Missense Mutations that Inactivate the *Aspergillus nidulans nrtA* Gene Encoding a High Affinity Nitrate Transporter.

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Running title: Mutational analysis of NrtA.

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ABSTRACT

The transport of nitrate into prokaryotic and eukaryotic cells, of considerable interest to agriculture, ecology and human health, is carried out by members of a distinct cluster of proteins within the major facilitator superfamily. To obtain structure/function information on this important class of nitrate permeases, a collection of chemically-induced mutations in the nrtA gene encoding a 12 transmembrane domain, high affinity nitrate transporter from the eukaryote Aspergillus nidulans were isolated and characterized. This mutational analysis coupled with protein alignments, demonstrates the utility of the approach to predict peptide motifs and individual residues important for the movement of nitrate across the membrane. These include the highly conserved nitrate signature motif (residues 166 to 173) in Tm 5, the conserved charged residues Arg87 (Tm 2) and Arg368 (Tm 8) as well as the aromatic residue Phe47 (Tm 1) all within transmembrane helices. No mutations were observed in the large central loop (Lp 6/7) between Tm 6 and Tm 7. Finally, the study of a strain with a conversion of Trp481 (Tm 12) to a stop codon suggests that all 12 transmembrane domains and/or the C-terminal tail are required membrane insertion and/or stability of NrtA.
INTRODUCTION

Nitrate is a key source of nitrogen for a large number of microorganisms and plants. While nitrate limitation frequently confines the growth of these organisms especially in natural environments, its use in fertilizers to improve plant crop yield may give rise to (i) eutrophication of natural water systems and (ii) human and animal health concerns (reviewed by CRAWFORD and GLASS 1998; DANIEL-VEDELE et al. 1998; WILLIAMS and MILLER 2001). Furthermore, the central importance of nitrate is highlighted by reports that it is involved in certain plant metabolic and morphogenic processes (SCHIEBLE et al. 1997; ZHANG and FORDE 1998).

The influx of nitrate into cells is an active process since it occurs against a nitrate gradient (BROWNLEE and ARST 1983; FORDE 2002, VIDMAR et al. 2000). There are at least two classes of nitrate transport systems, high and low affinity, that have been identified (TRUEMAN et al. 1996). The Aspergillus nidulans nrtA (formerly crnA) gene (BROWNLEE and ARST 1983) encodes a membrane protein that belongs to a family of high affinity nitrate transporters (FORDE 2000; UNKLES et al. 1991, 2001). A typical secondary structure (Figure 1) was proposed for the 57 kDa (507 amino acids) NrtA protein in which 12 hydrophobic transmembrane domains (Tm) in alpha-helical conformation, pass through the membrane and connect by hydrophilic loops (Lp). The NrtA homologues belong to a distinct cluster, namely the Nitrate-Nitrite Porter Family (NNP; TC 2.A.1.8), within the major facilitator superfamily (MFS; TC 2.A.1) which comprises a range of functionally diverse proteins including mammalian and bacterial sugar transporters (NELISSEN et al. 1997; SAIER et al. 1999). In common with other MFS transporters, a motif Gly X X Asp X X Gly X Arg can be observed around Lp 2/3 with a less well conserved version around Lp 8/9 probably reflecting the proposed duplication of the first six
Tms which is thought to have occurred early in the evolution of MFS proteins (PAO et al. 1998). One unique feature of high affinity nitrate transporters is the presence of a consensus sequence Ala Ala Gly X Gly Asn X Gly Gly Gly, residues 163 to 172 (Figure 1) in Tm 5 described as the nitrate signature with a similar sequence repeated within Tm 11 (TRUEMAN et al. 1996; FORDE 2000). Second, conserved charged residues within otherwise hydrophobic helices, Arg87 (Tm 2) and Arg368 (Tm 8), or aromatic residues Phe47 (Tm 1) can be observed within NrtA (FORDE 2000). A final feature of A. nidulans NrtA (although common amongst MFS transporters) is its substantial (around 96 residues) central loop (Lp 6/7) which, although present, is much reduced in the corresponding plant proteins (maximum length of 32 residues). Instead, a long hydrophilic C-terminus of 69 residues is observed in plant NrtA-like proteins (TRUEMAN et al. 1996; FORDE 2000).

Complex hydrophobic membrane transporters are inherently difficult to purify and crystallize, clearly an obstacle to the study of their structure. Notwithstanding, three high resolution MFS protein structures have recently been solved for the oxalate-formate transporter OxlT (HIRAI et al. 2002), the glycerol-3-phosphate-inorganic phosphate transporter GlpT (HUANG et al. 2003) and the lactose transporter LacY (ABRAMSON et al. 2003). A striking feature of these structures is that the overall architecture of each of the proteins representing different MFS families is very similar, even superimposable (ABRAMSON et al. 2004; HIRAI and SUBRAMANIAM 2004). A general theme is the presence of a central substrate binding site within a hydrophilic pore access to which alternates from outside to inside and vice versa by the flexible movement of Tms. Details of the substrate binding site define the substrate specificity. In this respect and together with the fact that virtually nothing is known about NrtA function, we have used chemical mutagenesis to
change NrtA residues and study their effect on protein expression and transport. Such a strategy has yielded valuable information on the *E. coli* lacY permease (BAILEY and MANIOL 1998). The NrtA permease from the lower eukaryote *A. nidulans* is a particularly useful model for studying nitrate transport structure/function relationships due to the amenability of this organism to combined genetical and biochemical approaches. Furthermore, as MFS transporters make up a considerable proportion of membrane proteins in eukaryotes (WARD 2001), information on the NrtA branch of this important family, should be of wider significance in our understanding of eukaryotic transport proteins.

**MATERIALS AND METHODS**

*Aspergillus nidulans strains and media:* Standard wild-type (with regard to nitrogen regulation) strains with various color markers used for the isolation of *nrtA* mutants by chemical mutagenesis in this study were (i) *biA1*, (ii) *yA2 pyroA2*, (iii) *fwA1* and (iv) *chA1*. A multi-copy *nrtA* strain, designated SS1, was identified (in an attempt to generate a transformant over-expressing the nitrate transporter) on the basis of super-sensitivity to chlorate toxicity at a concentration of chlorate (10 mM) not toxic to the wild-type with urea (10 mM) as the sole nitrogen source (S. E. UNKLES unpublished results). Strain *nrtAgfp8* contains NrtA fused at the C-terminus to green fluorescent protein (CORMACK *et al.* 1997) encoded by a single copy construct integrated at the *argB* locus (D. A. ROUCH and S. E. UNKLES unpublished results). Routine *Aspergillus* growth media and handling techniques were as described before (CLUTTERBUCK 1974).

*Escherichia coli strains, plasmids and media:* Standard procedures were used for propagation of plasmids as well as for subcloning in *E. coli* strain DH5α. *E.*
coli strain BL21 (DE3) (Novagen, Inc., Madison, WI) was used to express a Lp 6/7 protein (residues 219-317) from a PCR-generated product ligated into pET21a (Novagen) (E. R. DASILVA unpublished results).

**Molecular methods:** DNA was isolated using a Nucleon BACC2 Kit (Amersham Pharmacia Biotech, Little Chalfont, UK). The conditions used during Southern blot analysis were as described previously (McCABE *et al.* 1990). Following PCR amplification of overlapping fragments using primer pairs (nrtA1.1 and nrtA1.2, nrtA2.1 and nrtA2.2, nrtA3.1 and nrtA3.2, nrtA4.1 and nrtA4.2, nrtA5.1 and nrtA5.2, nrtA6.1 and nrtA6.2) shown in Table 1, the entire nucleotide sequence of the *A. nidulans nrtA* mutants was determined by automated sequence analysis as described before (UNKLES *et al.* 1997).

**Isolation of chemically induced nrtA mutants:** Wild-type strains (with regard to nitrate assimilation) were treated with chemical mutagens which preferentially induce single mutations in GC nucleotide base pairs. After mutagenesis with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NTG) (ADELBERG *et al.* 1965) or 4-nitroquinoline-1-oxide (NQO) (BAL *et al.* 1977), mutants were isolated on the basis of resistance to (200 mM) chlorate toxicity with uric acid as the sole source of nitrogen (COVE 1976a). This approach takes advantage of the fact that mutations in the untranscribed strand are much less efficiently repaired than those in the transcribed strand thereby favoring changes, for example, in glycine (G-rich codons) rather than proline (C-rich) residues. In most mutant isolation experiments, sodium deoxycholate (0.08%) was included in the medium to reduce colony size and increase the number of colonies screened per Petri dish. *nrtA* mutants were distinguished from other chlorate resistant nitrate assimilation defective strains, by their ability to utilize nitrate (COVE 1976b).
The generation of \textit{nrtA nrtB} double mutants: A number of \textit{nrtA} mutant strains were crossed to \textit{nrtB}110, a mutant strain which contains a deletion in the gene encoding the other nitrate transporter, NrtB, in \textit{Aspergillus nidulans} (Unkles \textit{et al.} 2001). Similar to \textit{nrtA} loss-of-function mutants, strain \textit{nrtB}110 grows normally on 10 mM nitrate as the sole source of nitrogen. Putative \textit{nrtA nrtB} double mutants (i.e. strains that grew poorly on nitrate, were verified by Southern blot analysis of the \textit{nrtB} to detect the deletion mutation \textit{nrtB}110 (Unkles \textit{et al.} 2001) and PCR amplification followed by DNA sequencing of the \textit{nrtA} mutant gene to ensure the presence of the \textit{nrtA} mutation.

Net nitrate transport assays: Strains were grown for 6.5 to 7.5 h at 37$^0$ in liquid minimal medium with 5 mM urea as the sole source of nitrogen (COVE 1966). The inducer of \textit{nrtA} expression, sodium nitrate (10 mM) was added 100 min prior to harvesting by filtration. Assays were carried out as described by Brownlee and Arst (1983).

Western blotting: Growth and induction conditions were as above. The resulting spore germlings were washed with cold sterile distilled water and frozen in liquid nitrogen. Crude plasma membrane preparations were made by grinding around 300 mg pressed wet weight of the spore germlings in liquid nitrogen and suspending the powder in 10 ml extraction buffer (250 mM sucrose, 5 % (v/v) glycerol, 1 mM magnesium chloride, 1 mM EDTA, 25 mM MOPS, pH 7.2) at 4$^0$ containing 100 µM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol and one Complete Mini protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany) per 10 ml extraction buffer. The suspension was centrifuged at 2000 x g for 10 min at 4$^0$, the supernatant collected and centrifuged at 18000 x g for 30 min at 4$^0$. The gelatinous pellet was resuspended in 150 µl extraction buffer and stored at -80$^0$. Protein samples
(50 µg) were electrophoresed on 10 % acrylamide gels (LAEMMLI 1970) and transferred to nitrocellulose membrane (TOWBIN et al. 1979). Blots were blocked by incubation overnight at 4°C in TBS (137 mM sodium chloride, 20 mM Tris, pH 7.5) containing 5 % (w/v) membrane blocking agent (Amersham Pharmacia Biotech). NrtA was detected by incubation of the blot with 1:500 anti-NrtA antibody (UNKLES et al. 2004) in TBS containing 0.5 % (w/v) membrane blocking agent for 2 h at room temperature and, after washing with TBST (TBS containing 0.1 % (v/v) Tween 20), with 1:40000 horseradish peroxidase conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, USA) for 1 h at room temperature. After further washing with TBST, peroxidase activity was visualized using ECL Plus reagents (Amersham Pharmacia Biotech) and Hyperfilm ECL (Amersham Pharmacia Biotech). For GFP detection, peroxidase-conjugated polyclonal anti-GFP antibodies were purchased from Santa Cruz Biotechnology and used at a concentration of 1:500.

RESULTS

Conserved residues: An alignment of 52 available NrtA sequences was analysed using Clustal W (Thompson et al. 1994) and refined by eye. These sequences included proteins from a diverse range of organisms from eubacteria and archaeabacteria to algae, fungi and plants. Sixteen very highly conserved residues (defined on the basis of 50 of 52 conservation (i.e. greater than 95%) are indicated in red (Figure 1). These residues are Phe47 (50/52), Arg87 (52/52), Phe140 (52/52), Gly157 (52/52), Gly165 (51/52), Gly167 (51/52), Gly170 (52/52), Tyr323 (52/52), Gly328 (52/52), Arg368 (52/52), Gly371 (52/52), Gly372 (52/52), Asp376 (52/52), Gly452 (52/52), Gly458 (51/52) and Gly461 (51/52).
**nrtA mutant isolation:** The nrtA gene from 57 nrtA mutants was completely sequenced and of these, 38 were found to be missense mutants, the remainder represented by eight deletions, six chain termination, two intron boundary and three insertions. The location and distribution of residues altered in the 38 missense mutants are presented in Table 2 and Figure 2. Amino acid replacements in all Tm domains except Tm 3 and Tm 6 were observed and as predicted (see Materials and Methods) a substantial number of glycine and arginine alterations (33/38) were observed. A large proportion of missense mutations (i.e. ten) occurred within Tm 5 and all these (26% of the entire collection of missense mutants) were found to locate within the nitrate signature motif. Nine of these replacements were found in just three glycine residues including residues Gly167 and Gly170 very highly conserved in diverse organisms (Figure 1). Interestingly, Gly172 which is conserved amongst the eukaryotes only is altered in four mutants (to cysteine, serine or aspartate in two independent mutants). There are 46 glycine residues in the entire protein and so with 28 glycine-altering mutations it would be expected that three glycine residues would be “hit” with a combined frequency of 1.8 on a random basis (or less than one mutation in each of the 3 codons). This estimation is based on the assumption that mutation events are entirely random (i.e. independent of DNA sequence conservation) and that only those resulting in a phenotype are recognized.

One missense mutation was found to affect Tm 1, four located within Tm 2, including two in the highly conserved residue Arg87, two in Tm 4 and three in Tm 7. Seven mutations occurred within the nucleotides encoding residues of Tm 8, including the highly conserved Arg368. The other six mutants were represented by alterations to glycine residues; Gly361 and the highly conserved Gly371 and Gly372 residues, with Gly371 being changed in three independent mutants. Of the remaining
mutants, one mutation located within Tm 9 and two each were observed in Tms 10, 11 (including highly conserved Gly458) and 12. With regard to the loops, one missense mutation was observed to affect Lp2/3 (a MFS motif), two mutations altered the highly conserved Gly157 in Lp 4/5 and one change affected Lp 5/6 whilst none was observed within the large central Lp 6/7.

Six chain termination mutants were observed in our collection of mutants. These were distributed somewhat randomly across the gene and included a mutant, \textit{nrtA40P}, in which a stop codon replaced Trp481 in Tm 12. This strain was the subject of expression analysis (see below).

Finally, 12 deletions or insertions were identified and included strains used in previous studies \textit{vis-à-vis nrtA1}, formerly \textit{crnA1}, (TOMSETT and COVE 1979; BROWNLEE and ARST 1983; UNKLES \textit{et al.} 1991) and \textit{nrtA747} (UNKLES \textit{et al.} 2001) both of which were found to have a single bp deletion - \textit{nrtA1} at nucleotide position 481, codon 125 (Lp 3/4) and \textit{nrtA747} at nucleotide position 361, codon 85 (Tm 2). Other deletions included \textit{nrtA1009}, \textit{nrtA1010}, \textit{nrtA17}, \textit{nrtA65P}, \textit{nrtA343P}, \textit{nrtA2039F} and \textit{nrtA24Y}, while insertions occurred in mutants \textit{nrtA3} and \textit{nrtA97P}. Noteworthy is mutant \textit{nrtA52P} which has a single base pair addition near the 3’ end of the coding region (described in the footnote of Table 2) and was used in antibody studies.

\textbf{Resistance to chlorate toxicity:} Missense mutants isolated on the basis of chlorate resistance with urea as the nitrogen source, were tested for resistance to 200 mM chlorate on other nitrogen sources. All mutants remained similarly resistant as the loss-of-function strains \textit{nrtA1} and \textit{nrtA747} with proline and urea and equally sensitive as the wild-type strain (and as sensitive as \textit{nrtA1} and \textit{nrtA747}) on 10 mM glutamate or 10 mM arginine (UNKLES \textit{et al.} 2001).
**Net nitrate uptake:** Most of the missense mutants showed similar net transport basal levels (Table 2) as the deletion strains \(nrtA1\) and \(nrtA747\), (i.e. around 3-4 nmol/min/mg compared with around 10-12 nmol/min/mg in the wild-type strain), this basal level in loss-of-function \(nrtA\) mutants being due to the nitrate uptake contribution by the NrtB transporter (UNKLES et al.2001). Consequently, such missense mutations were regarded as NrtA loss-of-function. In addition, a number of mutants, (namely \(nrtA2015F, nrtA2036F, nrtA7P, nrtA2049F, nrtA946, nrtA2002F, nrtA14P, nrtA2044, nrtA14M, nrtA23P, nrtA19P\) and \(nrtA2059F\)) possessed net nitrate uptake above the basal level (Table 2).

**\(nrtA\) \(nrtB\) double mutants:** To determine if the higher level of net nitrate uptake in \(nrtA\) single mutants strains \(nrtA2015F, nrtA2036F, nrtA7P, nrtA2049F, nrtA946, nrtA2002F, nrtA14P, nrtA2044, nrtA14M, nrtA23P, nrtA19P\) and \(nrtA2059F\) was due to NrtA activity per se, the mutants were crossed one by one to a deletion mutant (\(nrtB110\)) in the other \(A\).nidulans nitrate transporter NrtB (UNKLES et al.2001). Double mutants were identified and verified by molecular technology (see Materials and Methods). These failed to grow on 10 mM or even 100 mM nitrate, similar to double deletion mutant strain \(nrtA747 nrtB110\) (Figure 3). Furthermore, negligible net nitrate uptake (i.e. 0 nmol/min/mg values) was observed in these double mutant strains, similar to double deletion mutant strain \(nrtA747 nrtB110\).

**NrtA expression:** To determine if mutant proteins were stably expressed, we assayed steady state levels in 6.5 h grown germlings (induced by nitrate) by Western blotting (Figure 4). Low levels of NrtA protein (around 50 kDa instead of the predicted 57 kDa) could be observed using anti-NrtA antibodies in the wild-type (Figure 4A lane 1) but none was observed in chain termination mutants \(nrtA747\).
The anti-NrtA antibodies also detected Lp 6/7 protein expressed in E. coli (S. E. UNKLES and E. R. DASILVA unpublished results). In contrast, the chain termination mutants nrtA747 (lane 2) and nrtAI (S. E. UNKLES unpublished results) lacked the 50 kDa signal as might be expected since these proteins, if expressed, would be devoid of the antibody recognition sites. However, neither chain termination mutant nrtA40P (lane 3) nor mutant nrtA52P (S. E. UNKLES unpublished results), with an addition of 44 codons following codon 489 at the 3’ end, possessed detectable NrtA protein. In a preparation from strain nrtAgfp8, which contains a single copy of the NrtA:GFP fusion construct at the argB locus, a protein of around 72 kDa (instead of the expected 81kDa) was revealed (lane 5). Anti-GFP antibodies also detected this 72 kDa protein in the preparation from nrtAgfp8 (lane 7) but not, as expected, in the wild-type protein sample (lane 6). Confirmation that the 50 kDa band was correctly identified as NrtA was obtained with the observation of a high intensity signal of that molecular size in transformed strain SS1 containing multiple copies nrtA (lane 4). Using these anti-NrtA antibodies, all missense mutants such as nrtA8 (Figure 4B lane 1), nrtA2 (lane 2) and nrtA2082 (lane 3) were shown to have similar low but observable levels of NrtA protein as in the wild-type (lane 4). Again, no 50 kDa protein was observed in the negative control sample for this blot, i.e. nrtA747 (lane 5).

**DISCUSSION**

A mutational survey of NrtA has been accomplished which gives clues to the residues that may play a role in nitrate transporter function, not only for fungi, but also for higher plants which are less amenable to such genetical approaches. Chemical mutagens were employed to target GC base pairs yielding mutant strains.
with a recognizable phenotype, i.e. chlorate resistance, thus identifying possibly important residue positions for activity of NrtA.

One clear hotspot for mutations was observed within the first nitrate signature of Tm 5, where residues Gly167, Ala169, Gly170 and Gly172 were represented by a total of ten substitutions. This relatively high frequency of change, together with the very high level of conservation of Gly167 and Gly170, suggests that this motif is crucial for function and even conservative substitutions, for example glycine to serine or cysteine, result in loss-of-function. The preponderance of such compact residues with small side chains and intolerance of even moderate increase in side chain bulk suggests a requirement for particularly tight helix packing in the region of the nitrate signature of Tm 5.

There are two arginine residues, Arg87 and Arg368, which are found within the otherwise hydrophobic helices of Tm 2 and Tm 8, respectively. These are the only positively charged residues conserved within Tm regions in all nitrate transporters and both were represented by mutations in this study, two resulting in Arg87 conversion to Gln and one in which Arg368 was changed to Cys. Since such mutations involved the loss of charge, it would appear that a positive charge at these positions is necessary for transport function.

Interestingly, helical wheel analysis of Tms in which several mutations have been identified (Tm 2, Tm 5, Tm 7 and Tm 8), showed that all of the mutations in Tms 2, 7 and 8 locate on one face of the Tm (S. E. UNKLES unpublished results). This distribution of mutations fits well within the context of known MFS transporter structures where Tms 1, 2, 4 and 5 of the N-terminal half of the protein, along with Tms 7, 8, 10 and 11 of the C-terminal portion, form the substrate translocation pore. Thus in NrtA also, Tms 2, 7 and 8 appear to have a specific orientation with those residues altered in this study probably facing the substrate channel. Of key interest
among the conserved residues targeted by our chemical mutagenesis are Arg87 in Tm 2 and Arg368 in Tm 8 since their positively charged side chains have the potential to interact directly with the nitrate anion. Unlike Tms 2, 7 and 8, however, residue positions of Tm 5 mutations are scattered throughout the helix supporting a structural role for these glycine and alanine residues, by virtue of their small side chain volume, rather than a specific function in nitrate translocation. Finally, it is noteworthy that although Tm 3 possesses three glycine residues, no mutants in this helix were recovered which may reflect the non-essentiality of these residues.

Indeed, in terms of known MFS structures, Tm 3 along with Tms 6, 9 and 12 are embedded within the membrane. As such they are unlikely to participate directly in transport and perhaps therefore are more tolerant of minor or localized conformational changes which would not have been recognized by our selection for loss-of-function.

The aromatic residue Phe47 is the only Tm 1 residue conserved in all species studied thus far and the only Tm 1 change (to Ser) present in our missense mutant collection. Interestingly, Tm 1 has a high proportion of aromatic residues (10/21) several of which are highly conserved within eukaryotic nitrate transporters, forming a motif Phe X X X Trp X X Phe X X X Phe X X X Phe/X X Phe/Tyr from position 36 to 51. Helical wheel analysis again places Phe47 on the same face as the other aromatic residues in this motif suggesting that these bulky side chains may have a function equivalent to aromatic residues of GlpT whose role is to close the translocation pore following binding of substrate (HUANG et al. 2003). Alternatively, Phe47 (and its symmetrical equivalent, the highly conserved aromatic amino acid Tyr323 in Tm 7) may be positioned in such a way as to constrain the flexibility of the long side chains of Arg87 and Arg368 within the translocation pore. Either way, alteration of the
bulky Phe47 to a compact residue such as serine might be expected to lead to loss-of-function.

With regard to the functionality of loops, not unexpectedly perhaps, alterations were observed in the sequence Gly X X Asp X Gly X Arg (residues 91 to 100 in *A. nidulans*) of Lp 2/3 and vicinity, a well conserved motif in the MFS superfamily. A conservative polar alteration to Ser of the highly conserved residue Gly91 (predicted to lie near the border of Tm 2 and the first residue of the conserved Lp2/3 MFS motif) results in concomitant reduction of transport activity suggesting that this residue is essential in NrtA. In the lactose transporter of *E. coli*, replacement of the equivalent glycine residue with amino acids of increased bulk (other than alanine) resulted in marked reduction of activity (JESSEN-MARSHALL *et al.* 1995). A similar sensitivity to bulk could account for the mutant phenotype observed with Gly372 (to Asp) within the repeated MFS motif at Lp 8/9 and possibly also for the adjacent Gly371 (represented by three mutations to Glu or Asp) although the effect may be due to charge introduction. In addition, a mutant was isolated in this study in which an Asp95 to Asn modification was represented. This aspartate residue is thought to be critical for the function of this MFS motif as a conformationally versatile region (PAZDERNIK *et al.* 2000). Within Lp 4/5, Gly157 is conserved (among a peptide stretch of lower similarity) in all nitrate transporters studied and modification of this residue (two independent changes to Arg or Glu) was noted. Although six glycine and four arginine residues are present in the poorly conserved large central loop (Lp 6/7), the fact that no missense mutations were found in the stretch of DNA encoding this loop might suggest that individual residues within Lp 6/7 play no crucial functional role.

Of the remaining mutants, alterations were observed in two of the glycine residues (Gly137 and Gly138) of Tm 4 which comprise a motif somewhat loosely
conserved in many MFS proteins including sugar transporters (HENDERSON 1991). Further glycine residues altered within Tms included the conserved residues Gly328 in Tm 7, Gly361 in Tm 8 (represented by two independent mutations), Gly433 in Tm 10, two glycines of the repeated nitrate signature motif of Tm 11 (Gly458 and Gly462 with two mutations) and Gly484 in Tm 12 as well as the non-conserved Gly396. However, all of these changes introduce a bulkier residue with a positive or negative charge into the Tm which may cause major local structural perturbation, and so it cannot be deduced whether these glycine residues per se are necessary for transport. The exception is Gly484 for which one of the changes was to Cys which might be expected to be tolerated.

The NrtA protein was expressed in all missense mutants (but not in chain termination mutants, see below), as detected by Western blots, at approximately similar levels although it is difficult to be precise due to the low levels of NrtA expression. Extensive in vitro mutagenesis of the E. coli LacY protein has revealed that only 5 % of missense mutations result in lack of expression of the lacY protein (BAILEY and MANIOL 1998; FRILLINGOS et al. 1998) and so expression of all the mutant proteins generated in this study, representing just 5 % of NrtA residues, might be expected. Among the six chain termination mutants observed, the mutant phenotype obtained by alteration of Trp481 within Tm 12 (mutant nrtA40P) was of particular interest. Together with nrtA52P in which the C-terminal 18 residues was replaced by 44 residues these were the only mutants encoding epitopes recognized by our anti-NrtA antibodies which had no detectable protein in Western blots. This suggests that all 12 transmembrane domains and/or the short C-terminal tail are required for NrtA insertion into the membrane and/or protein stability.

Whilst most of the missense mutants showed net transport basal levels of around 3-4 nmol/min/mg similar to deletion strains nrtA1 or nrtA747, (compared
with around 10-12 nmol/min/mg in the wild-type strain), certain missense mutants possessed activity above the basal level. Surprisingly, double mutants of these and strain \textit{nrtB110} (a deletion mutation within the other nitrate transporter NrtB) showed neither growth even on high contrations of nitrate, nor significant detectable net nitrate transport. Clearly therefore, these \textit{nrtA} mutants are NrtA loss-of-function, enhanced levels of activity observed in the single missense mutants perhaps being due to increased NrtB expression or possibly to a gain-of-function mutation in another transporter, alternatives which are currently being investigated. The results suggest that the selection of nitrate transport mutants on the basis of resistance to chlorate toxicity (at least at a concentration of 200 mM chlorate with uric acid as the sole source of nitrogen) yields \textit{nrtA} mutants which all appear to be loss-of-function. This is in contrast to \textit{nrtB} mutants which are sensitive to chlorate toxicity at a range of concentrations and with various single nitrogen sources.

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SCHEIBLE, W.R., A. GONZALES-FONTES, M. LAURER, B. MULLER-ROBER, M.


Table 1. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
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<tr>
<td>nrtA1.1 and nrtA1.2</td>
<td>TTCTACGAACTGCAGTTCC and TCCTTCAGTCCGTTGTC</td>
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<tr>
<td>nrtA2.1 and nrtA2.2</td>
<td>TGCTTGCATTCCTCTCATG and TGAGGTAACGAGGCCG</td>
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<td>nrtA6.1 and nrtA6.2</td>
<td>CAATGGGTTTCTCAGATCC and CCAAAAGCCTCTTTGAG</td>
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Sequences are shown 5’ to 3’.
Table 2. Characteristics of \( nrtA \) mutants.

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<th>Nucleotide change</th>
<th>Residue substitution</th>
<th>Position$^a$</th>
<th>Type of change$^b$</th>
<th>Net nitrate uptake$^c$</th>
<th>Expression$^c$</th>
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<td>( nrtA348P )</td>
<td>NTG</td>
<td>TTC→TCC</td>
<td>F47S</td>
<td>Tm1</td>
<td>Non-polar→Polar</td>
<td>3.50 ± 1.06</td>
<td>+</td>
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<td>( nrtA26P, (nrtA19F)^{NTG (NQO)} )</td>
<td>NTG</td>
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<td>R87Q</td>
<td>Tm 2</td>
<td>Loss of Charge</td>
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<td>NTG</td>
<td>TGC→TAC</td>
<td>C90Y</td>
<td>Tm 2</td>
<td>SPolar</td>
<td>3.26 ± 1.38</td>
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<td>( nrtA2015F )</td>
<td>NQO</td>
<td>GGC→AGC</td>
<td>G91S</td>
<td>Tm 2</td>
<td>Conservative</td>
<td>5.13 ± 1.20</td>
<td>+</td>
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<tr>
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<td>GAT→AAT</td>
<td>D95N</td>
<td>Lp 2/3</td>
<td>Loss of Charge</td>
<td>6.48 ± 0.90</td>
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<td>GGC→GAC</td>
<td>G137D</td>
<td>Tm 4</td>
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<td>Tm 4</td>
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<td>GGG→CGG</td>
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<td>Gain of Charge</td>
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<td>G157E</td>
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<td>GGT→AGT</td>
<td>G167S</td>
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<td>Tm 5</td>
<td>Conservative</td>
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<td>NTG</td>
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<td>4.60 ± 0.65</td>
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<td>10.31 ± 0.60</td>
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aPositions are predicted as described in the legend to Figure 1.

bThe presence or absence of NrtA protein estimated by Western blot is indicated by + or -, respectively.

cNA or ND, not applicable or not done.

dThe basal level in a loss of function nrtA mutant is around 3-4 nmol min/min/mg under the standard employed for net nitrate uptake (BROWNLEE and ARST, 1983). The results are the mean of three independent experiments ± standard deviation.

eTwo independently isolated mutants with identical changes. The first is the strain for which transport activity was determined.

fA base pair addition (C:G) at residue 489 results in an altered amino acid sequence after residue Ala489. This altered sequence reads
RVYLCLLGSACAEKSDEGVVDRVIGFSRGVWVRFCVLSDMIP*
Figure Legends

FIGURE 1.- Provisional secondary structure model of the high affinity nitrate transporter NtrA of *A. nidulans*. Model predicted through assessing the distribution and pattern of charged and hydrophobic amino acids using the TopPred program of Claros and von Heijne (1994) as implemented by Deveaud and Schuerer (Pasteur Institute: http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html). Predicted Tm segments of NrtA were refined by reference to the 52 sequence multiple alignment. Residues indicated in red denote very high conservation (greater than 95 %) observed by comparison of the 52 amino acid sequences from bacteria (21), fungi (5), algae and plants (26). Yellow dashes enclose the MFS motifs and blue dashes surround the nitrate signatures. The accession numbers of the 52 sequences used in alignments from which the data in Figure 1 is compiled are T51836, CAC05338, AAC35884, AB015472, AAF00053, AAF00054, Y08210, AY038800, AF0047718, AJ292342, AAC49531, AF091115, AF288688, AF332214, AF091116, AY053452, U34290, AB008519, ABO15472, T48982, AAF78499, AAK59570, Z25438, AY026523, AF135038, AF135039, AJ238664, Z69783, P22152, AF453778, B8G12-170, AAG45172, NP_241478, X15996, P37758, Z70792, CAB72205, AE004604, AAD22549, AAK22599, P42432, AAC06542, Z81360, AF149772, T37042, BAB58550, U40014, P46907, YI5252, AAG34371, CAC48822 and CAB65479.

FIGURE 2.- Position of altered residues. Red denotes residues which are very highly conserved (see Figure 1). Yellow dashes represent the position of MFS motifs and blue bars the position of nitrate signatures.
FIGURE 3.- Mutant growth tests. nrtA nrtB double mutant strains were compared with the wild-type strain in their ability to grow on minimal medium containing 100 mM nitrate as sole nitrogen source. Strains representative of the growth response of all the double mutants are shown.

FIGURE 4.- Analyses of NrtA expression by Western blot. (A) Lane 1 wild-type bia1, lane 2 nrtA747, lane 3 nrtA40P, lane 4 SS1, lane 5 nrtAgfp8, lane 6 wild-type bia1, lane 7 nrtAgfp8. (B) Lane 1 nrtA8, lane 2 nrtA2, lane 3 nrtA2082F, lane 4 wild-type bia1, lane 5 nrtA747. Growth of strains is described in the Materials and Methods. A band of 30 kDa was observed with the anti-NrtA antibody preparation in all strains and was also present when pre-immune serum was used in Western blots. The positions of molecular size markers (kDa) are shown.
nrtA2015F nrtB110
nrtA2036F nrtB110
nrtA7P nrtB110
nrtA2059F nrtB110
nrtA747 nrtB110
wild-type