the nitrate reduction pathway, the reduction rates of nitrate to nitrite and nitrite to ammonium by whole cells of the wild type, *ΔnapA*, *ΔnapB*, *ΔnapAΔnapB*, *ΔnrfA*, *ΔcymA* and *ΔnapBΔnrfA* strains were quantitatively calculated and compared. The rates were normalized to protein concentration of samples and presented in Table 3 and reduction dynamics were presented in Figure 4. In agreement with the finding earlier reported (Cruz-Garcia *et al.*, 2007), reduction of nitrate to ammonium in the wild type was in fact a two-step process, in which nitrite reduction would not start until

Table 2 Transcript profile of genes encoding *c*-type cytochromes

Genes	*Operon*[a]	*SIN (N)*[b]	*SIN (F)*[c]	*Ratio*[d]	*Location*[e]	*Function*
so0264 (scyA)		3764 ± 580	5532 ± 397	−0.56	P	Cytochrome *c*
so0479		586 ± 80	1321 ± 114	−1.17	P	Cytochrome *c*, putative
so0610 (petC)	*so0608-10*	2387 ± 720	7554 ± 2181	−1.66	CM	Ubiquinol-cytochrome *c* reductase,
so0714	*so0714-7*	505 ± 71	1030 ± 232	−1.03	P	Monoheme cytochrome *c*
so0716	*so0714-7*	825 ± 220	1461 ± 367	−0.82	P	Monoheme cytochrome *c*, putative
so0717	*so0714-7*	503 ± 88	1128 ± 201	−1.17	P	Monoheme cytochrome *c*
so0845 (napB)	*so0845-9*	14052 ± 1463	1414 ± 268	3.31	P	Cytochrome *c*-type protein NapB
so0939	*so0938-9*	698 ± 116	1224 ± 148	−0.81	P	Cytochrome *c*, putative
so0970		4870 ± 824	22912 ± 3870	−1.61	P	Fumarate reductase flavoprotein subunit
so1233 (torC)	*so1228-34*	777 ± 126	1775 ± 322	−1.19	CM	Tetraheme cytochrome *c*
so1413	*so1413-4*	1766 ± 2036	2296 ± 2368	−0.38	P	Tetraheme cytochrome *c*, putative
so1421 (ifcA-1)	*so1420-2*	3496 ± 528	7959 ± 850	−1.19	P	Fumarate reductase flavoprotein subunit
so1427	*so1427-30*	18735 ± 3429	33445 ± 4089	−0.84	P	Decaheme cytochrome *c*
so1659		1945 ± 170	4337 ± 466	−1.16	OM	Decaheme cytochrome *c*
so1777 (mtrA)	*so1776-82*	11415 ± 2816	14264 ± 2373	−0.32	P	Decaheme cytochrome *c* MtrA
so1778 (omcB)	*so1776-82*	40129 ± 8574	38532 ± 5552	0.06	OM	Decaheme cytochrome *c*
so1779 (omcA)	*so1776-82*	27585 ± 4753	29156 ± 3857	−0.08	OM	Decaheme cytochrome *c*
so1780 (mtrF)	*so1776-82*	1976 ± 301	5779 ± 858	−1.55	OM	Decaheme cytochrome *c* MtrF
so1782 (mtrD)	*so1776-82*	513 ± 83	1200 ± 217	−1.23	P	Decaheme cytochrome *c* MtrD
so2178 (ccpA)		28649 ± 6681	31323 ± 7079	−0.13	P	Cytochrome *c551* peroxidase
so2361 (ccoP)	*so2358-64*	3740 ± 565	4603 ± 709	−0.30	CM	Cytochrome *c* oxidase, *cbb3*-type, subunit III
so2363 (ccoO)	*so2358-64*	6954 ± 2053	6195 ± 1397	0.17	CM	Cytochrome *c* oxidase, *cbb3*-type, subunit II
so2727		6289 ± 1004	5978 ± 626	0.07	P	Cytochrome *c3*
so2930	*so2930-1*	734 ± 138	1860 ± 333	−1.34	P	Hypothetical diheme *c* protein
so2931	*so2930-1*	947 ± 185	2229 ± 298	−1.23	OM	Hypothetical diheme *c* protein
so3056	*so3056-8*	6711 ± 1994	6606 ± 1797	0.02	P	Tetraheme cytochrome *c*
so3300	*so3300-1*	3129 ± 644	3639 ± 783	−0.22	P	Cytochrome *c*
so3420	*so3420-1*	3949 ± 759	15547 ± 2243	−1.98	P	Cytochrome *c′*
so3623	*so3623-4*	1137 ± 164	1568 ± 230	−0.46	CM	Tetraheme cytochrome *c*
so3980		40603 ± 8196	7990 ± 581	2.35	P	Cytochrome *c552* nitrite reductase
so4047	*so4047-8*	8295 ± 1888	18965 ± 4231	−1.19	P	Cytochrome *c* family protein
so4048	*so4047-8*	19019 ± 4232	49309 ± 11985	−1.37	P	Cytochrome *c* family protein
so4142	*so4142-4*	760 ± 134	1353 ± 200	−0.83	P	Cytochrome *c* family protein
so4144	*so4142-4*	1148 ± 227	2631 ± 327	−1.20	P	Cytochrome *c*, putative
so4360	*so4357-60*	2329 ± 425	2677 ± 262	−0.20	P	Decaheme cytochrome *c*
so4484	*so4483-8*	10680 ± 4293	18022 ± 6453	−0.75	P	Cytochrome *c*-type protein Shp
so4485	*so4483-8*	3752 ± 1076	5921 ± 1835	−0.66	P	Diheme cytochrome *c*
so4570	*so4570-2*	408 ± 68	874 ± 156	−1.10	CM	Conserved domain protein
so4572	*so4570-2*	1501 ± 350	1907 ± 308	−0.35	CM	Cytochrome *c*, putative
so4591 (cymA)		37197 ± 5868	28154 ± 3821	0.40	CM	Tetraheme cytochrome *c*
so4606	*so4606-9*	1471 ± 286	5356 ± 418	−1.86	CM	Cytochrome *c* oxidase, subunit II
so4666 (cytcB)		19861 ± 3609	23408 ± 2669	−0.24	P	Cytochrome *c*

Abbreviations: CM, cytoplasmic membrane; P, periplasm; OM, outer-membrane.
[a]Structure of operons is based on operon prediction at www.microbesonline.org except those determined experimentally.
[b]Signal intensity from nitrate samples, the average: 6520.
[c]Signal intensity from fumarate samples, the average: 5521.
[d]Ratio of expression (nitrate/fumarate).
[e]Location of the protein.

nitrate was completely consumed (Figures 4a–c). When nitrate was used, nitrate reduction dynamics and rates of all *napA*$^+$ strains but *ΔnapB* were the same (Table 3). The *ΔnapB* strain was about 15% slower than other *napA*$^+$ strains in nitrate reduction (Figure 4a). It is particularly worth noting that the *ΔnapBΔnrfA* strain reduced nitrate as fast as the wild type (Figure 4a). Surprisingly, nitrite was not detected in the *ΔnapB* strain, along with all *napA*$^-$ strains (Figure 4b). Ammonium, however, reached the detectable levels in the *ΔnapB* strain sooner than in the wild type and was accumulated to the same level in the end as observed in the wild type (Figure 4c). This result suggests that the higher biomass of the *ΔnapB* strain at the early stage of growth may be because of the absence of nitrite toxicity. In addition, the nitrite reduction rate in the wild type was higher than the nitrate reduction rate. When nitrite was used, all strains except those without NrfA reduced nitrite at the same rate, including the *ΔnapB* strain whose nitrite reduction rate cannot be assessed when nitrate was supplemented as the sole electron acceptor (Table 3) (Figure 4d). This result indicated that the turnover rate of nitrite to ammonium was higher than that of nitrate to nitrite, which underlay that nitrite was below the detectable level in the *ΔnapB* culture when nitrate was used. In addition, this IC analysis on the *ΔcymA* culture samples confirmed that the strain indeed retained the ability to reduce nitrate although extremely weak but was unable to reduce nitrite at all (Figures 4a–d). Taken together, it is clear

Table 3 Nitrate and nitrite reduction rates in wild type and a variety of mutants[a]

EA	*Strain*	*Mean nitrate consumption rate*	*Mean nitrite production rate*	*Mean nitrite consumption rate*
NO_3^-	MR-1	27.52 ± 1.37	26.44 ± 1.82	37.64 ± 2.31
	ΔnapA	—	—	—
	ΔnapB	23.61 ± 1.46	ND	ND
	ΔnapAnapB	—	—	—
	ΔnrfA	28.15 ± 1.93	27.38 ± 1.85	—
	ΔnapBnrfA	27.72 ± 1.65	27.19 ± 1.28	—
	ΔcymA	1.82 ± 0.10	1.43 ± 0.12	—
NO_2^-	MR-1	NA	NA	35.03 ± 2.47
	ΔnapA	NA	NA	35.57 ± 2.02
	ΔnapB	NA	NA	34.99 ± 2.83
	ΔnapAnapB	NA	NA	36.00 ± 2.09
	ΔnrfA	NA	NA	—
	ΔnapBnrfA	NA	NA	—
	ΔcymA	NA	NA	—

Abbreviations: NA, not applicable; ND, no data.
[a]Rates are expressed as μmol of chemicals per mg proteins per hour.

that nitrite reduction of *S. oneidensis* is independent of either NapA or NapB, ruling out the possibility that NapB is able to work with both oxidoreductase NapA and NrfA.

NapB provides a fitness gain in nitrate reduction for S. oneidensis

Although the *ΔnapB* strain grown on nitrate exhibited a significant biomass increase, nitrate reduction rate in the *ΔnapB* strain was lower than that observed in the wild type. The competition assay has been widely used to determine whether an organism benefits from a gene in its genome, especially under the circumstance that deletion of the gene does not elicit a significant phenotype (Winzeler *et al.*, 1999; Giaever *et al.*, 2002). To test whether NapB provides an advantage in the bacterial growth on nitrate, a competition assay was carried out between the wild type and the *ΔnapB*

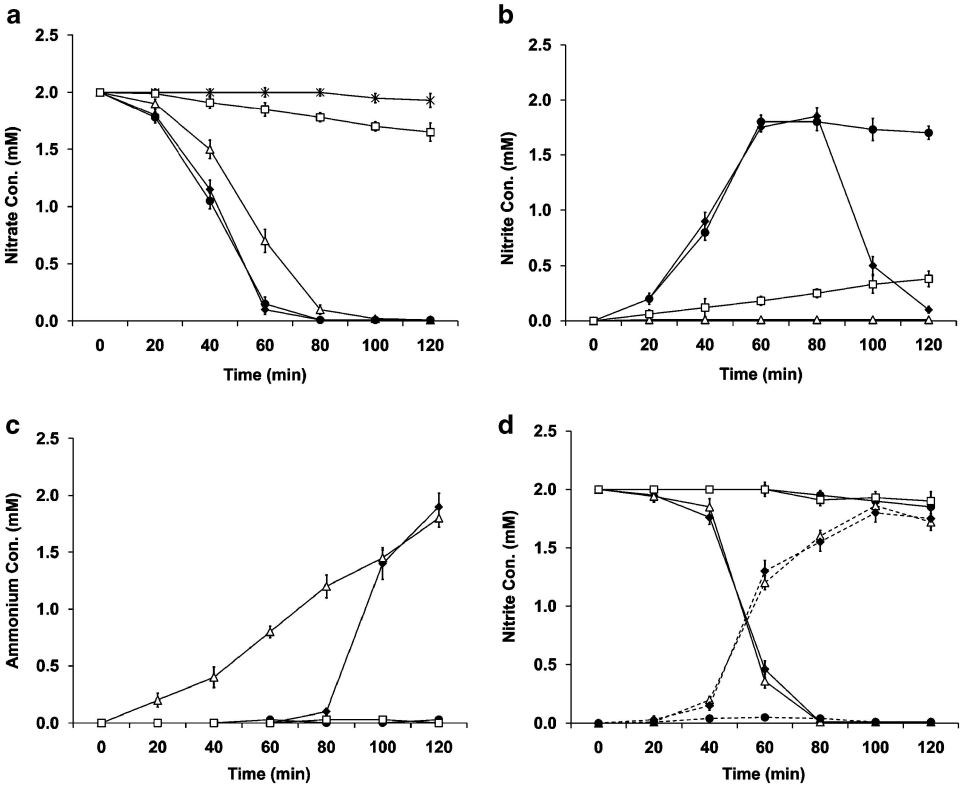

Figure 4 Nitrate and nitrite reduction by whole cells of the wild type and mutant strains. In all panels, MR-1 (♦), *ΔnrfA* (●), and *ΔnapB* (△) are common. Data presented in the panels ABC are from the same samples on 2 mM nitrate and presented in three panels for clarity. (**a**) The disappearance of nitrate from the assay media was measured by IC. In addition to the common, the panel includes *ΔnapA* (∗) and *ΔcymA* (□). In this panel, *ΔnapAΔnapB* = *ΔnapA* and *ΔnapBΔnrfA* = *ΔnrfA*, therefore *ΔnapAΔnapB* and *ΔnapBΔnrfA* were omitted for clarity. (**b**) The appearance of nitrite from the assay media was measured by IC. In addition to the common, the panel includes *ΔcymA* (□). In this panel, neither *ΔnapAΔnapB* nor *ΔnapA* is able to produce nitrate and *ΔnapBΔnrfA* = *ΔnrfA*, thus *ΔnapAΔnapB*, *ΔnapA*, and *ΔnapBΔnrfA* were omitted for clarity. (**c**) The appearance of ammonium from the assay media was measured by IC. In addition to the common, the panel includes *ΔcymA* (□). In this panel, *ΔnapAΔnapB* = *ΔnapA* = *ΔnrfA* = *ΔnapBΔnrfA* = *ΔcymA*. (**d**) The disappearance of nitrite (solid lines) and appearance of ammonium (dash lines) from the assay media on 2 mM nitrite were measured by IC. In addition to the common, the panel includes *ΔcymA* (□). In this panel, *ΔnapAΔnapB* = *ΔnapA* and *ΔnrfA* = *ΔnapBΔnrfA* = *ΔcymA*. Experiments were performed in triplicate, and error bars indicate the standard deviation from the mean.

strains. The results of competition experiments were presented in Table 4. In T0 samples, the average number of colonies was 276, of which 47.7% were identified by colony PCR (100 colonies examined per plate) to be the wild type. After 1-day competition, the percentage of the wild type increased to ~54.3%. After 5 days, the wild type made up to 81% of the population. The relative fitness values from T1 vs T0, T5 vs T0 and T5 vs T1 were 1.052, 1.069 and 1.074, respectively. This result indicates that NapB provides *S. oneidensis* a fitness gain in utilizing nitrate.

Discussion

Although *S. oneidensis* cells are able to employ both NAP and NRF to carry out a two-step process for reducing nitrate to nitrite and nitrite to ammonium, both systems are atypical, missing membrane-bound components NapC of NAP and NrfH of NRF. In this study, we verified that CymA completed two systems by transferring electrons to NapA through NapB or directly and NrfA. Interestingly, the in-frame *cymA* deletion strain still retained a noticeable capability of reducing nitrate to nitrite. Consistently, an *S. oneidensis ΔmenC* strain, which is defective in the synthesis of menaquinone, was able to grow on nitrate although the capability was impaired (Newman and Kolter, 2000). In contrast, growth did not occur in the presence of manganese oxide (MnO_2), fumarate, thiosulphate, sulphite, dimethyl sulfoxide or ferrihydrite ($Fe(OH)_3$) although reduction of all these electron acceptors depends on CymA. All these results suggest that (1) CymA transports electrons from the menaquinol pool to terminal reductases eventually, and (2) *S. oneidensis* possesses an alternative electron transfer pathway for nitrate reduction in the absence of CymA. A simple explanation is that *S. oneidensis* is able to use NapGH to transfer electrons to NapAB although much less efficiently. In *E. coli*, both NapG and NapH are not involved in menaquinol oxidation and instead form a quinol dehydrogenase that transfers electrons from ubiquinol through NapC to NapAB (Brondijk *et al*., 2002, 2004). However, NapGH of *Wolinella succinogenes* forms a menaquinol dehydrogenase as this organism does not synthesize ubiquinol (Kern and Simon, 2008). Therefore, a further investigation is much needed to explore the role of NapG and NapH in *S. oneidensis*.

One of the most striking findings was that the strain devoid of *napB* grew to the maximum cell density sooner but exhibited a slower nitrate reduction rate than the wild type. Given that NapB functions as an electron transfer subunit without a catalytic activity (Richardson, 2000), we propose that NapB can work with NapA only and is a preferred electron accepting protein from CymA. As shown in the conceptual model, CymA passes electrons to NapB when nitrate is available regardless of the presence of nitrite (Figure 5a). Under this condition, while accepting electrons from NapB, NapA is able to reduce nitrate to nitrite. Once nitrate is exhausted, electron flow to NapA either through NapB or directly will be blocked because of unavailability of the substrate (Figure 5a). In this case, CymA passes electrons to NrfA directly, enabling the latter to reduce nitrite to ammonium in the presence of nitrite. This explanation accounts for the two-step reduction of nitrate to ammonium observed in the wild type. In the absence of NapB, CymA delivers electrons to both NapA and NrfA simultaneously, resulting in a continuous reduction of nitrate to ammonium (Figure 5b). This proposal also explains the fast nitrate reduction in the *ΔnapBΔnrfA* stain.

Table 4 Relative fitness of strains as measured by competition assays

Samples	*No. of colonies*	*Percentage of MR-1*[a]	*Percentage of ΔnapB*[a]	*Relative fitness*
Day 0	276 ± 16	47.7	52.3	1.052 ± 0.012
Day 1	321 ± 23	54.3	45.7	1.069 ± 0.022
Day 5	288 ± 15	81	19	1.074 ± 0.019

[a]The averaged percentage of either the wild-type or mutant colonies identified by PCR (100 colonies per plate).

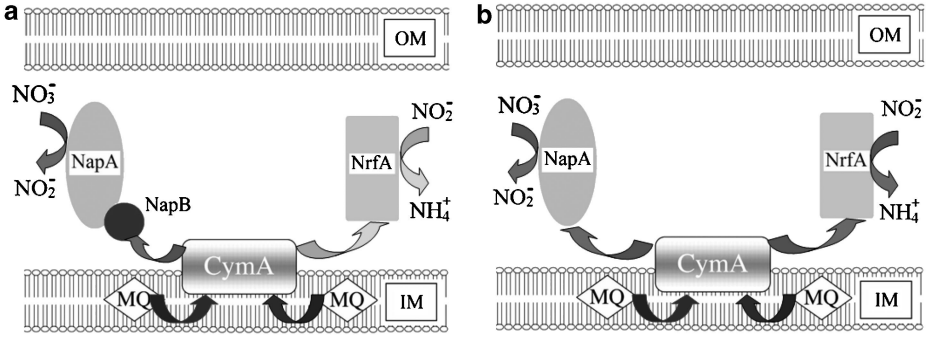

Figure 5 Model for reduction of nitrate to ammonium in *S. oneidensis*. Arrows represent pathway of electron flow. (**a**) The wild-type strain with nitrate or nitrite as the sole electron acceptor. The dark gray arrows represent the electron flow when nitrate is available. The light gray arrows represent the electron flow when nitrate is consumed completely or nitrite is used as the sole electron acceptor. (**b**) The *ΔnapB* strain with nitrate as the sole electron acceptor. The dark gray arrows represent the electron flow when nitrate is available.

When NrfA is not available, competition for electrons from CymA between NapA and NrfA collapses regardless of the presence of NapB.

Nitrite accumulation in *S. oneidensis* cells is dependent on NapB. Given that nitrite is much more toxic than nitrate to cells in general, it seems unexpected that *S. oneidensis* cells benefit from *napB* in the genome as showed by the competition assay. However, it may be perfectly reasonable in real environments where the amount of nitrate is extremely limited. By routing electrons to NapA only, NapB helps *S. oneidensis* to scavenge nitrate. Meanwhile, it is almost impossible for nitrite to reach a level higher enough to exert its negative influence on the physiology from nitrate reduction.

Microorganisms are generally able to utilize a variety of inorganic and organic matters in environments because of the availability of corresponding terminal enzymes and pathways (Schmidt *et al.*, 2003). In most cases, membrane-bound electron transport proteins are highly specific for their terminal enzymes, that is, NapC for NapAB, NirT for NirS, NrfH for NrfA, to name a few. Although *S. oneidensis* is renowned for its unusually diverse respiratory metabolism, the number of specific membrane-bound electron transporters to terminal reductases is surprisingly small. To solve this dilemma, the bacterium sets CymA in a branch-point position for multiple pathways. Unlike specific NapC or NrfH, promiscuous CymA seems to evolve an ability to interact with several terminal reductases evidenced by that *S. oneidensis* can utilize both fumarate and dimethyl sulfoxide simultaneously (unpublished results). In this study, we clearly showed that NapB enables CymA to determine the hierarchy of electron acceptor use. This is presumably because of the biochemical characteristics of NapB. In *Haemophilus influenzae*, the midpoint reduction potentials of two haem groups of NapB are unexpectedly low, resulting in a large thermodynamic advantage for drawing electrons from the menaquinone pool (Brigé *et al.*, 2001). In *Rhodobacter sphaeroides*, the association of NapA with NapB results in a structural arrangement such that heme I of NapB is left exposed to NapC for electrons. Heme I has a lower midpoint potential and thus is a more favorable pathway for electron flow from NapC (Arnoux *et al.*, 2003). In the case of *S. oneidensis*, NapB diverts electrons from CymA to NapA exclusively and resulting in a more effective NapA. Such a mechanism may help the bacterium in scavenging low concentrations of nitrate in the environment. The scenario may be more general given that several other pathways recruit small *c*-type cytochromes to get electrons from CymA (Schwalb *et al.*, 2002).

The fact that multiple pathways share CymA is common among studied *Shewanella* (Murphy and Saltikov, 2007). Interestingly, the numbers of pathways sharing CymA in *S. oneidensis*, *Shewanella* sp. ANA-3 and *Shewanella putrefaciens* CN-32 are 6, 5 and 4, respectively, with those for metal reduction in common (Murphy and Saltikov, 2007). This offers a possibility that *Shewanella* may use CymA for many pathways at the beginning and acquire dedicated electron transfer proteins for some of these pathways with time. If this holds, *S. oneidensis* may represent a preliminary with respect to pathway evolution.

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