Insights on nitrate respiration by *Shewanella*

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Shewanellae are well known for their ability to utilize a number of electron acceptors and are therefore considered to have important roles in element cycling in the environment, such as nitrogen cycling through dissimilatory nitrate reduction to ammonia (DNRA) and denitrification. Possessing two periplasmic nitrate reductase systems (NAP-α and NAP-β) is a special trait of the *Shewanella* genus, and both enzymes are likely to provide selective advantage to the host. This review relates the current knowledge and aspects of the nitrate respiration system of *Shewanella*. Specifically, the potential physiological functions and regulation mechanisms of the duo-NAP system are discussed in addition to the evolution of anaerobic respiration systems of *Shewanella*.

Keywords: *Shewanella*, NAP, nitrate

INTRODUCTION

Shewanella species are widespread in diverse environments and have major environmental relevance (Fredrickson et al., 2008). They are commonly found in food, freshwater, hydrothermal vents, cold water, deep-sea sediments, and even fields contaminated with arsenic, chromium (VI), and explosives (Viamajala et al., 2004; Saltikov et al., 2005; Zhao et al., 2005; Gao et al., 2006). *Shewanella* utilize a number of electron acceptors for energy sources, such as nitrate, nitrite, thiosulfate, elemental sulfur, iron oxide and manganese oxide (Fredrickson et al., 2008). Among these electron acceptors, nitrate is considered to be the preferred acceptor for anaerobic respiration by microorganisms due to its relatively high standard redox potential, with a powerful ability to generate energy, and its abundance in aquatic environments (Richardson, 2000).
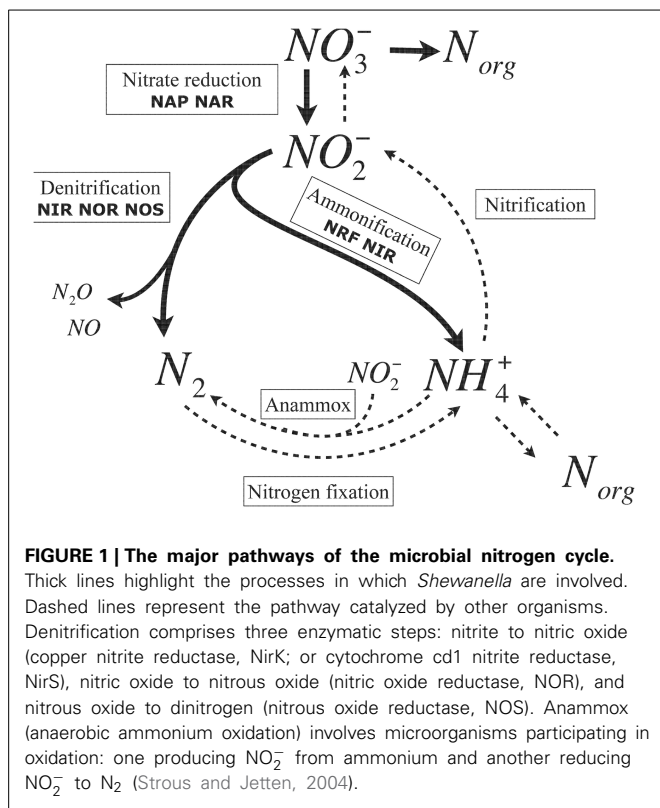
Briefly, the microbial nitrogen cycle involves biotic chain reactions comprising nitrogen fixation, dissimilatory nitrate reduction to ammonia (DNRA), denitrification, nitrification and anammox (anaerobic ammonium oxidation) (**Figure 1**). Denitrification has been intensely studied in recent decades and is largely assumed to be the major pathway for nitrate removal (Wallenstein et al., 2006; Kraft et al., 2011). In contrast to denitrification, knowledge on DNRA is mostly lacking.

To the best of our knowledge all *Shewanella* species are able to reduce nitrate. Most species reduce nitrate to nitrite and then to ammonia via the DNRA pathway, though a few species, such as *S. denitrificans* and *S. amazonesis*, carry out respiratory denitrification where nitrate is reduced to dinitrogen via nitrite, nitric oxide and nitrous oxide (Venkateswaran and Dollhopf, 1998; Brettar et al., 2002). Moreover, a notable number of Shewanellae are amenable to genetic manipulation, which makes detailed genetic studies on nitrate reducing pathways feasible. At the time of the writing of this review, 25 complete genomes of *Shewanella* were available at NCBI.

Many genes of the nitrate respiration system in *Shewanella* are identified as having multiple copies. The reduction of nitrate to nitrite is catalyzed by one of two distinct enzyme systems: the periplasmic nitrate reductase system (NAP) and the membrane-bound nitrate reductase system (NAR). Most *Shewanella* have duo-NAP (Nap-α and Nap-β) systems, which have been documented (Simpson et al., 2010b). Additionally, five strains (*S. halifaxensis* HAW-EB4, *S. sediminis* HAW-EB3, *S.* sp. MR-4, *S* sp. MR-7 and *S. woodyi* ATC51908) have both NAP and NAR systems, as listed in **Table 1**. Moreover, many genomes of *Shewanella* contain several nitrite reductase genes; these will be discussed below.

At the genetic level, horizontal gene transfer (HGT), gene duplication and loss of gene function are common strategies by microorganisms during evolution to adapt to environmental stress. Previous studies have suggested that *Shewanella* might acquire many new adaptive strategies through step-wise processes: the acquisition of mobile islands, selection of islands carrying ecologically important genes and loss of mobile and ecologically unimportant genes (Konstantinidis et al., 2009). Many

FIGURE 1 | The major pathways of the microbial nitrogen cycle. Thick lines highlight the processes in which *Shewanella* are involved. Dashed lines represent the pathway catalyzed by other organisms. Denitrification comprises three enzymatic steps: nitrite to nitric oxide (copper nitrite reductase, NirK; or cytochrome cd1 nitrite reductase, NirS), nitric oxide to nitrous oxide (nitric oxide reductase, NOR), and nitrous oxide to dinitrogen (nitrous oxide reductase, NOS). Anammox (anaerobic ammonium oxidation) involves microorganisms participating in oxidation: one producing NO_2^- from ammonium and another reducing NO_2^- to N_2 (Strous and Jetten, 2004).

genes of the nitrate respiration system in *Shewanella*, e.g., duo-NAP (Nap-α and Nap-β) systems, are identified as multi-copy (Simpson et al., 2010b), and the additional gene clusters that encode the same enzymes are likely to confer selective advantages to the host rather than be functionally redundant (reviewed and discussed below).

In this review, the current knowledge of the *Shewanella* nitrate respiration system will be reviewed with a specific focus on physiological functions, regulation mechanisms and evolutionary origin. This knowledge will certainly help in the better understanding of nitrate reduction pathways and the impact of HGT on the evolution of anaerobic respiration.

DENITRIFICATION AND AMMONIFICATION BY THE *SHEWANELLA* GENUS

Denitrification is a respiratory process in which nitrite is first reduced to nitric oxide by nitrite reductase (NIR), nitric oxide reductase (NOR) then catalyzes nitric oxide reduction to nitrous oxide, and dinitrogen is synthesized from the reduction of nitrous oxide by nitrous oxide reductase (NOS). This process occurs in three separate reactions, and a proton-motive force is independently generated by each reaction. *S. denitrificans* possesses all sets of genes involved in denitrification in its genome (Genebank accession: NC_007954.1), and its capability of denitrification was confirmed by physiological analysis (Brettar et al., 2002). In addition to *S. denitrificans*, two other *Shewanella* species are potentially capable of denitrification. Although no growth on

Table 1 | Membrane-bound nitrate reductase (NarG), periplasmic nitrate reductase (NAP) and nitrite reductase (denitrification, NirK; ammonification, NrfA, NirB) in the genomes of *Shewanella* species.

Strains	Nitrate reduction NAR	Denitrification NAP	DNRA NirK	NOR	NOS	NRF	NirB
S. amazonensis SB2B		αβ	+	+		+	
S. baltica OS155		αβ				+	
S. baltica OS183		αβ				+	
S. baltica OS185		αβ				+	
S. baltica OS195		αβ				+	
S. baltica OS223		αβ				+	
S. benthica KT99		αβ		+		+	+
S. denitrificans OS217		α	+	+	+		+
S. frigidimarina NCIMB 400		αβ		+		+	+
S. halifaxensis HAW-EB4	+	α		+		+	
S. loihica PV-4		αβ	+	+	+	+	
S. oneidensis MR-1		β				+	
S. pealeana ATCC 700345		αβ				+	+
S. piezotolerasn WP3		αβ		+		+	+
S. putrefaciens 200		αβ		+		+	
S. sediminis HAW-EB3	+	αβ		+		+	+
S. sp. ANA-3		αβ		+		+	
S. sp. HN-41		αβ		+		+	
S. sp. MR-4	+	αβ		+		+	
S. sp. MR-7	+	αβ		+		+	
S. sp. W3-18-1		αβ		+		+	
S. violacea		α		+			+
S. woodyi ATCC 51908	+	αβ		+		+	+

"+" Represent these genes are identified from the genome.

nitrate or nitrite was observed, *S. loihica* possesses the entire set of genes for denitrification (Gao et al., 2006). *S. amazonesis* was reported to denitrify nitrate to dinitrogen (Venkateswaran and Dollhopf, 1998); however, the genome of *S. amazonesis* lacks the gene encoding the NOS enzyme, which catalyzes the last step of denitrification, reducing nitrous oxide to dinitrogen. As the enzymatic mechanisms of dinitrogen synthesis in *S. amazonesis* have not yet been established, *S. amazonesis* is not a typical denitrifying bacterium. In addition, the nitrite reductase gene *nrfA* within DNRA pathway has been discovered in the genomes of *S. amazonesis* and *S. loihica*. Advanced studies on the function of the *nrf* gene in these two potential denitrifying bacteria will be interesting because to the best of our known, no strain reported so far is able to perform both denitrification and DNRA.

In DNRA, nitrite is reduced directly to ammonia by nitrite reductase (NrfA or NirB) without any intermediate; energy is also obtained by generating an electrochemical proton potential across the membrane. The pentaheme cytochrome NrfA is a membrane-associated respiratory enzyme that couples to membrane-associated formate-oxidizing enzymes via quinones to generate the membrane potential (Simon, 2002). NirB is an iron-sulfur protein that carries FAD and a siroheme cofactor (Jackson et al., 1981). The function of NirB was assigned primarily to the detoxification of nitrite produced by cytoplasmically oriented membrane-bound nitrate reductase (NarGHI) (Cole, 1996). However, the NirB enzyme may function as a nitrite-scavenger rather than a respiratory enzyme because energy is not conserved during nitrite reduction by NADH (Simon, 2002). For example, in *E. coli*, the *nrfA* operon is only induced under low-nitrate concentrations, whereas the *NirB* nitrite reductase is optimally synthesized only when nitrate or nitrite is in excess (Wang and Gunsalus, 2000). Most shewanellae carry out DNRA because their genomes have either the *nrfA or nirB* gene (or both) and lack genes for denitrification.

Notably, there are some indications that nitrous oxide is also generated as a byproduct of DNRA (Cruz-García et al., 2007; Vermeiren et al., 2009), though the mechanisms by which non-denitrifying bacteria generate nitrous oxide have not been revealed. One reasonable interpretation is that nitrous oxide is generated by nitrate-dependent iron reduction because the abiotic reduction of nitrite to nitrous oxide was observed in *S. putrefaciens* when both nitrate and iron (III) were available (Coby and Picardal, 2005).

PERIPLASMIC AND MEMBRANE-BOUND NITRATE REDUCTASE (NAR) IN *SHEWANELLA*

Although the subsequent reactions involve different enzymes and intermediates, the first steps of DNRA and denitrification are identical and can be carried out by the same enzyme: periplasmic nitrate reductase (NAP) or membrane-bound nitrate reductase (NAR). All the available *Shewanella* genomes contain NAP, and only 5 strains out of 23 (*S. halifaxensis* HAW-EB4, *S. sediminis* HAW-EB3, *S.* sp. MR-4, *S* sp. MR-7 and *S. woodyi* ATC51908) possess both the NAP and NAR systems (**Table 1**). Although not well understood, NAR and NAP may also be of physiological significance in addition to nitrate reduction. In *Shewanella* species, the understanding of the NAP system is more

comprehensive and therefore is selected for discussion in greater detail in this review.

Nap genes are widely distributed in gram-negative bacteria, and our understanding on the NAP system of *Shewanella* is mainly derived from research in model organisms such as *E. coli*. The current understanding of the subunits involved in the NAP system of the *Shewanella* genus is that the NAP system comprises seven compounds: NapA-H (except for NapF). NapA is the catalytic subunit where nitrate is reduced to nitrite, whereas NapB serves as the direct electron transporter, without a catalytic function. NapC is a quinol dehydrogenase protein, transporting electrons from both ubiquinol and menaquinol to terminal reductase NapAB (Simon and Kern, 2008). When NapC is missing, protein CymA performs the function of NapC, which will be discussed later. The electron transport model of NapABC and CymA is shown in **Figure 3**. NapGH couples electron transfer from the quinol pool via NapB to NapA *in E. coli* (Brondijk et al., 2002, 2004). However, it seems functionally redundant to retain NapGH in the few species of *Shewanella* that also possess NapC and/or CymA. It was suggested that NapGH might mediate the alternative pathway for nitrate reduction in addition to NapC or CymA (Gao et al., 2009), though the physiological significance of this alternative pathway is unclear.

GENE ARRANGEMENT AND PROTEIN STRUCTURE DIFFERENCES BETWEEN NAP-α AND NAP-β

One of the apparent differences between NAP-α and NAP-β is the arrangement of their gene clusters. Briefly, NAP-α (*napED-ABC*) could be defined as a *napC*-dependent system, with NAP-β (*napDAGHB*) being *napC* independent. In *S. oneidensis,* which lacks NapC, the NapC homolog CymA mediates electrons from the quinone pool to both NapAB-β and NrfA (Cruz-García et al., 2007; Gao et al., 2009). In *S. piezotolerans*, which possesses both NAP-α and NAP-β, CymA is involved in both the reduction of nitrate to nitrite and nitrite ammonification (Chen et al., 2011). Furthermore, the different gene arrangements indicate the structural distinction between NAP-α and NAP-β proteins, which will be discussed below.

NAP could be divided into two types: monomeric and heterodimeric NAP. Both NAP-α and NAP-β are components of heterodimeric NAP, which is considered more evolved than monomeric NAP. In heterodimeric NAP, all NapA monomers form a heterodimeric complex with NapB. In contrast, monomeric NapA does not form a complex with the redox partner NapB; instead, a small tetraheme cytochrome C (∼10 kDa), called NapM, may be the direct electron donor for NapA (Marietou et al., 2005). The monomeric class of NapA is considered to be the evolutionary link between monomeric NAS (assimilatory nitrate reductase) and heterodimeric NAP and thus a more ancient protein compared with heterodimeric NAP (Stolz and Basu, 2002; Jepson et al., 2006).

PHYSIOLOGICAL SIGNIFICANCE OF THE duo-NAP SYSTEM OF *SHEWANELLA*

The existence of multiple nitrate reductases within *Shewanella* is not as functionally redundant as it appears. By investigating the NAP system of *Shewanella* and other nitrate reducers, we can find

some clues to the functional importance of duo-NAP, especially when the host is under environmental stress.

SCAVENGING A LIMITED CONCENTRATION OF NITRATE

Despite the fact that the majority of organic matter degradation in marine shelf sediments is coupled to sulfate reduction, the reduction of nitrate yields more energy than that of sulfate, and the former is therefore consumed preferentially (Jørgensen, 1982). It has been proposed that NAP may assist bacteria in scavenging nitrate in environments in which the amount of nitrate is extremely limited. For example, in *E. coli,* which possesses two NAR (NarGHJI and NarZYWV) and one NAP (NapFDAGHBC) enzyme, the NAP enzyme is primarily expressed at low nitrate concentrations and may assist nitrate respiration via NarGHI when the bioenergetic efficiency of nitrate respiration via the NAR enzyme is limiting (Potter and Cole, 1999; Stewart et al., 2002). In addition to *E. coli,* five species of *Shewanella* harbor both NAP and NAR, as mentioned above (**Table 1**), and similar mechanisms may be utilized by shewanellae.

Furthermore, NapB and NapC are suggested to support the scavenging of nitrate by transferring electrons specifically to NapA. For example, *S. oneidensis* MR-1 cells have only one NAP (NAP-β), and the function of NapB has been proposed as the scavenging of nitrate by routing electrons to NapA only, therefore gaining a benefit during a competition assay test (Gao et al., 2009). Another example is *S. piezotolerans* WP3, which harbors a duo-NAP system (NAP-α and NAP-β). The NapC of NAP-α in WP3 transports electrons to NapAB exclusively, specifically providing a growth advantage to the host (Chen et al., 2011). Overall, both NapB and NapC may support the host in coping with nitrate-limiting conditions by routing electrons exclusively to NapA.

MAINTAINING REDOX BALANCE

During chemoheterotrophic growth on reduced carbon compounds, the substrate must be oxidized to a level at which it can be assimilated (Richardson, 2000). During the oxidation process of carbon compounds, reductants are released and partially utilized for ATP synthesis. However, when reductants are in excess, a deposition system must be available. The NAP-α of the duo-NAP system is thought to help maintain this redox balance (Simpson et al., 2010a,b), as implicated in early studies on denitrifying bacteria. For example, in *Paracoccus denitrificans, Paracoccus pantotrophus* and *Rhodobacter capsulatus,* the expression of *NapABC* was found to be stimulated when reduced carbon compounds (such as butyrate), rather than oxidized carbon compounds (such as succinate and malate), were supplied as a carbon source (Sears et al., 2000; Gavira et al., 2002). Thus, the NAP-α system controls the redox balance by using nitrate as an ancillary oxidant to dissipate any excess reductant.

FACTORS REGULATING THE duo-NAP SYSTEM

The distinct physiological functions of the NAP system indicate that NAP-α and NAP-β may have multiple regulation mechanisms. Studies with *S. oneidensis* suggest that anaerobic respiration systems containing NAP are regulated by the electron transport regulator (EtrA) and cyclic AMP receptor protein (CRP),

which control anaerobic respiratory gene expression individually (Maier and Myers, 2001; Beliaev et al., 2002; Saffarini et al., 2003).

EtrA is a global transcriptional regulator involved in the regulation of aerobic and anaerobic metabolism, and it shares 73.6% amino acid sequence similarity with the oxygen-sensing regulator Fnr in *E. coli* (Saffarini and Nealson, 1993). In mutant strains of *S. oneidensis* with *etrA* gene deletion, the expression of *nap* genes (*nap*-β) was significantly repressed (Cruz-García et al., 2011). CRP is a well-known regulator of carbon metabolism (Botsford and Harman, 1992), but CRP is essential for anaerobic growth in *S. oneidensis,* and a *crp* gene-deleted strain is defective in nitrate reduction (Saffarini et al., 2003; Gao et al., 2008). According to previous studies, CRP lacks an apparent redox-sensing domain, and it is still unclear which CRP is activated under anaerobic conditions (Fredrickson et al., 2008). Moreover, the *nap, nar, nrf,* and *nir* gene operons are further controlled by the NarQ-P two-component system. NarQ senses the presence of nitrate/nitrite, the response regulator NarP is then phosphorylated; finally, phospho-NarP activates the transcription of *nap* gene operons (Darwin and Stewart, 1995). Other environmental factors that may also influence duo-NAP system regulation include the carbon source, temperature, and oxygen concentration.

CARBON SOURCE

We investigated the upstream regions of *nap* gene operons from 23 genomes of *Shewanella* and confirmed that there were no apparent Crp sites in the upstream region of *nap*-α (**Figure 2**). In all cases, the upstream control regions of *napDAGHB (nap*-β*)* contain NarP, EtrA, and Crp sites, whereas *napEDABC (nap*-α*)* contain sites for NarP and EtrA. The above findings suggest that NarP-Q, CRP, and EtrA are likely to be involved in the regulation of the duo-NAP system and that the *nap*-α operon may not belong to the CRP regulation system, which is a well-known regulator associated with carbon metabolism (Botsford and Harman, 1992). Therefore, the transcription of the *nap*-β gene operon may respond to different types of carbon sources, such as the reduced carbon compounds and oxidized carbon compounds mentioned above.

LOW TEMPERATURE

Almost all species of *Shewanella* can thrive under cold temperature (2~4°C), which is one of the hallmark features of this genus. Recently, the influence of low temperatures on the duo-NAP system was studied in *S. piezotolerans* WP3 (Chen et al., 2011). Under conditions of 4°C anaerobic nitrate respiration, the transcription of *napDABC (nap*-α*)* was fivefold higher relative to its transcription at 20°C. Moreover, the transcription of *napDAB (nap*-β*)* was dramatically reduced compared to that at 20°C. However, no typical cold-shock box sequence was found in the potential control regions of the *nap* gene operons (Chen et al., 2011). A gene encoding a DEAD box RNA helicase (HrpA) is located upstream of the *nap*-α operon in almost all *Shewanella* species (excluding *S. denitrificans* and *S. frigidimarina*) (Chen et al., 2011). RNA helicases have been observed to be overexpressed at low temperature in many psychrophilic microorganisms and help to unwind
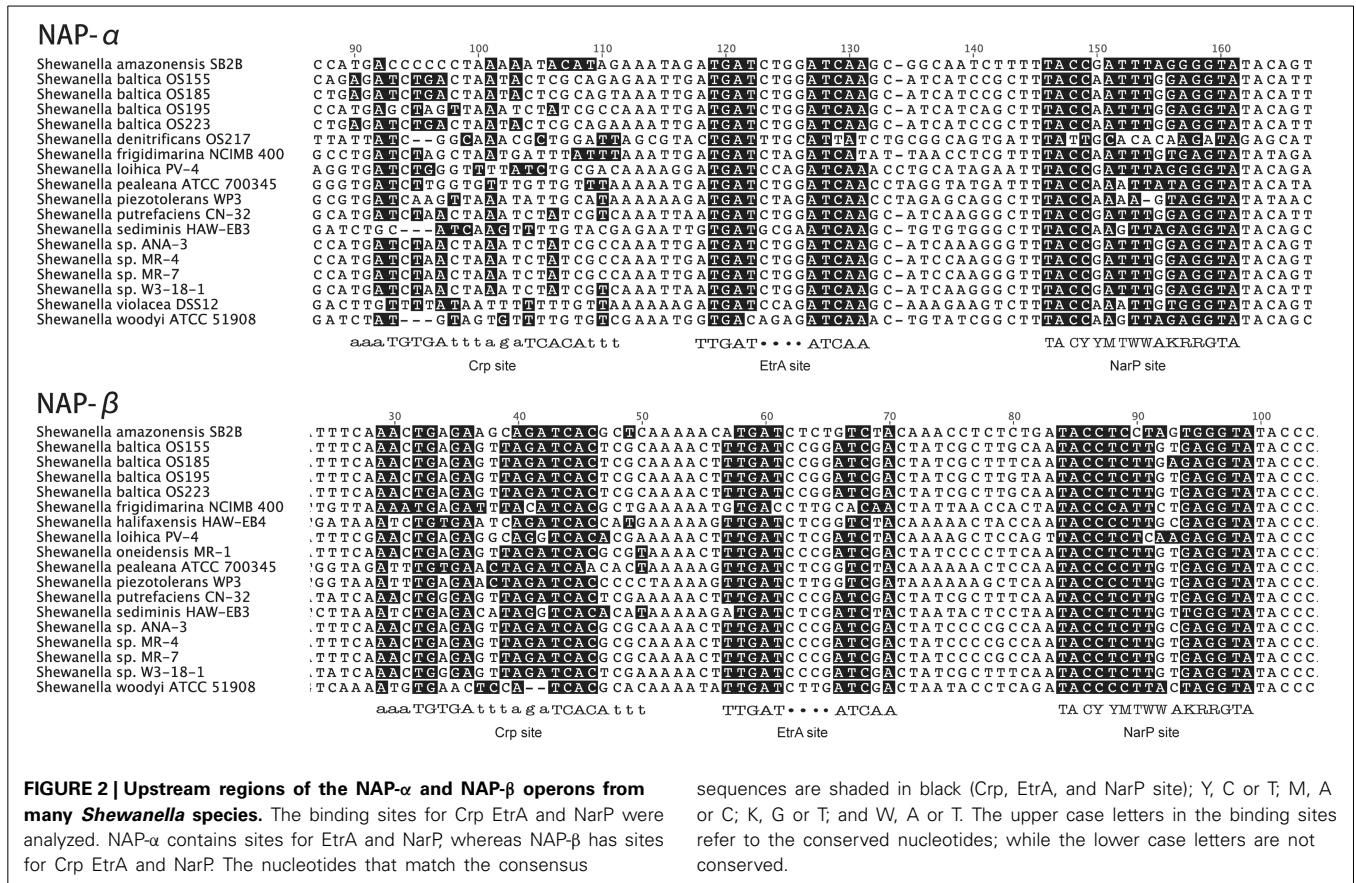
FIGURE 2 | Upstream regions of the NAP-α and NAP-β operons from many *Shewanella* species. The binding sites for Crp EtrA and NarP were analyzed. NAP-α contains sites for EtrA and NarP, whereas NAP-β has sites for Crp EtrA and NarP. The nucleotides that match the consensus sequences are shaded in black (Crp, EtrA, and NarP site); Y, C or T; M, A or C; K, G or T; and W, A or T. The upper case letters in the binding sites refer to the conserved nucleotides; while the lower case letters are not conserved.

RNA secondary structures for efficient translation under cold conditions (Cartier et al., 2010; Piette et al., 2011). Therefore, the HrpA protein of *Shewanella* may be involved in the regulation and mRNA processing of NAP-α at low temperatures. Under low temperature, the rates of biochemical reactions usually decrease. And the NAP-α is proposed a more efficient system on electron transporting (Chen et al., 2011). As a consequence, up-regulation of NAP-α may confer advantages to Shewanella in the cold environment.
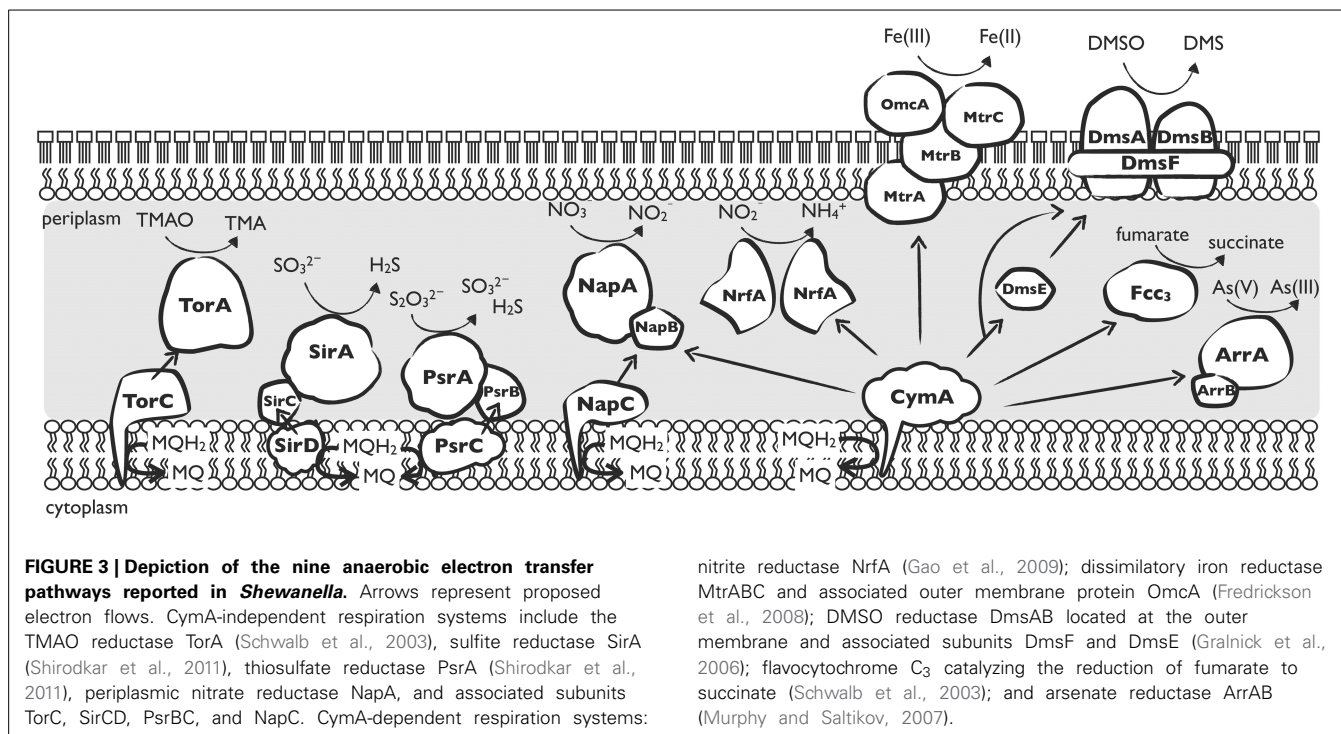
OXYGEN

Most *Shewanella* are facultative anaerobes, and oxygen levels are proposed to be another factor regulating the expression of the duo-NAP system (Simpson et al., 2010b). Studies of *Shewanella* genomes show that most *Shewanella* species have both ubiquinones and menaquinones. Ubiquinones are considered to be "aerobic quinol" (Soballe and Poole, 1999), and menaquinones are suggested to be "anaerobic quinol" (Maier and Myers, 2001) (Simpson et al., 2010b). *S. oneidensis* mutants deficient in menaquinone and methylmenaquinone, but having wild-type levels of ubiquinone, lose the ability to utilize nitrate, iron(III) and fumarate as electron acceptors (Myers and Myers, 1993). These results indicate that the single NAP-β of *S. oneidensis* may draw electrons from menaquinone. Aerobic nitrate reduction *via* NAP is of physiological significance. It could be a self defense mechanism for the cell converting nitrate to nitrite that is very toxic. And it also may provide poorly coupled route for the

oxidation of highly reduced carbon sources in order to poise the redox balance (Sears et al., 2000). Studies on the sources of electrons and the transporter of electrons in NAP-α and NAP-β may further elucidate the influence of oxygen on the activation of the duo-NAP system.

In summary, the duo-NAP system in Shewanella has distinct physiological functions, would confer the cells advantages in scavenging nitrate when it's limited. Various environmental factors including carbon sources, temperature, and oxygen might influence the regulation of duo-NAP system. It's generally understood that these regulations are required to maintain/control the redox balance within the cells, such as using nitrate as an ancillary oxidant to dissipate any excess reductant. The detail molecular mechanisms are still waiting to be elucidated.

THE ORIGIN OF THE duo-NAP SYSTEM

As discussed above, intensive studies have focused on the regulating and operating mechanisms of the duo-NAP system. However, little is known about the origin of this system; for example, how did the duo-NAP system of the *Shewanella* genus arise during evolution? Based on current understanding, the duo-NAP system is more likely to have resulted from HGT as opposed to gene duplication. Indeed, on the phylogenetic tree constructed with NapA protein sequences, NapA-β of *Shewanella* forms a relatively independent branch, while NapA-α are more closely related to the NapA of *Vibrio* species and *Photobacterium profundum*, indicating that the duo-NAP system

FIGURE 3 | Depiction of the nine anaerobic electron transfer pathways reported in *Shewanella*. Arrows represent proposed electron flows. CymA-independent respiration systems include the TMAO reductase TorA (Schwalb et al., 2003), sulfite reductase SirA (Shirodkar et al., 2011), thiosulfate reductase PsrA (Shirodkar et al., 2011), periplasmic nitrate reductase NapA, and associated subunits TorC, SirCD, PsrBC, and NapC. CymA-dependent respiration systems:

nitrite reductase NrfA (Gao et al., 2009); dissimilatory iron reductase MtrABC and associated outer membrane protein OmcA (Fredrickson et al., 2008); DMSO reductase DmsAB located at the outer membrane and associated subunits DmsF and DmsE (Gralnick et al., 2006); flavocytochrome C$_3$ catalyzing the reduction of fumarate to succinate (Schwalb et al., 2003); and arsenate reductase ArrAB (Murphy and Saltikov, 2007).

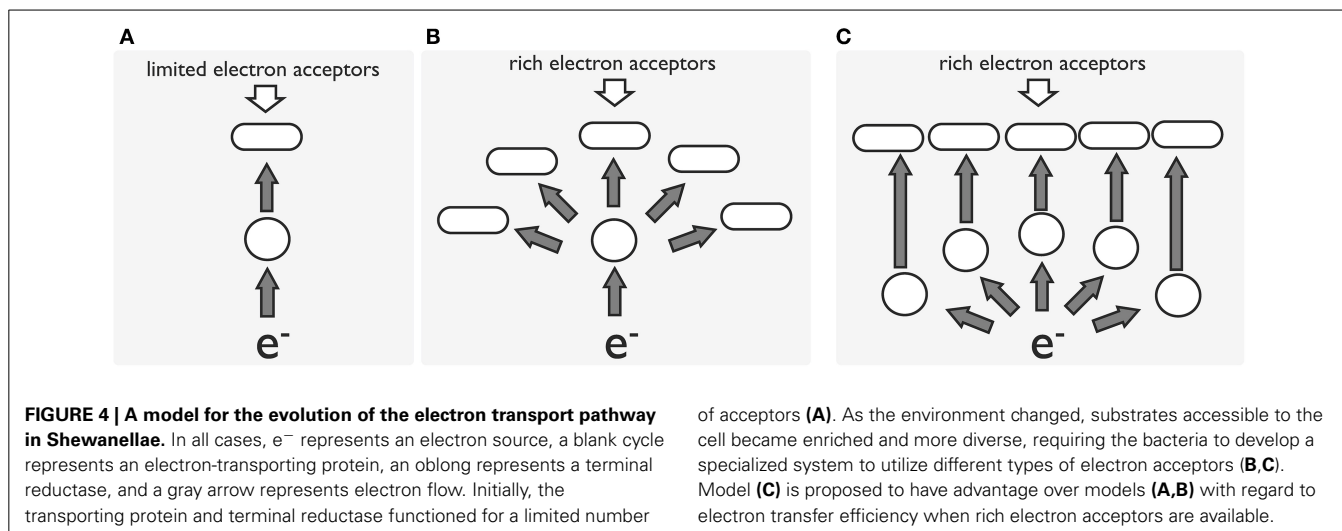is the result of HGT rather than gene duplication (Chen et al., 2011).

To understand why most *Shewanella* species have acquired NAP-α, we should take a close look at NapC and CymA. Both NapC and CymA are tetraheme c-type cytochromes belonging to the NapC/NirT family of proteins. The distribution of NapC/NirT proteins is relatively less widespread (they are found only in several subgroups of proteobacterial), which suggests that they may be evolutionarily more recent (Sharma et al., 2010). Members of this protein family play a role in the mediation of electrons in several anaerobic respiration pathways: NrfH and NirT for nitrite reduction, TorC for TMAO reduction, DorC for DMSO reduction, and FccC for methacrylate reduction (Simon and Kern, 2008). The proposed electron transport network of CymA and other specific transfers is illustrated in **Figure 3**. Sequences among the members of this family have a high degree of similarity, and they are suggested to have a common ancestor prior to recruitment into their specific anaerobic respiration system (Bergmann et al., 2005). This common ancestor is likely to function in the most ancient microbial metabolism, such as iron (III) reduction because iron reduction is widely agreed as one of the most ancient microbial metabolisms on the early Earth (Vargas et al., 1998). Alternatively, the ancestor may be a universal component of many electron transport pathways. If this is correct, CymA may have developed various functions at the time, and CymA may have also inherited multi-function capacities from the common ancestor. In addition, *E. coli* NapC has been reported to show ferric reductase activity (Gescher et al., 2008), suggesting that the specified electron transport proteins still share a commonality with their ancestor.

Based on multiple lines of evidence, we speculate that the origin of the duo-NAP system is the consequence of the co-evolution of microorganisms and environments. At the very beginning of its origin, *Shewanella* may have used metal as its sole electron acceptor. During evolution on Earth when extensive substrates became available, CymA may have developed its enzyme functions specifically to utilize alternative electron acceptors. By making use of the multifunctional protein CymA, the organism benefits by not increasing the burden of an expanding genome when selection pressure limits genome size (Huberts and Van Der Klei, 2010). However, for a protein that has multiple related functions, the additional roles might result in a low specificity of the active site (Jeffery, 1999). This model of respiration may have become less competitive as the environment changed. To cope with this deficiency, *Shewanella*, had to recruit specific electron-transport proteins (such as NapC, PsrC, SirC, and TorC, see **Figure 3**) by HGT. Nitrate is the most preferred electron acceptor in the natural environment for *Shewanella* because the cells can synthesize more energy for growth. Therefore, to primarily use nitrate, it is conceivable that *Shewanella* recruited the specific electron transport proteins NapB and NapC and routed electrons to NapA only.

Moreover, HGT was analyzed in several *Shewanella baltica* strains (Caro-Quintero et al., 2011), and the results indicate that the exchange rate of the genes, including *nap*, is rapid and that this gene-exchange contributes to successful adaptation to specific physicochemical conditions.

CONCLUDING REMARKS

According to genetic and physiological studies, most *Shewanella* species are ammonifiers, whereas only a few are denitrifiers.

FIGURE 4 | A model for the evolution of the electron transport pathway in Shewanellae. In all cases, e⁻ represents an electron source, a blank cycle represents an electron-transporting protein, an oblong represents a terminal reductase, and a gray arrow represents electron flow. Initially, the transporting protein and terminal reductase functioned for a limited number of acceptors (**A**). As the environment changed, substrates accessible to the cell became enriched and more diverse, requiring the bacteria to develop a specialized system to utilize different types of electron acceptors (**B,C**). Model (**C**) is proposed to have advantage over models (**A,B**) with regard to electron transfer efficiency when rich electron acceptors are available.

Genes for nitrate reductase (NAP and NAR) and nitrite reductase (NRF and NIR) are identified as multi-copies in the genomes of most shewanellae. The duo-NAP system (NAP-α and NAP-β) was probably derived through horizontal gene transfer and environmental adaptation, with NAP-α having apparent advantages over NAP-β with regard to nitrate scavenging. Moreover, environmental factors, such as carbon source, temperature and oxygen levels, may influence the regulation of the duo-NAP system of *Shewanella* via different mechanisms. Based on available evidence, a model for the evolutionary course of the anaerobic respiration system in *Shewanella* is proposed here (**Figure 4**). At the early point of their emergence, *Shewanella* species may have used CymA as a common electron transfer that served multiple pathways of respiration; afterward, the specific transport protein NapC was acquired via gene transfer, which provided competitive advantages to the host under stressed conditions.

CHALLENGES AND FUTURE WORK

Recently, DNAR was suggested to play an important role in N metabolism (Kraft et al., 2011), which has been long ignored in previous investigations, as it was generally assumed that most of the nitrate in nature is respired via denitrification. It would be important and challenging to quantify the ecological contributions, diversity, and controlling factors of different nitrate respiration processes (DNAR and denitrification) in different environments. Shewanellae are believed to be important players in environmental N cycling, though additional detail and quantitative evaluation on their contribution are still lacking. Possessing multiple genes in nitrogen metabolism pathways is believed to confer *Shewanella* with environmental adaptation flexibility and advantages, though these assumptions need to be tested in the future. In addition, the mechanisms by which the duo NAP system benefits the host need to be elucidated. Understanding the regulation and evolution of the NAP systems in *Shewanella* would help in the understanding of the adaptation and evolution of this environmentally important bacterial genus and their application in bioremediation.

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