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Functional, biochemical and genetic diversity of prokaryotic nitrate reductases

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Abstract. Prokaryotic nitrate reduction can serve a number of physiological roles and can be catalysed by a number of biochemically distinct nitrate reductases. Three distinct nitrate reductase classes can be indentified in prokaryotes, NAS, NAR and NAP. NAS is located in the cytoplasmic compartment and participates in nitrogen assimilation. NAR is usually a three-subunit complex anchored to the cytoplasmic face of the membrane with its active site located in the cytoplasmic compartment and is involved in anaerobic nitrate respiration. NAP is a two-subunit complex, located in the periplasmic compartment, that is coupled to quinol oxidation via a membrane anchored tetraheme cytochrome. It shows considerable functional flexibility by participating in anaerobic respiration or redox energy dissipation depending on the organism in which it is found. The members of all three classes of enzymes bind the bis-molybdopterin guanine dinucleotide cofactor at the active site, but they differ markedly in the number and nature of cofactors used to transfer electrons to this site. Analysis of prokaryotic genome sequences available at the time of writing reveals that the different nitrate reductases are phylogenetically widespread.

Key words. Nitrate assimilation; nitrate respiration; nitrogen cycle; denitrification; nitrate reductase; nitrite reductase.

Background

It has emerged that many bacteria can express multiple, functionally and biochemically distinct, nitrate reductases. For example, *Paracoccus pantotrophus* has three nitrate reductases [1]. One of these, NAS, is located in the cytoplasmic compartment, is ammonium repressible and participates in nitrogen assimilation (fig. 1). The other two, however, are both linked to respiratory electron transport systems, each ultimately taking electrons from the quinol pool (figs 2 and 3). One of the enzymes (NAR) is a three-subunit complex anchored to the cytoplasmic face of the membrane with its active site located in the cytoplasmic compartment (fig. 3) (see article by Blasco et al. for details of the biochemistry of this enzyme from *Escherichia coli*). The other respiratory enzyme (NAP) is a two-subunit complex, located in the periplasmic compartment, that is coupled to quinol oxidation via a membrane anchored tetraheme cytochrome (fig. 2) [2–4]. The members of all three classes of enzyme bind the bis-molybdopterin guanine dinucleotide (bis-MGD) cofactor at the active site, but they differ markedly in the number and nature of cofactors used to transfer electrons to this site. Analysis of the complete or partially complete prokaryotic genome sequences available at the time of writing reveals that the different nitrate reductases are phylogenetically widespread (table 1). However, biochemical analysis of nitrate metabolism in some bacteria has often been confused by a failure to distinguish between these different groups of enzymes, and physiological analysis has sometimes failed to recognise different roles for nitrate reduction in bacteria. This article will serve to review the nitrate reductase classes, assess their distribution amongst bacteria and comment on the physiological roles for the enzymes in different bacteria.

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The assimilatory nitrate reductases

The bacterial assimilatory nitrate reductases can be grouped into at least three subgroups, typified by the enzymes from *Synechococcus* sp. PCC7942, *Klebsiella oxytoca* and *Bacillus subtilis*. These systems differ in the nature and number of electron transfer cofactors bound by the catalytic bis-MGD subunit and by the nature of the probable electron donors to each enzyme. Biochemical studies on bacterial assimilatory nitrate reductases have not kept pace with genetic characterisation, and this situation needs to be redressed in future studies. An excellent analysis of the nitrate assimilation gene clusters, and the biochemical implications thereof, has recently been presented by Lin and Stewart [5], and the subject is thus only dealt with briefly here. It should be noted that none of the bacterial nitrate reductases described below shares any significant homology with the eukaryotic assimilatory nitrate reductase described in Campbell's review.

Klebsiella oxytoca

Analysis of the gene cluster (*nasFEDCBA*) of the *K. oxytoca* assimilatory nitrate reductase suggests that the enzyme is comprised of two subunits (fig. 1). The catalytic subunit (NasA) is predicted to bind bis-MGD, a N-terminal domain $[4Fe4S]^{1+,2+}$ cluster and a C-terminal domain $[2Fe2S]^{1+,2+}$ cluster [6, 7]. This latter domain has sequence homology to the $[2Fe2S]^{1+,2+}$ NifU ferredoxin. The second subunit (NasC) is predicted to bind an FAD cofactor and to mediate electron transfer from NAD(P)H (nicotinamide adenine dinucleotide phosphate) to NasA. The *K. oxytoca nas* gene cluster additionally includes the gene (*nasB*) encoding a siroheme nitrite reductase. The amino acid sequence of this enzyme also suggests the presence of two NifU-like [2Fe2S] centres, showing how integral these clusters may be to electron transfer processes involved in nitrate reduction to ammonium. A two-subunit assimilatory nitrate reductase that may resemble that of *K. oxytoca*

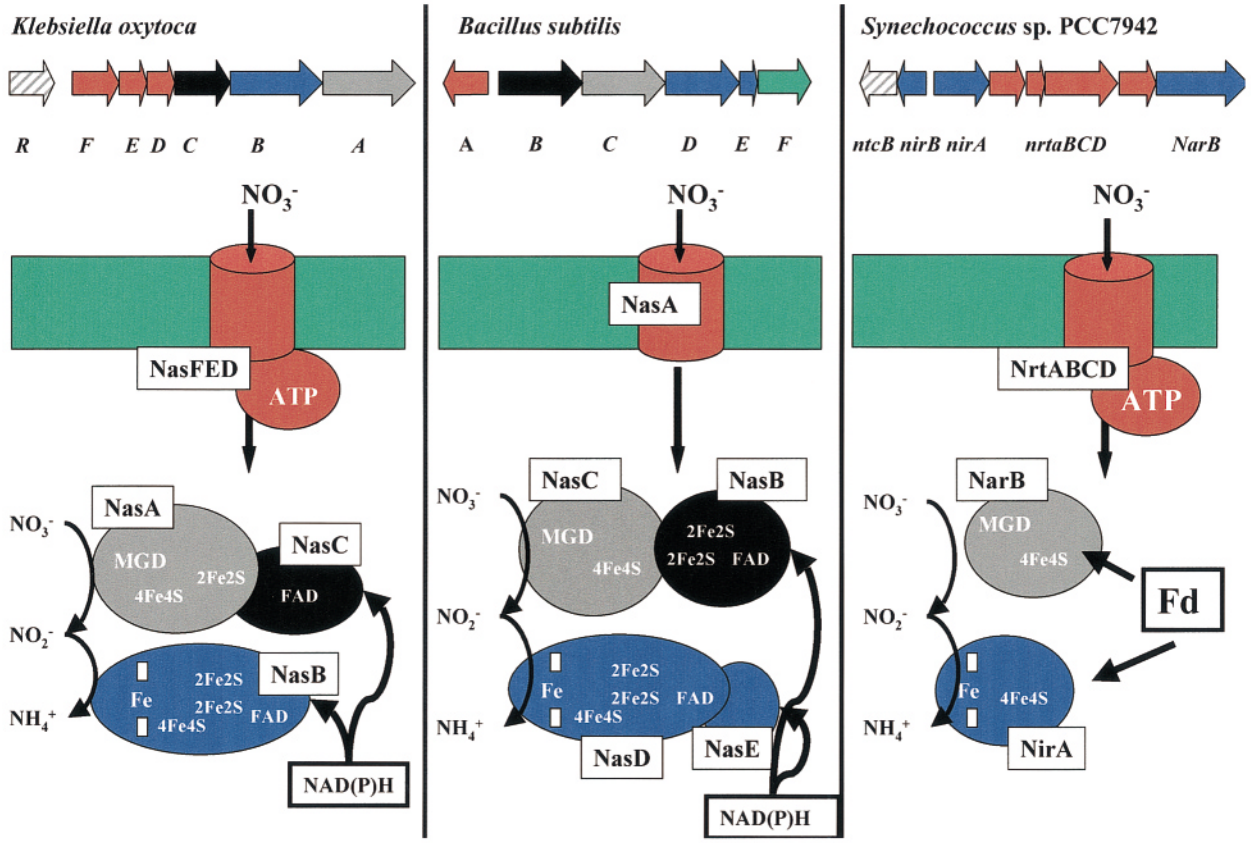

Figure 1. The organisation of the assimilatory nitrate reductase gene clusters and the predicted organisation of the proteins encoded by these genes in *Klebsiella oxytoca*, *Bacillus subtilis* and *Synechococcus* PCC7942.

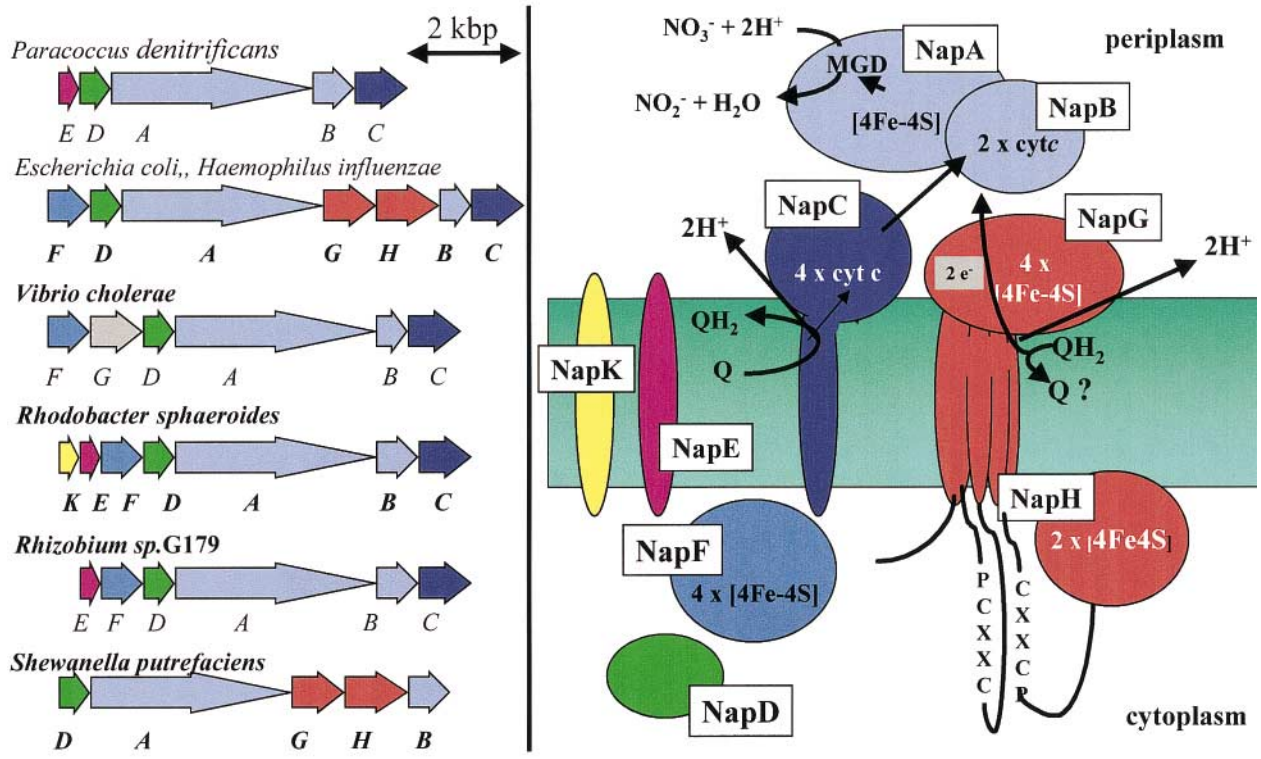

Figure 2. The organisation of the periplasmic nitrate reductase gene clusters and the predicted organisation of the proteins encoded by these genes in a number of different bacteria.

has recently been purified from the photosynthetic bacterium *Rhodobacter capsulatus* [8]. The *K. oxytoca nas* operon also includes genes (*nasFED*) encoding an ATP-dependent nitrate and nitrite transport system ([9], see article by Moir and Wood). The regulation of the *nasFEDCBA* gene cluster has been studied in some detail and is subject to dual control by ammonium and nitrate/nitrite. The former is elicited via general nitrogen regulation (Ntr) [10] and the latter by a nitrate/nitrite responsive transcription antiterminator protein (NasR) [fig. 1] which binds directly to *nasF* leader messenger RNA (mRNA) [10, 11].

Synechococcus sp. PCC7942

Sequence analysis of the *Synechococcus* sp. PCC7942 assimilatory nitrate reductase predicts it to be simpler than that of *K. oxytoca*, and comprising an $\sim$70-kDa polypetide that binds only the bis-MGD cofactor and an iron-sulphur centre [12] (fig. 1). The likely electron donor is reduced ferrodoxin, generated by photosystem I during photosynthesis. Hence light is indirectly important in regulating nitrate metabolism, though not in the manner discussed for the plant system in the article by MacKintosh and Meek. The *nirA* gene encoding a siroheme nitrite reductase also clusters with the NarB gene, as do the *nrtABCD* genes, which encode a putative ATP-dependent nitrate transporter (fig. 1) [12]. The complete genome sequence of the closely related *Synechocystis* sp. strain PCC6803 reveals that there are two putative nitrate transporters NtrABCD [13]. The genes for one copy cluster with *narB*. In *Synechocystis* sp. strain PCC6803, the siro-heme nitrite reductase gene, *nirA*, is not part of the *narB* gene cluster. The transcription of the nitrate/nitrite assimilation genes of cyanobacteria is responsive to cellular nitrogen status and is dependent on the NtcA protein, which is a member of the Fnr/Crp family of transcription factors (for a fuller discussion see Lin and Stewart [5]). Genes encoding proteins homologous to the nitrate-responsive NarX/L dual component sensor-regulator pair of *E. coli* are also present on the *Synechocystis* chromosome, raising the possibility that they mediate a nitrate/nitrite induction of NarB or NirA.

B. subtilis

The *B. subtilis nasABCDEF* gene cluster includes genes for a nitrate reductase (*nasB*), a siroheme-FeS nitrite

reductase (*nasD*) and a flavoprotein (*nasC*) that may act as electron donor to one or both of NasD and NasB (fig. 1) [14]. The C-terminal segment of the nitrite reductase may be transcribed separately (*nasE*). Also present is a gene for a putative nitrate transporter, NasA. This is homologous to NarK and distinct from the ATP-dependent systems used in the *Synechocystis* assimilatory system (these transporters are considered further in the review of Moir and Wood). Sequence analysis of the *nasC* gene product suggests that it binds bis-MGD and a [4Fe4S] cluster, but not the NifU-like [2Fe2S] cluster predicted to be present in the *K. oxytoca* enzyme. Instead, sequence analysis of the translated *nasB* gene, encoding the putative flavodoxin electron donor for NasC, reveals the presence of a tandom repeat of two NifU-like units followed by a carboxy-terminal segment that might bind a further [4Fe4S] or [3Fe4S] cluster [5]. The assertion that NasB is indeed the electron donor to NasC requires experimental validation. The regulation of the *B. subtilis nas* gene cluster is still emerging and appears complex. The nitrate and nitrite reductases of *B. subtilis* have two different physiological functions. Whilst *B. subtilis* has two distinct nitrate reductases, encoded by *narGHI* (see below) and *nasBC*, which function in respiratory and assimilatory nitrogen metabolism, the NADH-dependent, soluble nitrite reductase encoded by the *nasDE* genes is required for both respiratory and assimilatory processes. It has recently been shown that transcription of *nasDEF* is driven by both a *nas* operon promoter and an internal promoter that resides between the *nasC* and *nasD* genes [15]. Transcription from both promoters is activated by nitrogen limitation during aerobic growth by the nitrogen regulator, TnrA. However, under conditions of oxygen limitation, *nasDEF* expression and nitrite reductase activity are significantly induced. Anaerobic induction of *nasDEF* requires the presence of nitrite and the ResDE two-component regulatory system [16]. This system is also required for expression of the *narGHI* genes of respiratory nitrate reductase (discussed below) and demonstrates a coregulation with *nasDEF*.

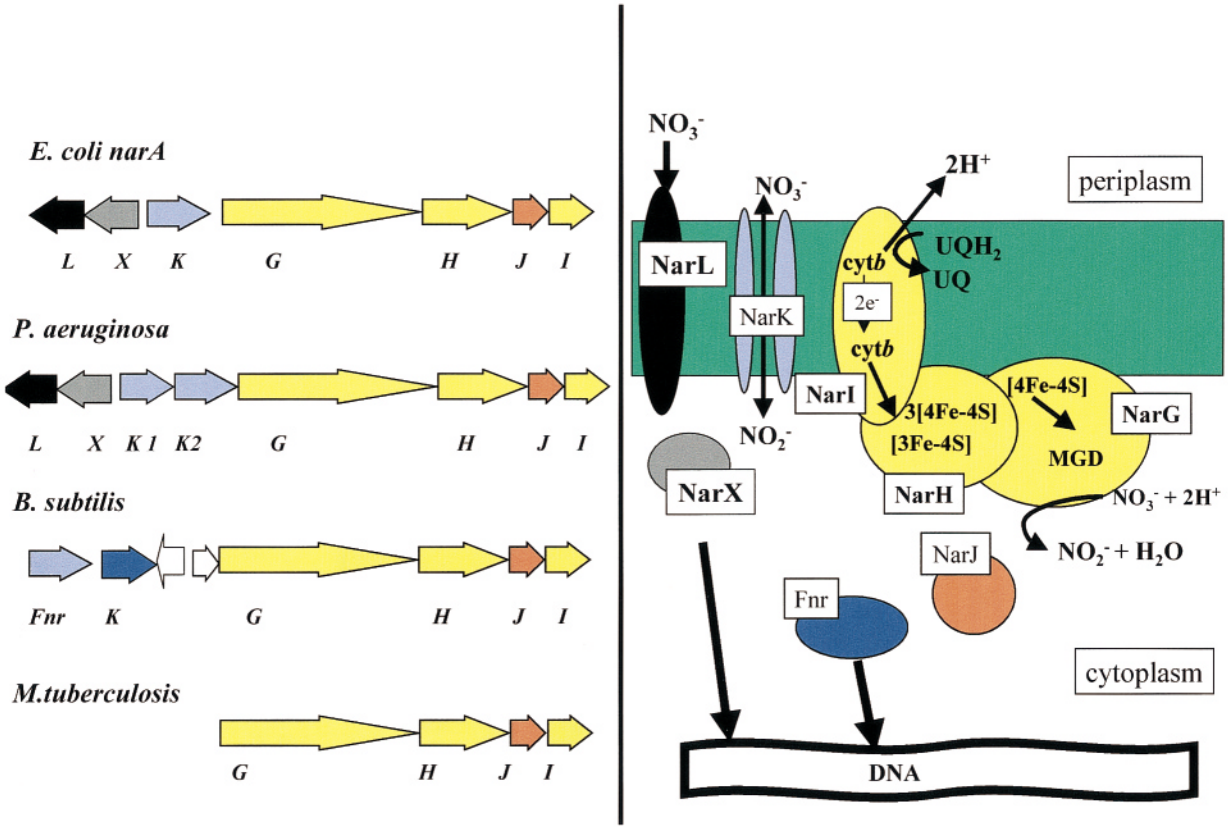

Figure 3. The organisation of the membrane-bound nitrate reductase gene clusters and the predicted organisation of the proteins encoded by these genes in a number of different bacteria.

Table 1. The distribution of nitrate reductases in Bacteria and Archaea.

	NAR	NAP	NAS
Alpha Proteobacteria			
Caulobacter crescentus			+
Paracoccus pantotrophus	+	+	+
Rhodobacter capusulatus AD2		+	+
Rhodobacter capusulatus BK5	+		+
Rhodobacter capusulatus E1F1			+
Rhodobacter sphaeroides f sp. denitrificans	+	+	+
Sinorhizobium meliloti		+	
Beta Proteobacteria			
Bordetella bronchiseptica		+	
Ralstonia eutropha	+	+	+
Gamma Proteobacteria			
Escherichia coli	++		+
Haemophilus actinomycetemcomitons		+	
Haemophilus ducreyi		+	
Haemophilus influenzae		+	
Klebsiella oxytoca	++		+
Legionella pneumophila		+	
Pasteurella multocida		+	
Pseudomonas aeruginosa	+	+	+
Pseudomonas putida			+
Salmonella paratyphimurium	++	+	
Salmonella typhimurium	++	+	
Shewanella putrefaciens		+	+
Vibrio cholerae		+	
Yersinia pestis		+	
Delta Proteobacteria			
Desulfovibrio desulfuricans		+	
Epsilon Proteobacteria			
Campylobacter jejuni		+	
Gram-Positive Bacteria			
Bacillus subtilis	+		+
Bacillus stearothermophilus	+		+
Corynebacterium diptheriae	+		
Mycobacterium tuberculosis	+		
Mycobacterium bovis	+		
Mycobacterium avium	+		+
Staphylococcus aureus	+		
Staphylococcus carnosus	+		
Streptomyces coelicolor	+		+
Cyanobacteria			
Synechococcus sp. PCC7942			+
Synechocystis sp. PCC6803			+
Anabaena			+
Oscillatoria			+
Deinococcus Group			
Thermus thermophilus	+		
Aquificales			
Aquifex aeolicus			+
Crenarchaeota			
Aerpyrum pernix	+		
Euryarchaeota			
Archaeoglobus fulgidus	(+)		

The table reports data derived from a BLAST analysis of 77 complete or unfinished prokaryotic genome sequences at the NCBI Blast Server (http://www.ncbi.nlm.nih.gov/; 19 June release). *P. denitrificans* NAP, *E. coli* NarA and *Synechoccus* NarB were blasted as representative examples of the NAP, NAR and NAS classes. Assignment of positive hits was made using the segment 3 signatures discussed in the text. The *R. capsulatus*, *P. pantotrophus*, *D. desulfuricans*, *Anabaena* and *Osciliatoria* data were not derived from this analysis, but are included because they are referred to in the text.

Azotobacter vinelandii

Historically, assimilatory nitrate reduction by *A. vinelandii* has been the subject of a considerable amount of study. However, final interpretation of the nature of the system, in the context of those described for *K. oxytoca*, *B. subtilis* and the cyanobacteria, requires publication of the gene sequences of the *nas*/*nir* clusters. Biochemical studies on the nitrate reductase suggest that the system will resemble that of *Synechococcus* with the catalytic subunit binding bis-MGD and an FeS centre and being dependent on electron transfer from small ferrodoxins or flavodoxins [17]. The electron paramagnetic resonance (EPR) signals originating from the Mo(V) state of this enzyme resemble those of the periplasmic nitrate reductase, consistent with a close evolutionary relationship between these two enzyme groups (see below) [18]. There is evidence of ammonium repression of *nas* gene expression via the Ntr system and of nitrate/nitrite induction. Two genes (*nasST*) involved in the mediating nitrate/nitrite induction have been sequenced, and Lin and Stewart have presented a model in which NasS acts as a nitrate sensor and NasT serves as a transcription antiterminator [5]. Thus, the combined action of NasST resembles that of NasR in *K. oxytoca*.

The respiratory nitrate reductases

In contrast to the assimilatory nitrate reductases, there is a large body of biochemical and spectroscopic information available for the respiratory nitrate reductases of bacteria, including a crystal structure for the periplasmic nitrate reductase of *Desulfovibrio desulfuromonas* [19]. It has also emerged that both the membrane-bound and the periplasmic nitrate reductases can have different physiological functions in different bacteria.

NAP

NAP is normally isolated as a two-subunit enzyme comprising an $\sim$90-kDa catalytic subunit (NapA) which binds bis-MGD cofactor and a [4Fe4S] cluster, and a $\sim$16-kDa electron transfer subunit (NapB) that binds two *c*-type hemes [2–4, 20–22]. The enzyme crystallised from *D. desulfuricans* comprised only NapA [19], but it cannot be excluded that the NapB subunit was lost during purification as the two subunits can be separated in the *Rhodobacter capsulatus* enzyme [23]. Consideration of *nap* gene clusters amongst different bacteria reveals considerable heterogeneity in their composition (fig. 2). With the exception of *S. putrefaciens*, all of the *nap* clusters have four genes in common:

napDABC. It is notable that in all cases *napD* overlaps with *napA*, suggesting translational coupling. The conservation of *napA*, *napB* and *napC* is expected, as they encode the terminal bis-MGD-containing reductase (NapA), the diheme (NapB) and tetraheme (NapC) components of the periplasmic electron transfer system (fig. 2). It is notable that in *S. putrefaciens* a NapC homologue CymA has been identified at a distinct locus [24, 25], but mutation of this gene causes loss of electron transport to nitrate, fumarate and Fe(III).

The postulated role of NapD is that of a 'private chaperone' involved in maturation of NapA prior to export to the periplasm [3, 26]. One intriguing problem with NapA and, indeed, a number of other periplasmic redox proteins has been the question of whether cofactor insertion takes place in the periplasm or cytoplasm. The current view is that cofactor insertion takes place in the cytoplasm and that a folded protein is then exported across the cytoplasmic membrane into the periplasmic compartment. The transport system responsible for this (Tat) has been identified and recognises a twin arginine-containing motif in the signal sequences of the substrate proteins [27]. A similar system is present in plant thylakoid membranes, and the molecular characterisation of the TAT system represents an important goal for future research in cell biology/biochemistry.

The five other *nap* genes, *napEKFGH*, are found in different combinations in different bacteria. NapK and NapE are monotopic integral membrane proteins of no currently known function. Sequence analysis of NapF predicts it to be a soluble 20-kDa cytoplasmic protein which binds four [4Fe4S] clusters. NapG (20 kDa) is also likely to bind four [4Fe4S] clusters, but may possess a signal peptide that directs it for export to the periplasm (fig. 2). NapH (32 kDa) is predicted to be an integral membrane protein with four transmembrane helices arranged so that both the N- and C-termini of the polypeptide are cytoplasmic. The proposed cytoplasmic C-terminal domain contains two 4-Cys motifs in a C-terminal domain that are typical of those binding [4Fe4S] clusters. NapH also contains two cytoplasmic Cys-$(Xaa)_3$-Cys-Pro motifs, one in the cytoplasmic loop between helices 2 and 3 and one after helix 4 (fig. 2). These motifs may be involved either in binding a metal centre, an FeS cluster, or in thiol redox interchange reactions. NapG and NapH show strong sequence similarity to two proteins, MauM and MauN, respectively, present in the methylamine dehydrogenase gene clusters of methylotrophs [4]. The NapH/MauN secondary structure can also be recognised in several other proteins, e.g. the *nosR* gene product of the nitrous oxide gene clusters of denitrifying bacteria, RdxA of photosynthetic bacteria and the *fixG*/*ccoG* gene products in the high-affinity cytochrome cbb_3 oxidase gene cluster [4]. Disruption of the *napG*, *napH* and *napF* genes in *E. coli* did not completely block physiological electron transfer to NapAB or eliminate *napAB* expression [26], and thus it is not yet possible to assign a function to the proteins. One possibility is involvement in electron transfer processes and/or regulation in response to a cellular redox signal. It may then emerge that there are distinct electron transport pathways to NapAB in some bacteria, one via NapC and one via NapGH (fig. 1). The case of *S. putrefaciens* having no *napC* in the *nap* cluster but utilising a homologue (*cymA*) encoded elsewhere on the chromosome raises the possibility that some bacteria could do likewise with respect to *napFGH*. For example, a NapH homologue (CcoG) is encoded in the *cco* gene cluster of *P. denitrificans*. This cluster encodes subunits of the cytochrome cbb_3 oxidase, and there is evidence to indicate that cyt cbb_3 is expressed at high levels under growth conditions where Nap is also expressed at high levels [28].

The membrane-bound nitrate reductase

The membrane-bound nitrate reductase is a complex, three-subunit quinol dehydrogenase. It contains a $\sim$140-kDa bis-MGD catalytic subunit (NarG), a $\sim$60-kDa electron transfer subunit (NarH) which binds four FeS clusters and a di-*b*-heme integral membrane quinol dehydrogenase subunit (NarI). The biochemistry and spectroscopy of this well-characterised enzyme will be reviewed in the article by Blasco et al. At the heart of all the bacterial *nar* gene clusters currently sequenced is a *narGHJI* operon (fig. 3), in which *narGHI* encode the enzyme complex and *narJ* may encode a private chaperone of analogous function to NapD in the NAP system. Upstream of *narGHJI* the gene content can vary. In *E. coli* it is preceded by the *narK* gene encoding a putative nitrogen oxyanion transporter (see review of Moir and Wood). Further upstream of *narK* there are genes encoding the two-component nitrate-responsive sensor-regulator system NarXL. There is some heterogeneity in the gene organisation of the region upstream of *narGHJI* in bacteria. In some gene clusters, e.g. *Pseudomonas aeruginosa* (EM:PSNARXL), there are two *narK* genes (see article by Moir and Wood). In *Mycobacterium tuberculosis* the *narK* gene does not cluster with the *narGHJI* genes, but there are four *narK*-like genes elsewhere on the chromosome. In *B. subtilis*, there is also a gene encoding a transcription factor of the Fnr family. In *E. coli*, Fnr serves to mediate the anaerobic induction of the *narGHJI* operon, acting in conjunction with the nitrate-responsive NarXL system to maximise nitrate reductase expression [29]. Consideration of the *B. subtilis* system reveals similarities and distinctions. A two-component regulatory system, ResD and ResE, in addition to Fnr, has been shown to be indispensable for

nitrate respiration [16, 30–32]. In *B. subtilis*, *fnr* transcription is highly induced by oxygen limitation, which is not the case for *E. coli*. *B. subtilis fnr* is transcribed from its own promoter as well as from a promoter located upstream of *narK* (fig. 2). ResDE may have a global role in *B. subtilis* anaerobic gene regulation and has been found to be required for transcriptional activation of *fnr* from the *fnr*-specific promoter, with FNR then being required for activation of *narK-fnr* transcription from the FNR-dependent *narK* operon promoter under anaerobiosis [14]. It is clearly dangerous to consider that all *fnr*-dependent *nar* operons will be regulated in the same manner, and separate studies on a number of different systems are now required.

The MGD subunits and evolutionary considerations

In the bis-MGD-binding subunits of various molybdopterin enzymes, the molybdenum can be coordinated by up to four thiolate ligands provided by the two MGD moieties (fig. 4). It can additionally be coordinated by –S, –O or –Se provided by cysteine, serine or selenocysteine residues in the polypeptide chain and a variable number of oxo (=O), hydroxy (–OH) or water groups [19, 33–36]. The reaction catalysed by nitrate, DMSO and TMAO (trimethylamine *N*-oxide) reductases is an oxo-transferase reaction in which an oxo group on the oxidised Mo(VI) ion is lost as OH^-/H_2O when the cofactor is reduced to the Mo(IV) state. Nitrate can bind to the reduced state and is reduced to nitrite, which is released leaving behind a nitrato oxygen to regenerate the oxo group on the Mo(VI) species.

The structure of some of the catalytic subunits of members of the MGD family have emerged in recent years, from formate dehydrogenase [33], DMSO reductase [35, 36], TMAO reductase [34], and NAP from the sulphate-reducing bacterium *D. desulfuromonas* [19]. All the subunits show a high degree of similarity in their structural organisation with the bis-MGD cofactor lying at the bottom of a deep substrate cleft (see article by Blasco et al.). However, there is some debate over the number of oxo groups bound to the Mo in these enzymes. Crystal structures and spectroscopic studies indicate one oxo group in the Mo(VI) form of *R. sphaeroides* DMSO reductase [36] and two in the Mo(VI) form of *R. capsulatus* DMSO reductase [35]. Similarly with nitrate reductases, EXAFS (extended X-ray absorption fine structure) studies on *P. pantotrophus* periplasmic nitrate reductase indicate a di-oxo Mo(VI) state [18], and crystal structure studies on *D. desulfuricans* [19] periplasmic nitrate reductase suggest a mono-oxo Mo(VI) state [19].

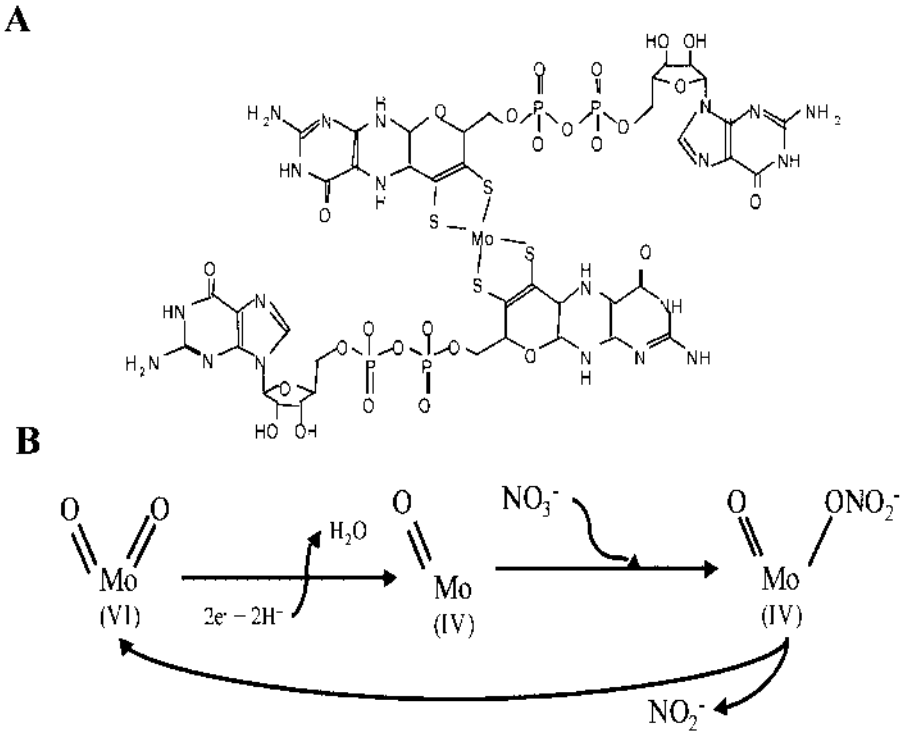

Figure 4. The Bis-MGD cofactor (*A*) and a basic oxo-transfer mechanism for nitrate reduction (*B*).

Figure 5. Multiple sequence alignments of the prokaryotic nitrate reductases, formate dehydrogenases, *S*-oxide/*N*-oxide reductases and selenate reductase. *Pp*, *Paracoccus pantotrophus*; *Ec*, *Escherichia coli*; *Hi*, *Haemophilus influenzae*; *Ha*, *Haemophilus actinomycetemcomitons*; *Vc*, *Vibrio cholerae*; *Spa*, *Salmonella paratyphi*; *St*, *Salmonella typhimurium*; *Yp*, *Yersina pestis*; *Re*, *Ralstonia eutropha*; *Cj*, *Campylobacter jejuni*; *Pa*, *Pseudomonas aeruginosa*; *Pm*, *Pasteurella multocida*; *Dd*, *Desulfovibrio desulfuricans*; *Sco*, *Synechococcus* sp.; *Scys*, *Synechocystis* sp.; *Bs*, *Bacillus subtilis*; *Bst*, *Bacillus stearothermophilus*; *Scl*, *Streptomyces coelicolor*; *Ko*, *Klebsiella oxytoca*; *Kp*, *Klebsiella pneumoniae*; *Ma*, *Mycobacterium avium*; *Sp*, *Shewanella putrefaciens*; *Mf*, *Methanobacterium formicicum*; *Mt*, *Methanococcus thermophilus*; *Rs*, *Rhodobacter sphaeroides*; *Rc*, *Rhodobacter capsulatus*; *Pf*, *Pseudomonas fluorescens*; *Tt*, *Thermus thermophilus*; *Sa*, *Staphylococcus aureus*; *Sc*, *Staphylococcus carnosus*; *Mb*, *Mycobacterium bovis*; *Cd*, *Corynebacterium diptheriae*; *Ts*, *Thauera selenatis*; *Aa*, *Aquifex aeolicus*. NapA, periplasmic nitrate reductase bis-MGD subunit; NasA/NarB, cytoplasmic assimilatory nitrate reductase bis-MGD subunit; Fdh/Fdo, formate dehydrogenase bis-MGD subunit; RsDmsA, RcDorA, periplasmic DMSO reductase bis-MGD subunit; HiDmsA, membrane-bound DMSO reductase bis-MGD subunit; TorA, periplasmic trimethylamine *N*-oxide bis-MGD subunit; SerA, selenate reductase bis-MGD subunit.

Comparison of the primary structures of the catalytic (MGD) subunits of the bacterial NAR, NAP and NAS enzymes suggests that NAP and NAS are most closely related to each other and to the MGD subunits of formate dehydrogenases [4]. This similarity is particularly well defined in the so-called segment 3 region of the polypeptide chain, which provides a Cys or SeCys ligand to the Mo (fig. 5). It is notable, though, that the *D. desulfuricans* NAP is the most divergent of the current NAP sequences and is rather more similar to the assimilatory enzymes. Given the apparent differences in Mo coordination between *D. desulfuricans* NAP and *P. pantotrophus* NAP, it may emerge that the NAP enzymes should be divided into two subgroups.

The MGD subunit of NAR is much larger than that of NAP and NAS (120–140 kDa compared with 80–90 kDa), and there is no conserved cysteine in the segment 3 region of the NARs. Rather, there are a number of conserved serine residues, which raises the possibility that NAR has a Mo-O-Ser ligand rather than the Mo-S-Cys ligand demonstrated for NAP [19] and predicted for NAS. In this respect, NAR appears more similar to the DMSO reductase subgroup of MGD enzymes than to the NAP/NAR/Fdh subgroup. This is also reflected in Mo(V) EPR spectra, which show that the Mo(V) environment of NAP and NAS is similar but is likely to be quite distinct in NAR, which exhibits signals that share more similarity with those seen in the *Rhodobacter capsulatus* DMSO reductase [18, 37]. These spectroscopic and primary structure analyses raise the possibility that nitrate reduction has evolved more than once in the bis-MGD-binding family.

Distribution of NAP, NAR and NAS amongst different bacterial species

Detailed biochemical and physiological analysis of nitrate reduction in bacteria is limited to a handful of species. However, given that primary sequences of the MGD subunits of NAP, NAR and NAS have clear distinguishing signatures, it is possible to analyse the emerging genome sequences from bacteria to map the distribution of these different enzymes. The clearest signature for distinguishing between NAP, NAR and NAS can be found in the segment 3 region of the polypeptide chain, described above, that provides an amino acid ligand to the molybdenum ion (fig. 5). The distinct sequence signatures that allow this type of analysis also facilitate differential analysis of the genes for these enzymes from community DNA samples, providing a useful molecular tool for studying the ecology of bacterial nitrate reductases [38, 39]. Few patterns are readily apparent as yet, though it is clear that many species have more than one nitrate reductase. Some have all three classes of nitrate reductase and some have two isozymes of the same class. The periplasmic nitrate reductase appears particularly prevalent in pathogenic γ proteobacteria, many of which do not appear to possess genes for any other type of nitrate reductase. As yet no respiratory nitrate reductase has been identified in cyanobacteria. The heterogeneous distribution of nitrate reductases amongst different strains of *R. capsulatus* illustrates how dangerous it is to assume that nitrate metabolism will be similar in all strains of a single species.

Different physiological roles for the membrane-bound and periplasmic nitrate reductases in *Paracoccus* species

The identification of two respiratory nitrate reductases in a single organism raises the question of their physiological roles. Studies on enzyme expression in *Paracoccus pantotrophus* and *Paracoccus denitrificans* has revealed that NAR was predominantly expressed under anaerobic denitrifying growth conditions, whilst NAP was predominantly expressed under aerobic growth conditions. Consideration of the bioenergetic properties of each system offers a physiological rationale for this. In the case of NAR, quinol is oxidised at the periplasmic face of the cytoplasmic membrane by the NarI subunit (fig. 3). Protons are ejected into the periplasm, whilst the electrons flow back across the membrane via the two stacked NarI hemes. These electrons then pass, via the multiple NarH FeS centres, to the cytoplasmically located NarG MGD cofactor where nitrate is reduced to nitrite with the associated consumption of two protons [21, 40] (fig. 3). This electron transfer process represents a classic Mitchellian electrogenic redox loop and ensures that the free energy in the QH_2/NO_3^- redox couple is conserved as protonmotive force. In NAP, quinol is also oxidised at the periplasmic face of the cytoplasmic membrane by NapC, but the electrons flow into the periplasm where they ultimately reduce nitrate to nitrite [41]. Thus, in contrast to NAR, the free energy in the QH_2/NO_3^- redox couple is not conserved as protonmotive force and is therefore dissipated (fig. 2).

In physiological terms, it makes bioenergetic sense to express the energy-coupled NAR system under anaerobic conditions when the organism is dependent on nitrate reduction for energy conservation. The expression of an energy-dissipating system under aerobic conditions suggests a role for NAP in redox balancing. During chemoheterotrophic growth on reduced carbon sources the carbon substrate must be oxidised to the level at which it can be assimilated. If this oxidation results in the release of more reductant than is needed for the generation of the ATP required for the metabolism of the carbon, then a means of disposing of the excess reductant must be available. If it is not, the growth rate will be slowed to that allowed by the reoxidation of NADH by cell maintenance reactions. The need to dissipate reductant appears to be most acute during the metabolism of a reduced carbon substrate under conditions that are both oxygen and energy sufficient. For example, expression of NAP and electron flux through the system is much greater during aerobic growth of *Paracoccus* sp. on the reduced carbon substrate butyrate compared with the more oxidised malate [42]. The genetic regulation that leads to an increase in enzyme expression in response to changes in the cellular redox state remains to be elucidated. However, our recent analysis has revealed that there are two promoters upstream of the *nap* gene cluster. One of these is more active during aerobic growth on oxidised carbon substrates, and the other is more active during aerobic growth on reduced carbon substrates. The activity of both promoters is repressed during anaerobiosis, when *nar* is expressed. Point mutations that lead to complete derepression of both promoters under all growth conditions have been constructed. This has allowed identification of a putative binding site for a redox-responsive transcription factor. The biochemical identification of this factor is an important goal for future research in this area [62].

Different physiological roles for the membrane-bound and periplasmic nitrate reductases in enteric bacteria

E. coli can express either NAR or NAP under anaerobic growth conditions. The $q^+/2e^-$ (positive charges moved across the membrane per two electrons trans-

ferred) for nitrate reduction by NAR would be 6 or 4 with NADH or formate as electron donor, but only 4 (NADH) and 2 (formate) when NAP is used to reduce nitrate. Given that NAR is more highly coupled than NAP, the question then arises as to when the NAP system is expressed and physiologically important. This has been addressed in recent competition experiments in continuous cultures where a strain expressing only NAR has been placed in competition with a strain expressing only NAP [43]. Under nitrate-limited conditions the strain expressing NAR is at a selective disadvantage, but the situation is reversed under carbon-limited conditions where the strain expressing NAP is at a selective disadvantage. This may reflect a lower K_s (higher affinity) for intact cells for nitrate when the NAP system is expressed. Thus, NAP may be important in scavenging nitrate from nitrate-limited environments, and consequently under these conditions coupling efficiency is sacrificed in favour of substrate affinity. Expression studies of Nap in *E. coli* support this viewpoint since the *nap* operon is induced at low nitrate concentrations but repressed at higher concentrations which induce the *narG* operon [44]. In this context it becomes significant that many of the pathogenic bacteria that may have to scavenge nitrate from the low levels present in many bodily fluids have the genetic information for NAP. Indeed in some of these, e.g. *Hemophilus influenzae*, NAP is the only nitrate reductase present (table 1). *E. coli* and *Salmonella typhimurium* can also express an isozyme of the NarGHI membrane-bound nitrate reductase. This second membrane-bound enzyme is termed NarZ. Recent work has indicated that this system is expressed in response to stress and is part of the RpoS regulon [45]. The physiological significance of this has yet to be established.

NAR in obligate aerobes

There are now a number of examples of genes for NAR being present in obligate aerobes, such as the pathogen *M. tuberculosis* or the commercially important antibiotic producing actinomycete *Streptomyces coelicolor*. Mycobacteria may need to deal with an anaerobic environment during infection. A *narG* mutant of *M. bovis* lacks the ability to reduce nitrate under anaerobic conditions and is compromised in its ability to colonise immunodeficient mice. In addition to the *narGHJI* operon, *M. tuberculosis* also has genes for a cryptic fused nitrate reductase, expression of which appears to be upregulated in anaerobic dormant mycobacteria [46]. The significance of nitrate reduction in the physiology of other 'obligate' aerobes will be important to establish in future research. In this respect, it is pertinent to note that our descriptions of a number of 'obligate' aerobes will undoubtedly need revising as more physiological studies are undertaken in response to the identification of genes involved in anaerobic metabolism in such organisms. A recent review by Nakano discusses this in the context of the long-held belief that the soil Gram-positive organism *B. subtilis* was a strict aerobe [47].

NAP in photosynthetic bacteria

Members of the *Rhodospirallaceae* family of α-proteobacteria, which includes *Rhodobacter* species, possess a cyclic photosynthetic electron transport system. This cyclic system relies on a single reaction centre, the cytochrome bc_1 complex, the QH_2/Q pool and one or more C-type cytochromes that mediate electron transfer between the two integral membrane complexes. Under illuminated conditions the photosynthetic reaction centre will only turn over if there is a supply of oxidised quinone (Q) as electron acceptor, whilst the protonmotive Q cycle of the cytochrome bc_1 complex requires the provision of both Q and QH_2. Consequently, the cyclic electron transport system is critically dependent on the QH_2/Q ratio in the Q pool. However, the cyclic electron transport system is not a closed system; there are a number of routes for electron input during photoheterotrophic metabolism. Thus, the UQ/UQH_2 pool can be coupled to low potential electron donors such as NADH. This could lead to extensive reduction of the UQH_2/UQ pool, restricting the rate of cyclic electron transport. Many strains of *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* have the ability to express NAP, and it has become clear that nitrate reduction can serve to repoise the cyclic electron transport system when it has become perturbed by over-reduction [48]. This may be particularly important during photoheterotrophic growth of *R. capsulatus* on reduced carbon substrates, such as butyrate. Use of auxilliary electron acceptors such as nitrate are actually critical to photosynthetic growth of some photosynthetic bacteria, such as *Roseobacter denitrificans* [49], even during metabolism of relatively oxidised substrates. Nitrate reduction may also be important following periods of darkness or prolonged periods of low light intensity, which can lead to a collapse of the light-dependent protonmotive force and consequently to the redox poise of the photosynthetic electron transport system becoming disturbed [48, 50].

As yet there is little information on the regulation of *nap* in *Rhodobacter* sp. The transcription start site of the *nap* operon has been mapped for cells grown with succinate as a carbon substrate, and the promoter appears to be more active under anaerobic photoheterotrophic conditions than aerobic chemoheterotrophic conditions [51]. However, the possibility of different

promoters being utilised during growth on oxidised and reduced carbon substrate, as observed for *nap* regulation in *P. pantotrophus*, has not been investigated.

NAP and anaerobic denitrification

In many of the best-characterised denitrification systems, NAR catalyses the first stage of anaerobic nitrate reduction to nitrite (e.g. *Pseudomonas stutzeri* and *Paracoccus* species). In *P. pantotrophus* the phenotype associated with a mutation in the structural genes of the *nar* operon could be suppressed by a second mutation causing derepression of *nap* under anaerobic conditions. Consequently, strains with the suppressor mutation could still grow under anaerobic denitrifying conditions using NAP, rather than NAR, in the first step [52]. The strain did, however, have a lower specific growth rate and growth yield [52]. Recently, it has become apparent that some *Rhizobia* species can express NAP and that disruption of the *nap* genes prevents growth under denitrifying conditions [53]. Also, in *Rhodobacter sphaeroides* f. sp. *denitrificans*, which can express both NAR and NAP, mutation of *nap* prevents anaerobic denitrification [54]. Thus in these organisms one of the physiological roles of NAP is in anaerobic denitrification. When considered in isolation, the energy coupling of NAR and NAP appear markedly different; $q^+/2e^- = 6$ (NAR) and 4 (NAP) with NADH as electron donor, and 2 (NAR) and 0 (NAP) with succinate as electron donor. However, when considered in the context of the entire denitrification pathway, the $q^+/2e^-$ ratio is 24 (NAR) or 22 (NAP) with NADH, and 8 (NAR) or 6 (NAP) with succinate. Thus, the energetic loss of using NAP rather than NAR is only 8% when NADH is the electron donor to the respiratory system.

Nitrate reduction in Archaea

Almost all the characterisation of bis-MGD enzymes has been carried out in bacteria. However, nitrate-reducing Archaea are known, for example the hyperthermophile *Pyrobaculum aerophilum* has been demonstrated to utilise nitrate or oxygen (at low partial pressures) as growth-supporting respiratory substrates [55]. Putative bis-MGD binding enzymes, including nitrate reductases, can be identified from primary struc-

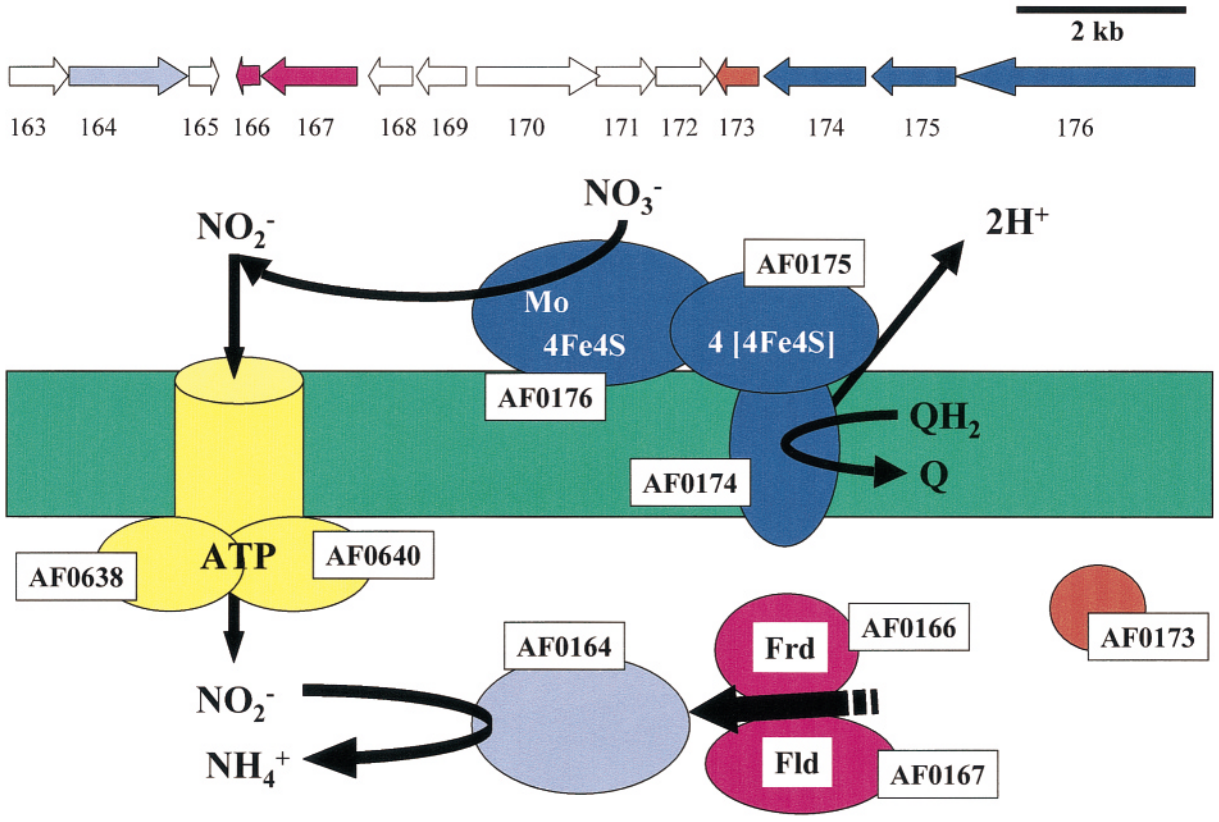

Figure 6. A gene cluster encoding a putative nitrate and nitrite reductase system in *Archeoglobus fulgidus*.

ture analysis of open reading frames in the genome sequences of *Archaeoglobus fulgidus* and *Aeropyrum pernix*. It should be noted that no biochemical data are available on these enzymes, and the possibility that they bind tungsten rather than molybdenum at the active site cannot be excluded. Indeed, the inclusion of tungstate in the growth medium of *P. aerophilum* stimulates anoxic growth with nitrate [55].

A putative nitrate reductase gene cluster has been assigned in the *Archaeoglobus fulgidus* genome [56]. As yet, there is no biochemical evidence to support this, yet the predicted organisation of the system is intriguing and merits comment. The gene cluster comprises four genes, Af173–Af176 (fig. 6). Af176 encodes an 80-kDa protein that has 42% similarity (31% identity, 41 gaps) to NarG of *E. coli*. It is, however, much smaller (~ 90 kDa). Significantly, it has a twin arginine signal peptide that suggests it is exported across the cell membrane. Af175 is predicted to bind four [4Fe4S] clusters, and Af174 is predicted to be an integral membrane complex. There are no suitable candidates for heme-binding ligands in the transmembrane helices of Af174. The final gene is predicted to encode a homologue of the NarJ private chaperone found in all *nar* gene clusters. This point alone strongly suggests that Af176 is a nitrate reductase subunit. Sequence analysis cannot exclude the possibility that Af176 is a membrane-bound DMSO reductase [27, 57] or membrane-bound selenate reductase [58, 59], both of which are known to be MGD enzymes. However, the sequence of the segment 3 region predicted to be involved in Mo-ligation clusters most closely to the segment 3 region of membrane-bound nitrate reductases (fig. 6). The enzyme topology as depicted in figure 6 is most likely and would resemble that proposed for the MGD enzyme tetrathionate reductase [60]. Quinol oxidation by this system would not be coupled to energy conservation, but the $H^+/2e^-$ for $H_2 \rightarrow NO_3^-$ would be 2. The *A. fulgidus* genome also has a member of the ATP-dependent nitrate/nitrite translocators (described in Moir and Wood's review) and genes encoding a putative cytoplasmic nitrite reductase. It may then be that *A. fulgidus* possesses an ancient nitrate reductase system in which the some of the nitrite produced from respiratory nitrate reduction can be used for assimilation purposes. The complete genome of *Aeropyrum pernix* has also revealed the presence of a putative *narGHJI* gene cluster [61]. This organism is a hyperthermophilic obligate aerophilic member of Crenarchaeota. Thus, this nitrate reductase may serve to supplement O_2-dependent protonmotive force during growth in nitrate-containing environments. The biochemical investigation of nitrate reduction in these hyperthermophilic Archaea is an important goal in the prokaryotic nitrate reduction field.

Future perspectives

This short overview has summarised the biochemical and functional diversity of prokaryotic nitrate reductases. Much remains to be learnt about the regulation of nitrate reductase gene expression outside of the enteric bacteria. Comparative biochemical studies may also provide much needed information on the catalytic cycles of the enzymes and the regulation of electron flux through the systems. Crystal structures and application of kinetic spectroscopies and electrochemistry will be important in this respect. Finally, just as with the eukaryotic systems discussed by Campbell, there is interest in prokaryotic nitrate reductases from the point of view of nitrate biosensors and water-purification systems. The rich variety of nitrate reductase from prokaryotes, with differing temperature tolerances, stabilities and complements of redox centres, provide a great opportunity of screening for the most appropriate enzymes for these applications.

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