DOI: 10.1534/genetics.104.036590

Missense Mutations That Inactivate the *Aspergillus nidulans nrtA* Gene Encoding a High-Affinity Nitrate Transporter

James R. Kinghorn,*,†,[1] Joan Sloan,† Ghassan J. M. Kana'n,*,‡ Edisio R. DaSilva,*,[2] Duncan A. Rouch†,[3] and Shiela E. Unkles*,†

**School of Biology, University of Saint Andrews, Fife KY16 9TH, Scotland, United Kingdom,* †*Department of Microbiology, Monash University, Clayton, Victoria 3800, Australia and* ‡*School of Biology, University of Mu'tah, Karak, Jordan*

Manuscript received September 21, 2004
Accepted for publication November 15, 2004

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* was isolated and characterized. This mutational analysis, coupled with protein alignments, demonstrates the utility of the approach to predicting peptide motifs and individual residues important for the movement of nitrate across the membrane. These include the highly conserved nitrate signature motif (residues 166–173) 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 for membrane insertion and/or stability of NrtA.

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). Futhermore, the central importance of nitrate is highlighted by reports that it is involved in certain plant metabolic and morphogenic processes (Scheible *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). At least two classes of nitrate transport systems, high affinity and low affinity, 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 (Unkles *et al.* 1991, 2001; Forde 2000). A typical secondary structure (Figure 1) was proposed for the 57-kD (507-amino-acid) NrtA protein in which 12 hydrophobic transmembrane domains (Tm's) in α-helical conformation pass through the membrane and connect by hydrophilic loops (Lp). The NrtA homologs belong to a distinct cluster, namely the Nitrate-Nitrite Porter family (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, the motif Gly *X 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 Tm's, 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 the consensus sequence Ala Ala Gly *X* Gly Asn *X* Gly Gly Gly, residues 163–172 (Figure 1) in Tm 5, described as the nitrate signature with a similar sequence repeated within Tm 11 (Trueman *et al.* 1996; Forde 2000). Other characteristics are the conserved charged residues within otherwise hydrophobic helices, Arg87 (Tm 2) and Arg368 (Tm 8), or aromatic residue Phe47 (Tm 1), which can be observed within NrtA (Forde 2000). A final feature of *A. nidulans* NrtA (although common among MFS transporters) is its substantial (~96 residues) central loop (Lp 6/7), which, although present, is much re-

[1]*Corresponding author:* School of Biology, University of St. Andrews, Sir Harold Mitchell Bldg., Fife KY16 9TH, United Kingdom. E-mail: jrk@st-andrews.ac.uk

[2]*Present address:* Department of Biochemistry, Federal University of Pernambuco, Recife, Brasil.

[3]*Present address:* School of Agriculture and Food Systems, University of Melbourne, Victoria 3030, Australia.

TABLE 1

Oligonucleotides used in this study

Oligonucleotide	Sequence
nrtA1.1 and nrtA1.2	TTCTACGAACTGCAGTTCC and TCCTTCAGTCCGGTTGTC
nrtA2.1 and nrtA2.2	TGCTTGCATTCCTCTCATG and TGAGGTAACGAGGCCG
nrtA3.1 and nrtA3.2	AGTCTTTATCGGCCTACTG and GCAAGTGAAGAGCATGCC
nrtA4.1 and nrtA4.2	GTTGGGACAGCCAACTC and AGCAGGCGTAGGGGACT
nrtA5.1 and nrtA5.2	CTCCCGCAAGGAGGCTT and AGTAAGACCAAACATAGTCG
nrtA6.1 and nrtA6.2	CAATGGGTTTCTCAGATCC and CCCAAACGCCTCTTGAG

Sequences are shown 5′ to 3′.

duced 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 (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 Tm's. 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 *Escherichia coli* LacY permease (Bailey and Manoil 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 genetic 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

***A. 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 overexpressing the nitrate transporter) on the basis of supersensitivity 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 (GFP; 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).

***E. 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, 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 (MacCabe *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, and 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 chlorate toxicity (200 mm) 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 to 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 *nrtA nrtB* double mutants: A number of *nrtA* mutant strains were crossed to *nrtB110,* a mutant strain that contains a deletion in the gene encoding the other nitrate transporter, NrtB, in *A. nidulans* (Unkles *et al.* 2001). Similar to *nrtA* loss-of-function mutants, strain *nrtB110* grows normally on 10 mm nitrate as the sole sourse of nitrogen. Putative *nrtA nrtB* double mutants (*i.e.*, strains that grew poorly on nitrate) were verified by Southern blot analysis of the *nrtB* to detect

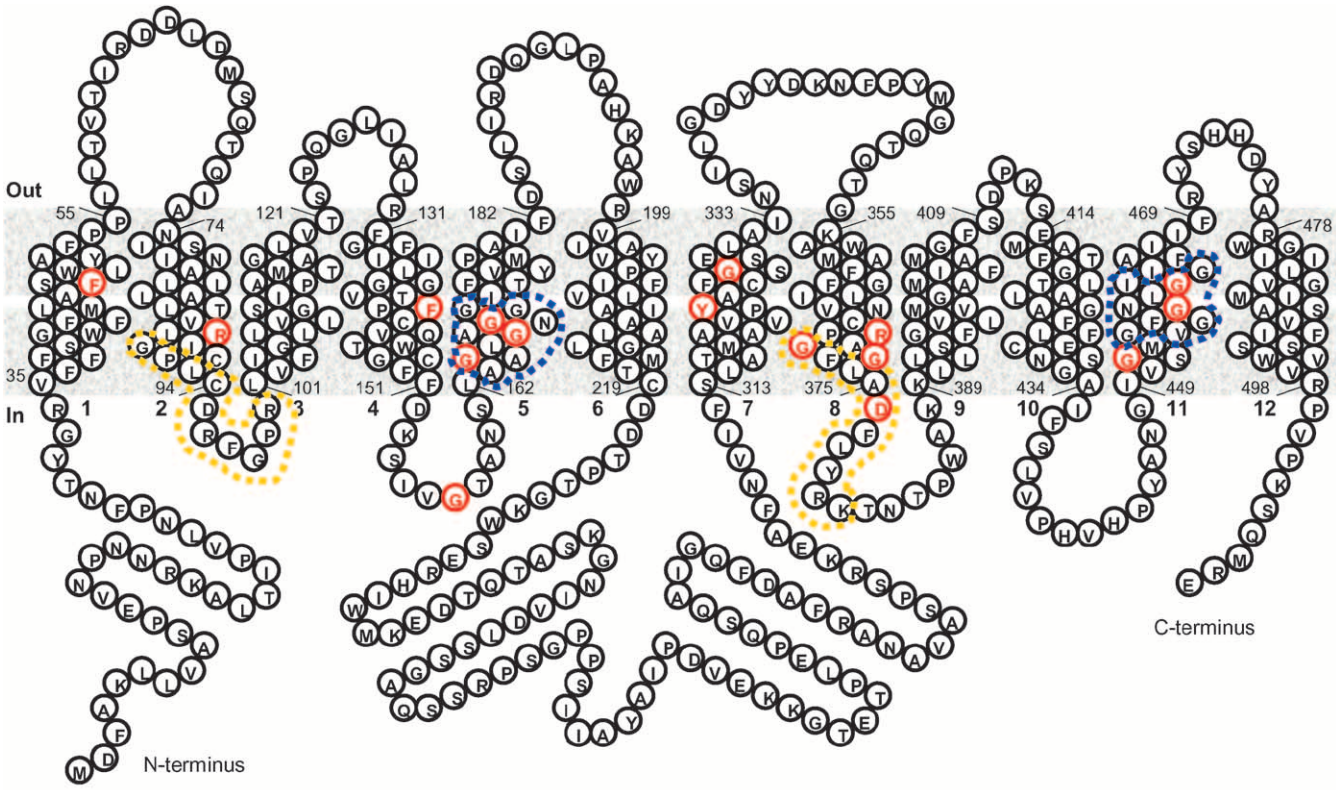

Figure 1.—Provisional secondary structure model of the high-affinity nitrate transporter NtrA of *A. nidulans*. Model predicted through assessing the distribution and pattern of charged and hydrophobic amino acids using the TopPred program of Claros and von Heijne (1994) as implemented by Deveaud and 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 (>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 are 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.

the deletion mutation *nrtB110* (Unkles *et al.* 2001) and PCR amplification followed by DNA sequencing of the *nrtA* mutant gene to ensure the presence of the *nrtA* mutation.

Net nitrate transport assays: Strains were grown for 6.5–7.5 hr at 37° in liquid minimal medium with 5 mm urea as the sole source of nitrogen (Cove 1966). The inducer of *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 ~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° 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 × *g* for 10 min at 4°, and the supernatant was collected and centrifuged at 18,000 × *g* for 30 min at 4°. The gelatinous pellet was resuspended in 150 μl extraction buffer and stored at −80°. 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° 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 hr at room temperature and after washing with TBST [TBS containing 0.1% (v/v) Tween 20] with 1:40,000 horseradish-peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hr 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 analyzed using Clustal W (Thomp-

TABLE 2

Characteristics of *nrtA* mutants

Allele	Mutagen	Nucleotide change	Residue substitution	Position[a]	Type of change	Net nitrate uptake[b]	Expression[c]
nrtA348P	NTG	TTC → TCC	F47S	Tm 1	Nonpolar → polar	3.50 ± 1.06	+
nrtA26P, (*nrtA19F*)[c]	NTG (NQO)	CGA → CAA	R87Q	Tm 2	Loss of charge	4.02 ± 0.35	+
nrtA334P	NTG	TGC → TAC	C90Y	Tm 2	SH → polar	3.26 ± 1.38	+
nrtA2015F	NQO	GGC → AGC	G91S	Tm 2	Conservative	5.13 ± 1.20	+
nrtA2036F	NQO	GAT → AAT	D95N	Lp 2/3	Loss of charge	6.48 ± 0.90	+
nrtA303P	NTG	GGC → GAC	G137D	Tm 4	Gain of charge	3.97 ± 0.58	+
nrtA94P	NTG	GGC → GAC	G138D	Tm 4	Gain of charge	3.66 ± 0.66	+
nrtA2020F	NQO	GGG → CGG	G157R	Lp 4/5	Gain of charge	2.29 ± 0.38	+
nrtA62P	NTG	GGG → GAG	G157E	Lp 4/5	Gain of charge	3.62 ± 0.59	+
nrtA7P	NTG	GGT → AGT	G167S	Tm 5	Conservative	6.89 ± 0.78	+
nrtA2107F	NQO	GCT → CCT	A169P	Tm 5	Conservative	3.36 ± 1.28	+
nrtA13	NTG	GGT → CGT	G170R	Tm 5	Gain of charge	3.66 ± 0.55	+
nrtA337P	NTG	GGT → AGT	G170S	Tm 5	Conservative	3.03 ± 0.45	+
nrtA2049F	NQO	GGT → TGT	G170C	Tm 5	Polar → SH	5.28 ± 1.75	+
nrtA2046F	NQO	GGT → TAT	G170Y	Tm 5	Conservative	3.72 ± 1.26	+
nrtA946, (*nrtA55P*)[d]	NTG (NTG)	GGT → GAT	G172D	Tm 5	Gain of charge	5.73 ± 0.80	+
nrtA2003F	NQO	GGT → TGT	G172C	Tm 5	Polar → SH	4.22 ± 0.03	+
nrtA2082F	NQO	GGT → AGT	G172S	Tm 5	Conservative	3.34 ± 0.61	+
nrtA2	NTG	CTC → CCC	L185P	Lp 5/6	Conservative	3.69 ± 0.24	+
nrtA2002F	NQO	GCC → CCC	A324P	Tm 7	Conservative	4.97 ± 0.11	+
nrtA14P, (*nrtA1087*)[d]	NTG (NTG)	GGG → GAG	G328E	Tm 7	Gain of charge	5.94 ± 0.10	+
nrtA2044 (*nrtA45P*)[d]	NQO (NTG)	GGG → AGG	G361R	Tm 8	Gain of charge	4.60 ± 0.65	+
nrtA8	NTG	CGT → TGT	R368C	Tm 8	Loss of charge	3.79 ± 0.79	+
nrtA301P (*nrtA9M*)[d]	NTG	GGT → GAT	G371D	Tm 8	Gain of charge	2.85 ± 0.67	+
nrtA2031F	NQO	GGT → CGT	G371R	Tm 8	Gain of charge	4.03 ± 1.10	+
nrtA335P	NTG	GGA → GAA	G372E	Tm 8	Gain of charge	3.28 ± 0.80	+
nrtA14M	NTG	GGT → GAT	G396D	Tm 9	Gain of charge	5.03 ± 1.90	+
nrtA2104	NQO	GGG → GAG	G433E	Tm 10	Gain of charge	3.90 ± 0.79	+
nrtA14F	NQO	GCA → GAA	A434E	Tm 10	Gain of charge	4.03 ± 0.30	+
nrtA23P	NTG	GGG → AGG	G458R	Tm 11	Gain of charge	7.06 ± 0.03	+
nrtA2043	NQO	GGT → CGT	G462R	Tm 11	Gain of charge	3.97 ± 1.17	+
nrtA19P	NTG	GGT → GAT	G484D	Tm 12	Gain of charge	5.13 ± 0.60	+
nrtA2059F	NQO	GGT → TGT	G484C	Tm 12	Polar → SH	4.98 ± 1.50	+
Wild type	NA	NA	NA	NA	NA	10.31 ± 0.60	+
nrtA1	NTG	GGA → ■GA[e]	125	Lp 3/4	NA	4.15 ± 0.05	−
nrtA747	NTG	CTA → ■TA[e]	85	Tm 2	NA	3.50 ± 0.20	−
nrtA40P	NTG	TGG → TGA	Y481*[f]	Tm 12	NA	ND	−
nrtA52P	NTG	GCC → GCCC	A489 →[g]	Tm 12	NA	ND	−

SH, sulfhydryl group of cysteine; NA or ND, not applicable or not done.

[a] Positions are predicted as described in the legend to Figure 1.

[b] The basal level in a loss-of-function *nrtA* mutant is ~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.

[c] The presence or absence of NrtA protein estimated by Western blot is indicated by + or −, respectively.

[d] Two independently isolated mutants with identical changes. The first is the strain for which transport activity was determined.

[e] The black box represents a single nucleotide deletion.

[f] The asterisk represents a stop codon.

[g] A base-pair addition (C:G) at residue 489 results in an altered amino acid sequence after residue Ala489. This altered sequence reads RVYLCLLGSACAEKSDEGVVDRVIGFSRGVWVRFCSVLFSDMIP*.

son *et al.* 1994) and refined by eye. These sequences included proteins from a diverse range of organisms, ranging from eubacteria and archaebacteria to algae, fungi, and plants. Sixteen very highly conserved residues (defined on the basis of 50 of 52 conserved, *i.e.*, >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), Gly-371(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 were

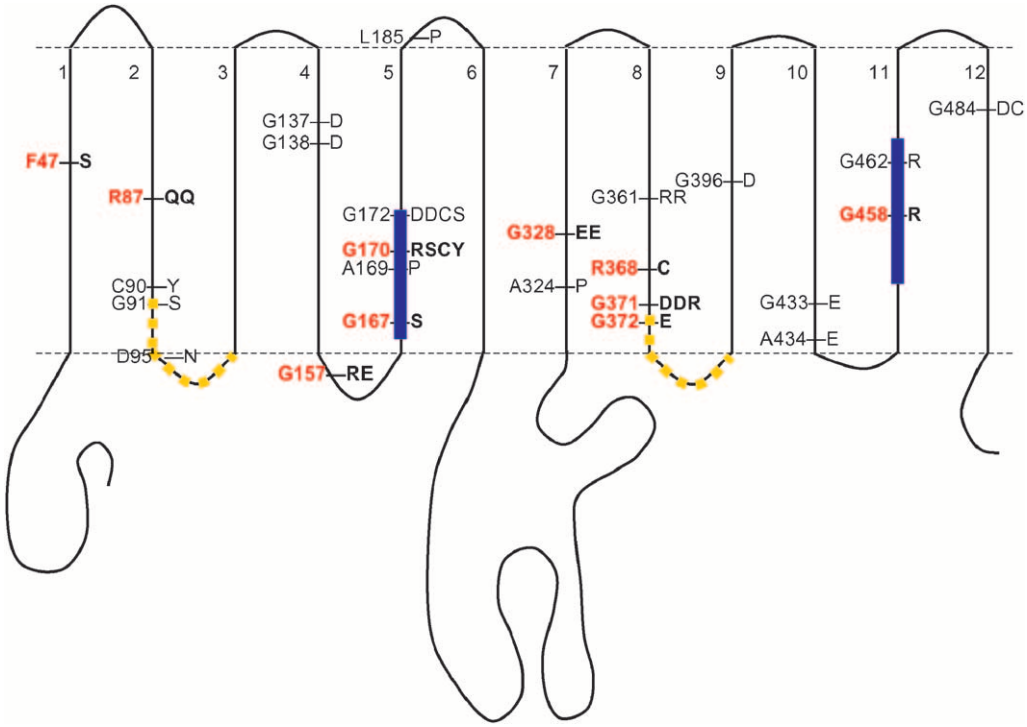

FIGURE 2.—Position of altered residues. Red denotes residues that are very highly conserved (see Figure 1). Yellow dashes represent the position of MFS motifs and blue bars the position of nitrate signatures.

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.*, 10) 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, which are very highly conserved in diverse organisms (Figure 1). Interestingly, Gly172, which is conserved among the eukaryotes only, is altered in four mutants (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 three 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, and four were located within Tm 2, including two in the highly conserved residue Arg87, two were located in Tm 4, and three were located 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 was located within Tm 9 and two each were observed in Tm's 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 while 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, *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 vis-à-vis *nrtA1*, formerly *crnA1* (TOMSETT and COVE 1979; BROWNLEE and ARST 1983; UNKLES *et al.* 1991), and *nrtA747* (UNKLES *et al.* 2001), both of which were found to have a single base-pair deletion—*nrtA1* at nucleotide position 481, codon 125 (Lp 3/4), and *nrtA747* at nucleotide position 361, codon 85 (Tm 2). Other deletions included *nrtA1009, nrtA1010, nrtA17, nrtA65P, nrtA343P, nrtA2039F*, and *nrtA24Y*, while insertions occurred in mutants *nrtA3* and *nrtA97P*. Noteworthy is mutant *nrtA52P*, which has a single-base-pair addition near the 3′-end of the coding region (described in Table 2, footnote *g*) and was used in antibody studies.

Resistance to chlorate toxicity: Missense mutants iso-

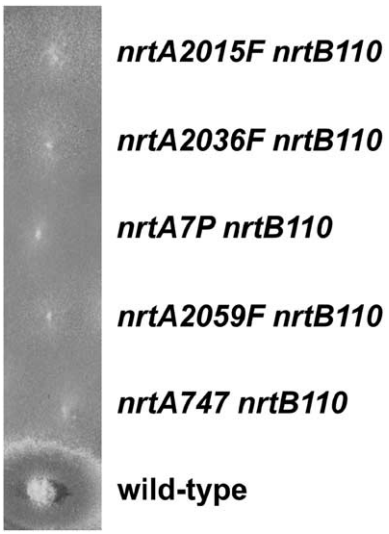

FIGURE 3.—Mutant growth tests. *nrtA nrtB* double-mutant strains were compared with the wild-type strain for their ability to grow on minimal medium containing 100 mM nitrate as sole nitrogen source. Strains representative of the growth response of all the double mutants are shown.

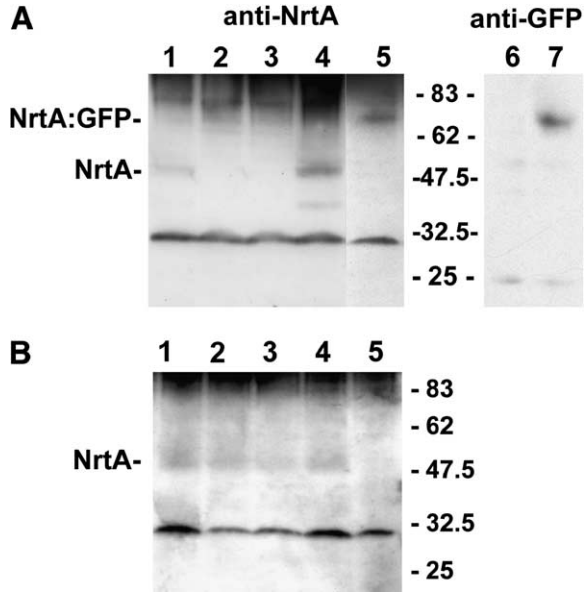

FIGURE 4.—Analyses of NrtA expression of by Western blot. (A) Lane 1, wild-type *biA1*; lane 2, *nrtA747*; lane 3, *nrtA40P*; lane 4, SS1; lane 5, *nrtAgfp8*; lane 6, wild-type *biA1*; lane 7, *nrtAgfp8*. (B) Lane 1, *nrtA8*; lane 2, *nrtA2*; lane 3, *nrtA2082F*; lane 4, wild-type *biA1*; lane 5, *nrtA747*. Growth of strains is described in MATERIALS AND METHODS. A band of 30 kD was observed with the anti-NrtA antibody preparation in all strains and was also present when preimmune serum was used in Western blots. The positions of molecular size markers (in kilodaltons) are shown.

lated 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 *nrtA1* and *nrtA747* with proline and urea and equally sensitive as the wild-type strain (and as sensitive as *nrtA1* and *nrtA747*) on 10 mM glutamate or 10 mM arginine (UNKLES *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.*, ~3–4 nmol/min/mg compared with ~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-mutant 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, by Western blotting, steady-state levels in germlings (induced by nitrate) grown 6.5 hr (Figure 4). Low levels of NrtA protein (~50 kD instead of the predicted 57 kD) could be observed using anti-NrtA antibodies in the wild type (Figure 4A, lane 1) but none were observed in chain termination mutants *nrtA747* (lane 2) or *nrtA40P* (lane 3). 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* (Figure 4A, lane 2) and *nrtA1* (S. E. UNKLES, unpublished results) lacked the 50-kD signal, as might be expected since these proteins, if expressed, would be devoid of the antibody recognition sites. However, neither chain termination mutant *nrtA40P* (Figure 4A, 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 ~72 kD (instead of the expected 81 kD) was revealed (Figure 4A, lane 5). Anti-GFP antibodies also detected this 72-kD protein in the preparation from *nrtAgfp8* (Figure 4A, lane 7) but not,

as expected, in the wild-type protein sample (Figure 4A, lane 6). Confirmation that the 50-kD 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 of *nrtA* (Figure 4A, 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-kD protein was observed in the negative control sample for this blot, *i.e.*, in *nrtA747* (lane 5).

DISCUSSION

A mutational survey of NrtA giving 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, has been accomplished. 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 10 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.

Two arginine residues, Arg87 and Arg368, 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 a loss of charge, it would appear that a positive charge at these positions is necessary for transport function.

Interestingly, helical wheel analysis of Tm's in which several mutations have been identified (in Tm 2, Tm 5, Tm 7, and Tm 8) showed that all of the mutations in Tm's 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 Tm's 1, 2, 4, and 5 of the N-terminal half of the protein, along with Tm's 7, 8, 10, and 11 of the C-terminal portion, form the substrate translocation pore. Thus in NrtA also, Tm's 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 Tm's 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, probably reflecting the nonessentiality of these residues. Indeed, in terms of known MFS structures, Tm 3 along with Tm's 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 the motif Phe *X X X* Trp *X X* Phe *X X X* Phe *X 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 X* Asp *X X* Gly *X* Arg (residues 91–100 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 with an Asp95-to-Asn modification was isolated in this study. 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 compose a motif somewhat loosely conserved in many MFS proteins including sugar transporters (Henderson 1991). Further glycine residues altered within Tm's 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 nonconserved 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 Manoil 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 were replaced by 44 residues, these were the only mutants encoding epitopes recognized by our anti-NrtA antibodies that 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.

While most of the missense mutants showed net transport basal levels of ~3–4 nmol/min/mg, similar to deletion strains *nrtA1* or *nrtA747* (compared with ~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 *nrtB110* (a deletion mutation within the other nitrate transporter NrtB) showed neither growth on even high concentrations of nitrate nor significant detectable net nitrate transport. Clearly, therefore, these *nrtA* mutants are NrtA loss-of-function, enhanced levels of activity observed in the single missense mutants perhaps 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 *nrtA* mutants, which all appear to be loss of function. This is in contrast to *nrtB* mutants, which are sensitive to chlorate toxicity at a range of concentrations and with various single nitrogen sources.

We appreciated the strains sent to us by J. Clutterbuck, University of Glasgow, United Kingdom. S.E.U. gratefully acknowledges the Australian Research Council for financial support. J.R.K. thanks The Royal Society (London) for funds to travel to Australia.

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Communicating editor: M. S. Sachs