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Nitrate and nitrite transport in bacteria

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Abstract. The topological arrangements of nitrate and nitrite reductases in bacteria necessitate the synthesis of transporter proteins that carry the nitrogen oxyanions across the cytoplasmic membrane. For assimilation of nitrate (and nitrite) there are two types of uptake system known: ABC transporters that are driven by ATP hydrolysis, and secondary transporters reliant on a proton motive force. Proteins homologous to the latter type of transporter are also involved in nitrate and nitrite transport in dissimilatory processes such as denitrification. These proteins belong to the NarK family, which is a branch of the Major Facilitator Superfamily. The mechanism and substrate specificity of transport via these proteins is unknown, but is discussed in the light of sequence analysis of members of the NarK family. A hypothesis for nitrate and nitrite transport is proposed based on the finding that there are two distinct types of NarK.

Key words. Nitrate; nitrite; transporter; dissimilatory; assimilatory; NarK.

Topology of nitrate and nitrite reduction

Distinct enzymes are synthesised in bacteria for the reduction of nitrate within different cellular compartments. The periplasmic nitrate reductase (NAP) has its active site outside the cytoplasm, and therefore no specific biological mechanism is required for transport of the nitrogenous substrate to its site of reduction. On the other hand, two other types of nitrate reductase have their active sites within the cytoplasm: the membrane-bound nitrate reductase (NAR), which is operative under anaerobic conditions as a respiratory enzyme, and the cytoplasmic assimilatory nitrate reductase (NAS) (see article by Richardson et al.). To reach the active sites of these enzymes, nitrate must cross the cytoplasmic membrane. The product of nitrate reduction, nitrite, can be reduced via nitrite reductases, which reside in the periplasm or in the cytoplasm. Hence, sometimes the nitrite produced from nitrate reduction must cross a membrane in order to reach the active site of its reductase, or alternatively the nitrite may be expelled from the cytoplasm into the extracellular milieu. Four organisational possibilities for the topological arrangements of nitrate and nitrite transport events in bacteria are illustrated in figure 1, and form the basis for the discussions presented in this review.

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Dissimilatory systems

With respect to the transport systems required for respiratory nitrogen oxyanion reduction, two main areas will be covered: (i) the mechanism of transport of nitrate and nitrite across the plasma membrane and its possible role in regulation of anaerobic respiratory metabolism and (ii) the role of putative transporter NarK (and other transporters) in nitrate and nitrite transport. Most consideration of the topology of nitrate and nitrite transport for dissimilation has concentrated on denitrification, in bacteria such as *Paracoccus denitrificans*, and ammonification in *Escherichia coli*. In these cases nitrate uptake into the cytoplasm is required for NAR activity, whereas concomitant nitrite efflux is not always required (see fig. 1a and b).

Do we really need transporter proteins for nitrogen oxyanions?

Nitrate and nitrite, being charged molecules, should not be able to cross biological membranes at fast rates.

However, the pK_a of nitrous acid (HNO_2) is 3.3 [pK_a of nitric acid (HNO_3) is -1.3], and therefore nitrite may be able to cross biological membranes at significant rates in its protonated form (HNO_2) even at neutral pH by passive diffusion. It is possible that specific transporters for nitrite may not be required. However, early evidence that nitrate transport does require a specific transporter came from the finding that whereas purified NAR can reduce both nitrate and chlorate, intact cells of *E. coli* and *P. denitrificans* can only reduce nitrate, i.e. transport of nitrate is mediated by a protein that discriminates against chlorate [1].

Nitrate reduction is controlled by oxygen at the level of nitrogen oxyanion transport

In addition to oxygen regulating nitrate reduction at the level of gene expression, respiratory nitrate reduction is also inhibited in intact cells in the presence of oxygen [1–3]. This effect can be mimicked using ferricyanide, indicating that the nitrate reduction pathway is sensitive to oxidising conditions, rather than molecular oxygen itself [4, 5]. Inverted vesicles of *P. denitrificans* are capable of reducing nitrate and oxygen simultaneously. Furthermore, treatment of anaerobically grown cultures of *P. denitrificans* with low concentrations of detergents such as Triton X-100 relieves the inhibitory effect of oxygen on nitrate reduction, whereas identical concentrations of the detergent allowed the intact cells to reduce chlorate [2]. These findings indicate that the oxygen inhibitory effect on nitrate reduction is related to the transport of nitrate and/or nitrite across the cytoplasmic membrane rather than the limitation of electron flow to the nitrate reductase itself due to diversion of electrons from nitrate reductase to the thermodynamically more favourable electron acceptor, oxygen. This supposition is buttressed by the finding that treatment with Triton X-100 leads to a slight increase in oxygen reduction, i.e. it would be expected to decrease further electron flow to nitrate reductase [2]. How does this oxygen inhibition of nitrate transport come about? In *P. denitrificans*, nitrous oxide (N_2O), like oxygen, inhibits the reduction of nitrate in intact cells. The inhibitory effects of oxygen and nitrous oxide on nitrate reduction are diminished by antimycin, which inhibits electron flow through the cytochrome bc_1 complex (resulting in a more highly reduced quinone pool). This indicates that transport of nitrogen oxyanions may be dependent upon the redox state of the respiratory chain, possibly the redox state of the quinone pool being sensed by the transport process [6], i.e. nitrate reduction occurs only when the respiratory chain is sufficiently reduced.

Denis and co-workers [7] found that inhibition of nitrate reduction by oxygen was greatly lessened in strains of *E. coli* unable to synthesise the respiratory oxidases (cytochromes *bo* and *bd*), supporting the idea that the inhibition of nitrate reduction under aerobic conditions is brought about by inhibition of transport when the respiratory chain becomes oxidised. However, consider-

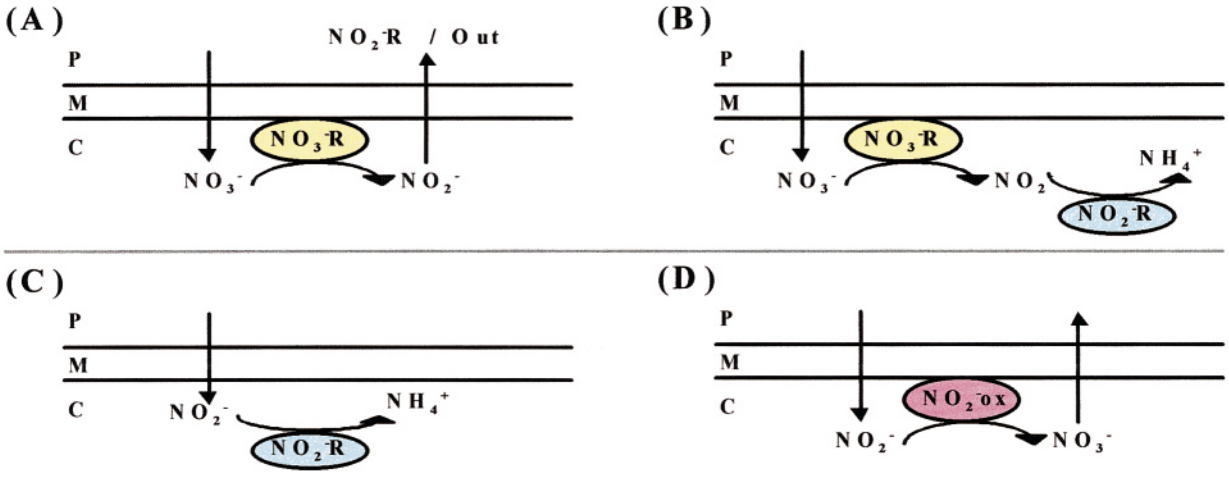

Figure 1. Topological arrangements for nitrate and nitrite transport. (*A*) Nitrate reduction in the cytoplasm, accompanied by nitrite extrusion into the periplasm. (*B*) Nitrate reduction in the cytoplasm; nitrite is not exported but may be further reduced to ammonium in the cytoplasm. (*C*) Nitrite uptake into the cytoplasm for reduction to ammonium. (*D*) Nitrite uptake into the cytoplasm followed by oxidation and extrusion of the resultant nitrate. This occurs in nitrite-oxidising lithotrophs. P, represents periplasm; M, membrane; and C, cytoplasm. R signifies reductase enzymes, and ox signifies oxidases.

able inhibition of nitrate reduction by oxygen in the absence of oxidases was still observed, which may be due to the activity of a third *E. coli* oxidase [8], or there may be a distinct effect of oxygen on nitrate/nitrite transport evident in these strains.

Kucera and co-workers [9] measured internally accumulated nitrate in whole cells of *P. denitrificans*. Provision of oxygen (via the catalytic action of catalase on hydrogen peroxide) actually led to an increase in the steady-state nitrate pool inside the cell. The conclusion of this work was that the control of nitrate reduction by oxygen could not be explained by inhibition of nitrate transport. Rather, regulation of nitrate reduction by oxygen may be controlled by both competition for electrons by the oxidases, and control at the level of nitrite export. The possibility that nitrite export may be the site of control by oxygen is explored below.

Energetic considerations

For nitrate to be taken up into an actively metabolising bacterial cell, it must be moved against a proton electrochemical gradient (Δp), which is likely to be of the order of 180 mV. Given that the k_m for nitrate of the isolated nitrate reductase is approximately 10 μM [10], a passive mechanism for nitrate uptake would yield a k_s for nitrate of around 10 mM, whereas in fact the nitrate affinity is similar to that of the isolated enzyme, showing that nitrate uptake is compensated for by movement of another charge to make the net effect electroneutral, or possibly resulting in a net inward movement of positive charge. This result is in keeping with the mechanism being nitrate/nitrite antiport or nitrate/$n\mathrm{H}^+$ symport (where $n \geq 1$).

An alternative mechanism for nitrate uptake would involve an ATP-driven nitrate uptake system (like those used for nitrate assimilation, below). However, such a system would be energetically costly and is therefore highly unlikely to operate. Experimental determinations of the P/2e$^-$ ratio calculated from medium acidification following pulses of NO_3^-, NO_2^- or N_2O, or from the specific rate of ATP synthesis in chemostats of *P. denitrificans* growing with NO_3^- or NO_2^- showed that the transmembrane charge displacement is the same (per electron) for nitrate reduction, nitrite reduction and nitrous oxide reduction [11, 12]. These values agree with theoretical calculations for these reductases based on the known properties of their electron transport chains, and indicate that the nitrate uptake process does not result in significant loss of Δp, i.e. nitrate uptake is not driven by ATP hydrolysis or the use of >1 proton during the mechanism of a putative nitrate/proton symporter.

Putative nitrogen oxyanion transport mechanisms

Nitrate/nitrite antiporter. In denitrification at steady state, an equal number of nitrate molecules are taken up into the cytoplasm as the number of nitrite molecules that must be released into the periplasm in order to be (i) reduced as part of the operation of the denitrification process or (ii) released into the extracellular medium to accumulate to a detectable amount of nitrite (fig. 1a). This is also the case during ammonification in *E. coli* when nitrite is reduced via the periplasmic nitrite reductase (NRF). Under these conditions the idea that a nitrate/nitrite antiporter is operating is attractive because of the equal stoichiometry of nitrate and nitrite transported, and the electroneutrality of such a process.

Nitrate/proton symporter. Under some conditions *E. coli* expresses a cytoplasmic nitrite reductase whose function is to detoxify nitrite rather than use its reduction to support growth. In this case nitrite does not need to be exported to the periplasm after reduction of nitrate by NAR (fig. 1b). A similar situation arises in pre-steady-state denitrification, i.e. before nitrite is available to act as a counterion. In these cases, it is appropriate to consider the possibility of nitrate/proton symport (nitrate/hydroxide antiport) as the uptake mechanism.

Evidence in support of both the nitrate/nitrite antiporter and the nitrate/proton symporter was provided by Boogerd and co-workers [11]. *P. denitrificans* cells were treated with a protonophore to dissipate Δp, and it was observed that there was a lag before nitrate reduction occurred in intact cells. This lag could be abolished by supplementing the medium with nitrite, indicating that nitrate uptake requires either nitrite as a counterion or a proton gradient, i.e. a nitrate/proton symporter.

Some workers have presented evidence that the sole mechanism for nitrate uptake is via a nitrate/proton symporter (see e.g. [13, 14]). Unfortunately, the conclusions of these studies have relied upon the use of protonophores FCCP and CCCP, which are capable of functioning as thiol reagents as well as protonophores [15, 16] and may therefore have been inhibiting transport processes directly, rather than simply dissipating Δp.

Nitrite transport

Import of nitrite may be necessary in *E. coli* for ammonification via the cytoplasmic nitrite reductase. This may occur by passive diffusion or be facilitated by proteins such as NirC from *E. coli* [17]. NirC has been predicted to serve as a bidirectional nitrite-specific

transporter, but mutants in *nirC* from *Staphylococcus carnosus* are unaffected in nitrite transport. However, this may be due to the existence of multiple ('redundant') pathways for nitrite transport [18]. It should be noted that any such passive transport system is moving nitrite against Δp such that the intracellular concentration will be $\sim 1000\times$ lower than the extracellular concentration if Δp is 180 mV. It is likely that if nitrite uptake is required for a cytoplasmic nitrite reductase, this will be actively driven by Δp as described for the putative nitrate/proton symporter.

Involvement of NarK

Although we do not know the mechanism of transport of the nitrogen oxyanions for sure, it is clear that proteins of the NarK family are involved. NarK was first identified in *E. coli*, and its location directly upstream from the structural genes (*narGHJI*) encoding the membrane-bound nitrate reductase suggested a role in nitrate reduction [19]. Studies using a nitrate-specific electrode demonstrated a decreased steady-state rate of nitrate reduction in a *narK* mutant (compared with wild-type) which was restored by treatment with detergent, indicating that the lesion in the *narK* mutant is transport of nitrogen oxyanions across the plasma membrane [19].

It was proposed that NarK was a nitrate/nitrite antiporter, thus providing nitrate to the nitrate reductase, and allowing nitrite to leave the cytoplasm [20]. However, Rowe and co-workers [14] have presented compelling evidence that NarK in *E. coli* is a nitrite efflux protein. Uptake of nitrate into an *E. coli narK* mutant appears to be as rapid as in the wild type using ^{13}N - nitrate, whereas using a nitrite-sensitive fluorophore [*N* - (ethoxycarbonylmethyl) - 6 - methoxyquinilonium bromide], it was found that NarK enhances nitrite transport in proteoliposomes, and that this process is unaffected by nitrate. What do these experiments tell us? First, they make it clear that in *E. coli*, protein(s) other than NarK can transport nitrate into the cell. At the time of the work of Rowe and co-workers it was not appreciated that *E. coli* contains a second copy of a NarK-like protein encoded by *narU* [21]. Perhaps this protein operates in nitrate uptake. Second, the results suggest that the *narK* phenotype is due to a lesion in nitrite efflux, and that this causes rates of nitrate reduction in intact cells to be low as a consequence of the accumulation of toxic nitrite inside the cell. Given that nitrite can cross biological membranes relatively freely as nitrous acid and that when $\Delta p = 180$ mV will partition across the membrane such that $[NO_2^-]_{out} = 1000 \times [NO_2^-]_{in}$, it seems unlikely that a lesion in nitrite efflux could have such a major impact upon nitrate reduction rates in intact cells. Furthermore, nitrite concentrations up to 50 mM do not inhibit nitrate reductase activity [D. J. Richardson, personal communication]. The remaining possible explanation for the phenotype of *E. coli narK* consistent with its role as nitrite efflux protein is that the intracellular accumulated nitrite inhibits nitrate uptake and thus leads to a considerable decrease in nitrate reduction rates in the intact cell.

A mutant has been made in a *narK* homologue named *narT* in the Gram-positive nitrate reducer *Staphylococcus carnosus* [22]. A *narT* mutant grows poorly on nitrate, but uptake of nitrate can be enhanced by treatment with benzyl viologen, which can act as an ionophore. The dependence of nitrate uptake on NarT suggests that NarT may be a nitrate uptake protein [22]. Alternatively, NarT may be a nitrite effluxer, and intracellular accumulated nitrite in a *narT* mutant may prevent biological nitrate uptake via a separate nitrate transporter protein.

Distribution of NarK

Multiple homologues of NarK have been found amongst phylogenetically diverse eubacteria capable of dissimilatory nitrate reduction. Organisms containing NarK homologues include the deeply branched eubacteria *Aquifex aeolicus* [23] and *Thermus thermophilus* (accession no. CAB65479). NarK does not seem to be represented in the Archaea. There is at least one copy of a *narK* homologue found upstream from *narGHJI* in most nitrate reducers which have been studied genetically so far.

Denitrifying bacteria *Pseudomonas aeruginosa* (accession no. Y15252), *Ps. stutzeri* [24] and *Paracoccus pantotrophus* [N. J. Wood, S. J. Ferguson, D. J. Richardson et al., unpublished] contain two copies of *narK*-like genes upstream from *narGHJI* arranged in series and under the control of the same *nar* promoter region. Two NarK homologues are also found in series in *Thermus thermophilus* (accession no. CAB65479), although in this case the nature of the downstream genes have not yet been revealed, but are likely to be nitrate reductase genes. This arrangement of pairs of *narK* genes is suggestive that two transporters are required for nitrogen oxyanion transport, which presumably have distinct biochemical functions.

The genome sequence of *Mycobacterium tuberculosis* has revealed the presence of four copies of *narK*, designated *narK1*, *narK2*, *narK3* and *narU* [25]. In this organism none of the NarK homologues is found upstream from *narGHJI*, although *narK2* is located upstream from a gene designated *narX* which may encode a single subunit ('fused') nitrate reductase (this is entirely distinct from nitrate/nitrite sensory gene *narX* from *E. coli*). *narK3* and *narU* are found in the vicinity of genes required for synthesis of a cytoplasmic assimi-

latory nitrite reductase. There are no genes known to be involved in nitrogen metabolism in the vicinity of *narK1*. The reasons for the multiple copies of *narK* homologues, and the absence of a *narK* homologue upstream from *narGHJI* are unclear, and work is hampered by our lack of understanding of the physiology of this organism. It is possible that a distinct type of transporter is involved in the nitrate uptake for nitrate reductase in *M. tuberculosis*. A candidate for this role is a hypothetical protein (Rv1159), which has similarity to transmembrane transporters, and is encoded upstream from *narGHJI* [25]. Other Gram-positive organisms that contain NarK homologues include *Bacillus subtilis*, which contains two homologues, designated NarK and NasA [26], and *Streptomyces coelicolor* (accession no. CAB53437).

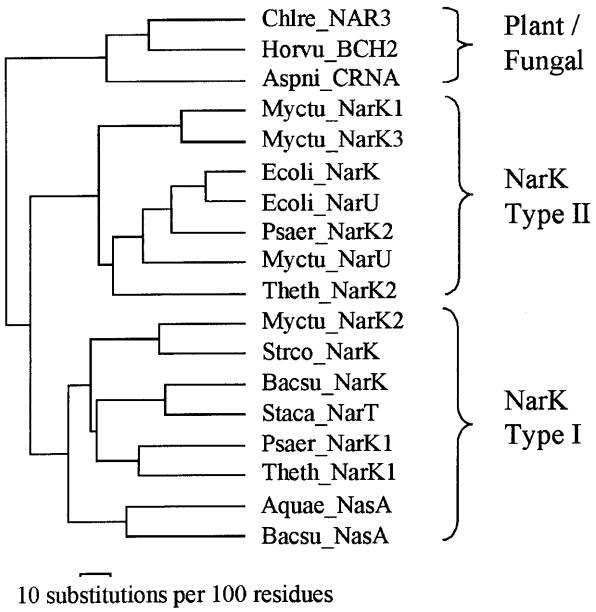

Figure 2. Phylogenetic tree of members of cluster 6 of the Major Facilitator Superfamily. A phylogenetic tree was constructed using a multiple sequence alignment created with the GCG programme pileup, calculating the difference between sequences using the Jukes-Cantor method and building a tree using the UPGMA algorithm. Members of the plant and fungal nitrate transporter group used were NAR3 from *Chlamydomonas reinhardtii* (Chlre), BCH2 from *Hordeum vulgare* (Barley) (Horvu) and CRNA from *Aspergillus nidulans* (Aspni). NarK homologues used were those from *Mycobacterium tuberculosis* (Myctu), *Escherichia coli* (Ecoli), *Pseudomonas aeruginosa* (Psaer), *Thermus thermophilus* (Theth), *Streptomyces coelicolor* (Strco), *Staphylococcus carnosus* (Staca), *Bacillus subtilis* (Bacsu) and *Aquifex aeolicus* (Aquae).

Phylogenetic relationships

NarK belongs to the Major Facilitator Superfamily (MFS) of transmembrane transporters [27]. On the basis of phylogenetic analysis the superfamily has been divided into six clusters; the NarK homologues belong to cluster 6 of the MFS [28]. The other members of this cluster are the so-called high-affinity nitrate transporters of plants and fungi (see also article by Galván and Fernandez). This latter group of proteins consists of Δp-driven transporters which import nitrate and/or nitrite into the cell [29]. The bacterial and the eukaryotic proteins clearly have a common ancestor distinct from members of the other clusters of the MFS [28]. The MFS cluster most closely related to that containing NarK is cluster 4, which contains phosphate/ester-phosphate antiporters [27], i.e. the most closely related group of transporters is also involved in anion transport.

Figure 2 is a phylogenetic tree showing the relationships within cluster 6 of the MFS transporters. The plant and fungal transporters cluster separately from the bacterial proteins. For the sake of simplicity, only three representatives of the plant/fungal group are shown in figure 2.

Within the bacterial NarK-like proteins there are two distinct subgroups. Type I includes those NarK homologues identified in *S. carnosus*, *B. subtilis* and copies of NarK1 from *Ps. aeruginosa*, *T. thermophilus* and *P. pantotrophus* (not shown) and NasA homologues from *B. subtilis* and *A. aeolicus*. Type II contains NarK and NarU from *E. coli*, copies of NarK2 from *Ps. aeruginosa*, *T. thermophilus* and *P. pantotrophus* (not shown) and three of the NarK homologues from *M. tuberculosis*.

Is there any functional significance to this division of NarK into two subgroups? The distinct types of NarK clearly diverged from a common ancestor in the distant evolutionary past, but in several cases members of each type are found, in series, in the same organism. For example, both types of NarK are found in the distantly related organisms *Ps. aeruginosa* and *T. thermophilus*. The divergent sequences of each NarK type demonstrates that two copies in series in an organism did not arise by a recent gene duplication event. The high degree of similarity between NarK1 from *Ps. aeruginosa* and *T. thermophilus* is suggestive of horizontal gene transfer between phylogenetically distant organisms. Why should two related, but distinct, types of transport protein be found in series upstream from nitrate reductase genes? The obvious suggestion is that they are both involved in nitrogen oxyanion transport but that they have distinct substrate specificities, i.e. Type I and Type II NarK are functionally divergent.

We hypothesise that type I NarKs are responsible for nitrate uptake, whereas the type II NarK proteins are nitrite exporters. Compelling evidence that *E. coli* NarK (a member of the type II group) is a nitrite effluxer has been discussed above. Other, more circumstantial evi-

dence supports the distinction between the two groups of NarK homologues. First, NasA from *B. subtilis* and *A. aeolicus* (which are clustered with the type I NarK) are encoded by genes located adjacent to the genes for assimilatory nitrate uptake. There is every reason to expect, therefore, that these gene products will be required for nitrate uptake and should have no need to function in either nitrite uptake or nitrite efflux. *M. tuberculosis* NarK2, which is located adjacent to gene for NarX (fused nitrate reductase), clusters with type I transporters, whereas the other NarK homologues from this organism are of type II. These latter genes include *narK3* and *narU*, which are located in the vicinity of nitrite assimilatory genes, suggesting they are involved in nitrite transport. The arrangements of NarK-like genes and the enzymes they may service are presented in figure 3.

The arrangement and identity of genes upstream from *narGHJI* in the denitrifier *Ps. stutzeri* is slightly different from *Ps. aeruginosa* (fig. 3). In *Ps. stutzeri* there are two genes encoding putative transporters, named *narK* and *narC* [24]. The N-terminal sequence of NarK is published, and our analysis shows it bears greatest similarity to *Ps. aeruginosa* NarK2, whereas NarC was found to be most similar to plant nitrate transporters [24], indicating that in this organism the role of type I NarK is replaced by a plant-type high-affinity nitrate uptake transporter, whereas the type II NarK is retained, presumably to function in nitrite efflux.

The high degree of similarity of *narU* and *narK* from *E. coli* clearly suggests that they arose recently through a gene duplication event, and that it is unlikely that they differ biochemically, but rather they are part of distinct nitrate reductase clusters which are differentially regulated (Regulation of gene expression section below). This leaves open the question of what is responsible for nitrate uptake in *E. coli* but, as suggested for *M. tuberculosis* (see above), other unrelated transmembrane proteins may be involved in nitrate uptake for dissimilation in *E. coli*.

NarK structure-function relationships

Consistent with a role in transmembrane transport, members of the NarK family are predicted to have 12 transmembrane helices. Hydrophobicity plots of members of the NarK family consistently show predicted helices 2 and 8 to be the least hydrophobic (our analysis), largely due to conserved arginine residues located within this predicted membrane span amongst all members of cluster 6 of the MFS (i.e. all NarK and the plant/fungal nitrate transporters). The location of these arginines within the membrane-spanning domains, and the finding that they are completely conserved, indicates that they may be crucial for anion transport. There are 15 totally conserved residues amongst the members of cluster 6 of the MFS, the two Arg residues, nine Gly residues, seven of which are found within predicted membrane spans, Pro, Phe and Tyr residues within membrane spans and a conserved Asp residue located in the loop predicted between helices 8 and 9 (fig. 4). Topology is predicted such that N- and C-termini are

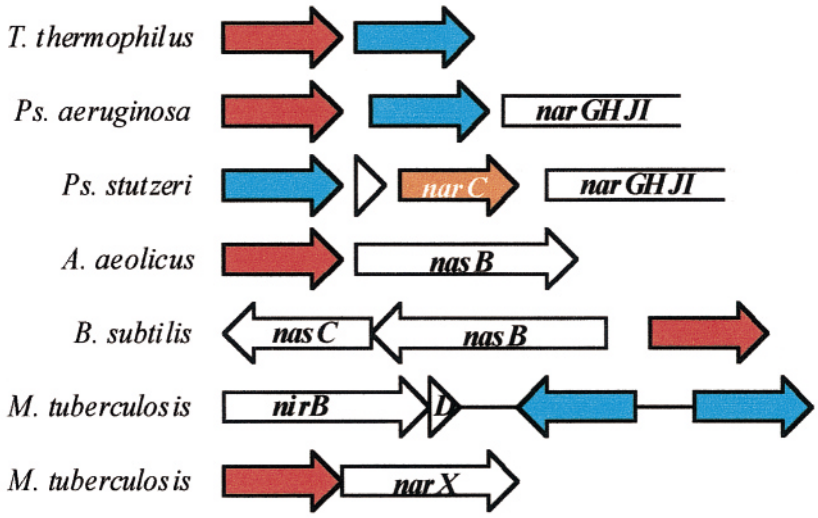

Figure 3. Genetic organisation of *narK*. Parts of gene clusters containing *narK* homologues are shown. Genes for members of the type I NarK group are shown in red and genes for members of the type II NarK group are shown in pale blue. *narC* from *Ps. stutzeri* is closely related to the eukaryotic members of the cluster 6 of the Major Facilitator Superfamily and is shown as orange.

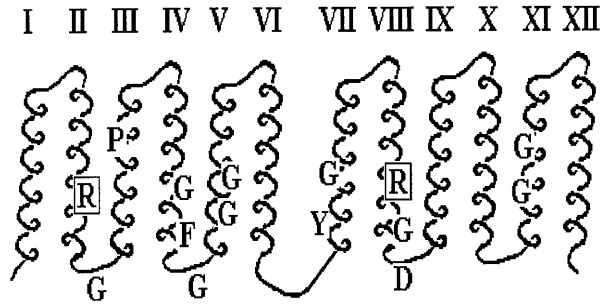

Figure 4. Transmembrane helices and conserved residues in NarK. The 12 predicted transmembrane helices of NarK are shown together with those residues which are completely conserved amongst the known members of the NarK family (and the plant/fungal nitrogen oxyanion transporters). The two conserved arginines found within predicted membraneous regions of the protein are boxed. Conserved Gly, Gly and Asp, outside of the predicted membraneous regions, are predicted to be located in the cytoplasm.

cytoplasmic. Thus, the conserved Asp is predicted to be cytoplasmic and may control access to (or egress from) a network of residues, including the Arg residues, within and crossing the membrane that allow transmembrane transport of anions. Bulky conserved residues Phe and Tyr may also help control substrate access to the transporter. Gly and Pro residues are associated with helix breaking, and may lend a flexibility to the membrane protein structure important for substrate movement.

Members of the MFS generally possess 12 transmembrane-spanning helices, which are considered to have arisen from ancient duplications of proteins containing 6 transmembrane helices [27]. Pairwise comparisons of the sequences of N-terminal and C-terminal halves of the members of cluster 6 of the MFS show them to be significantly similar to one another compared with members of other clusters of the MFS (our analysis), thus indicating that the duplication giving rise to cluster 6 occurred after evolutionary divergence from the other clusters of the superfamily. Interestingly, when the two halves of the proteins in the family are aligned, the conserved arginines from helix 2 and helix 8 align together, indicating that the arginine was present in the six-helix ancestor. Perhaps a prototypical nitrogen oxyanion transporter consisted of a dimer of identical six-helical proteins.

Is there any indication from the primary sequences of NarK for a mechanism of control of nitrate transport by oxygen? Transport has been demonstrated to be a controlling site for the effect of oxygen (redox state) on nitrate reduction in a limited number of organisms, including *E. coli* and *P. denitrificans* [1, 7]. The effect of oxygen on nitrate reduction in a wide variety of other organisms has been examined, and in most cases oxygen inhibited nitrate reduction in intact cells but not in extracts [3]. Unfortunately, that study used sonicated cell extracts, and it is not possible to differentiate between relief of inhibition at the level of nitrate transport and relief of inhibition due to competition for electrons. There are few completely conserved residues amongst the entire NarK family, and none of these are residues which might be expected to play a role in oxygen control. However, Cys residues, found within predicted transmembrane helices 4 and 10, are conserved between NarK and NarU from *E. coli* and NarK2 from both *Ps. aeruginosa* and *P. pantotrophus* (which is a very close relative of *P. denitrificans*). A redox-dependent disulfide bridge formation between these two cysteines, causing reversible inactivation of NarK under oxidising conditions, may be the cause of oxygen inhibition of nitrate/nitrite transport. Some support for the importance of redox-sensitive thiol groups in the transport process comes from findings that in *Ps. aeruginosa*, nitrate uptake is inhibited by *N*-ethylmaleimide [30]. It is possible that control of nitrogen oxyanion transport by oxygen is limited to the nitrate-reducing proteobacteria (e.g. *E. coli*, *Paracoccus* species, *Pseudomonads*). It will be of interest to specifically examine the impact of oxygen on transport and nitrate reduction in intact cells of a wider variety of organisms than have so far been rigorously tested. The possibility that the control of nitrogen oxyanion transport by oxygen is exerted at the level of nitrite transport (i.e. type II NarK) is lent support by the findings of Kucera and co-workers [9] that in the presence of oxygen intracellular nitrate concentration is higher than under anaerobic conditions.

A working model for nitrate and nitrite transport via type I and type II NarK, the effect of mutation of the type II transporter and the impact of oxidising conditions on nitrite accumulation and hence inhibition of nitrate uptake via the type I transporter is presented in figure 5.

Regulation of gene expression

Regulation of the nitrate reduction process by oxygen occurs at the level of inhibition of transport, but there also is regulation of gene expression. Specific regulation of *narK*-like genes is covered briefly here.

Preceding the *narK* locus of *E. coli* are two control motifs: one for the oxygen sensor protein FNR, the other responsive to the nitrate/nitirite sensor/regulator two-component system, NarXL [19]. The juxtaposition of these motifs ensures that *narK* expression only occurs when conditions suitable for anaerobic nitrate respiration are present, as the expression of *narL* is unaffected by anaerobiosis [31]. Similarly, the *Ps. stutzeri narK* region also bears FNR and NarL binding sites [24]. The

second *narK* homologue in this region (*narC*) does not, however, although this would be explained if the two *narK* genes were transcribed as a single message, forming an independent operon. In *E. coli* the *narXL*, *narK* and *narGHJI* genes are transcribed as three independent units. This also appears to be the case in *Ps. stutzeri*, as the largest *narG* transcript observed ($\sim$ 6.9 kb) would accommodate *narGHJI* but is of insufficient length to include *narK*/*narC* and/or *narXL* messages.

The α subunit of membrane-bound nitrate reductase contains a molybdenum cofactor which is at the active site. Transcription of molybdoenzyme genes in *E. coli* is regulated according to the availability of molybdenum. Ultimate control resides with the molybdenum sensor ModE, mutations in which have the effect of reducing expression from *narG* and *narK*. ModE binds to the *narXL-narK* intergenic region, and the reduced levels of *nar* expression in a *modE* strain are probably due to the reduced activity of the nitrate-responsive element NarL [32].

E. coli contains a second nitrate reductase encoded by the *narZ* locus. DNA sequence analysis revealed the absence of *narXL* homologues in the vicinity, and until recently the mode of regulation and a likely role for this apparently redundant operon remained a mystery. Evidence now points towards a role for *narZ* in stationary phase survival or in response to stress after the demonstration that it is under the control of the alternative sigma factor RpoS [33] (see also article by Richardson et al.). Whether the *narK* homologue, *narU*, present at the *narZ* locus is also regulated in a stationary phase/stress-responsive manner remains to be tested, but it would appear likely.

What can be concluded from the multitude of regulators that appear to have a part to play in control of nitrogen oxyanion transporter transcription? One of the implications is that the multiple regulators must interact closely either with each other or with the RNA polymerase, the concerted effect of which is to fine-tune the transcription of transport systems to prevailing environmental conditions. The situation in prokaryotes, while certainly complex, is nevertheless simpler than in eukaryotes, where signals have to traverse both the cytoplasmic and nuclear membranes and where repression by ammonium is also a common feature (see the review by Galvan and Fernandez).

Assimilatory nitrate transport

Bacteria take up nitrate, which is subsequently reduced to nitrite and then ammonia, and is then assimilated into cellular organic material. This process requires a transporter for nitrate. There are two types of transporters involved in nitrate uptake for assimilation in bacteria; members of the NarK family discussed above, and ATP-driven nitrate transporters that belong to the ABC transporter family [34].

B. subtilis is capable of growth using nitrate as sole source of nitrogen. Assimilatory nitrate and nitrite reduction is encoded in the locus-containing genes *nasA–F* [35]. Mutational analysis shows that *NasB* and *nasC* are required for nitrate reduction, and *nasDEF* are required for nitrite reduction. Mutants in *nasA* are incapable of growth on nitrate but are unaffected in growth on nitrite. The growth defect in the *nasA* strain is only explained if NasA is involved in nitrate transport, although nitrate uptake has not been directly measured. NasA is a member of the NarK family of nitrogen oxyanion transporters, thus supporting the demonstration from physiological analysis that NasA is a nitrate transporter. NasA homologues are also located in the close vicinity of genes for assimilatory nitrate reductase in *A. aeolicus* [23] and *Ps. aeruginosa* (accession no: AF112870).

A completely distinct type of nitrate transporter is involved in assimilatory nitrate reduction in some other bacterial species. In dissimilatory systems, it is crucial that metabolic energy is not wasted on the nitrate transport process, whereas this is not so important for assimilation. Therefore, systems in which ATP-driven

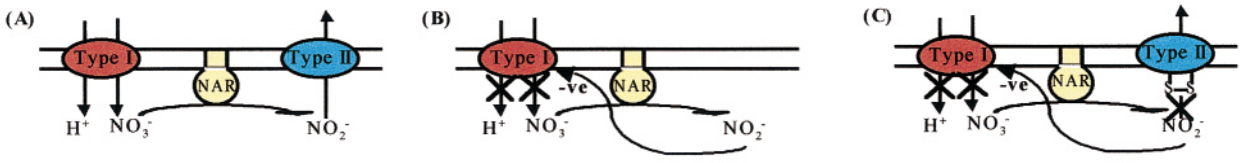

Figure 5. Type I and type II NarK. A proposed mechanism for oxygen inhibition. (*A*) Type I NarK is hypothesised to import nitrate into the cytoplasm, whereas type II NarK exports nitrite into the periplasm. (*B*) In the absence of type II NarK, nitrite builds up, which may inhibit further nitrate uptake via type I NarK in a feedback effect. (*C*) The proposed role of two cysteine residues found amongst type II NarK is to form a disulfide bridge under oxidising conditions, which prevents nitrite export and hence nitrate uptake by a feedback mechanism.

assimilatory nitrate uptake occurs have become successful, exemplified particularly by *Klebsiella* and *Synechococcus* species. Periplasmic binding-protein-dependent ATP-binding cassette (ABC) transporters are responsible for active nitrate (and nitrite) uptake in these organisms. The transport systems are encoded by *nasFED* in *K. oxytoca* [36] and by *nrtABCD* in *Synechococcus* sp. PCC7942 [37]. *nasD* and *nrtD* and *nrtC* encode the cytoplasmic protein subunits which possess the characteristic ATP binding motifs of ABC transporters. NasD is thought to form a homodimer, whereas NrtC and NrtD function as a heterodimer. *nasE* and *nrtB* encode the integral membrane subunits of the transporter which form the pore allowing nitrate (and nitrite) transport. *nasF* and *nrtA* encode nitrate-(and nitrite-) binding proteins in the periplasm and support high-affinity uptake of nitrate via these ABC transporters. It has been noted that NrtA is unusual in this class of binding proteins since it is anchored to the cell membrane [38]. Interestingly, mutations in *nasF* and *nrtA* are leaky, i.e. high concentrations of nitrate can support growth, indicating that the binding proteins are not obligatory for nitrate uptake, but rather that they are required for high-affinity nitrate uptake [36, 37]. It has been found that an *nrtD* mutant of *Synechococcus* sp. PCC7942 is capable of nitrite uptake at neutral pH but that the mutant (unlike the wild type) is deficient in nitrite uptake at pH 9.6 [39], indicating that the gene product is also involved in nitrite uptake. At neutral pH, passive diffusion of nitrous acid is kinetically significant, but at higher pH it is not.

Summary and future perspectives

A picture of the mechanisms by which nitrogen oxyanions are transported in bacteria is beginning to emerge. Nitrate and nitrite uptake via ATP-driven systems for assimilation is fairly well understood, but the precise functions of NarK homologues in both dissimilatory and assimilatory transport is still somewhat obscure. A hypothesis for different roles of phylogenetically distinct groups within the NarK family has been presented here, and this needs to be tested rigorously. Combining genetic and physiological analysis and by measuring accumulation of intracellular nitrate and nitrite, it may be possible to identify the roles of NarK homologues and identify whether they are very specific for certain substrates, or whether they are more promiscuous, being capable of, say, both uptake and excretion of nitrite (type II NarK), or both nitrate/proton symport and nitrate/nitrite antiport (type I NarK).

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