Apparent genetic redundancy facilitates ecological plasticity for nitrate transport

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Aspergillus nidulans possesses two high-affinity nitrate transporters, encoded by the *nrtA* and the *nrtB* genes. Mutants expressing either gene grew normally on 1–10 mM nitrate as sole nitrogen source, whereas the double mutant failed to grow on nitrate concentrations up to 200 mM. These genes appear to be regulated coordinately in all growth conditions, growth stages and regulatory genetic backgrounds studied. Flux analysis of single gene mutants using $^{13}\text{NO}_3^-$ revealed that K_m values for the NrtA and NrtB transporters were ~100 and ~10 μM , respectively, while V_{max} values, though variable according to age, were ~600 and ~100 nmol/mg dry weight/h, respectively, in young mycelia. This kinetic differentiation may provide the necessary physiological and ecological plasticity to acquire sufficient nitrate despite highly variable external concentrations. Our results suggest that genes involved in nitrate assimilation may be induced by extracellular sensing of ambient nitrate without obligatory entry into the cell.

Keywords: *Aspergillus nidulans*/ecological plasticity/genetic redundancy/nitrate assimilation/nitrate transport

Introduction

When two or more gene products carry out the same function, and mutation in one of these genes has no detectable effect on the biological phenotype, the genes are said to be redundant (Brookfield, 1997). It is likely that true redundancy does not exist since it is generally regarded that natural selection acts to retain only discretely functional proteins (Nowak *et al.*, 1997). Rather, apparent redundancy may actually serve to implement changes in spatial or developmental regulation (Cadigan *et al.*, 1994; Christophides *et al.*, 2000; Patterson *et al.*, 2000).

Nitrate is the major nitrogen source for most crop plants (Wray and Kinghorn, 1989; Crawford and Glass, 1998; Daniel-Vedele *et al.*, 1998; Forde, 2000). Nitrate also acts as a signal for the control of many metabolic and morphogenic processes (Scheible *et al.*, 1997; Zhang and Forde, 1998). In the natural environment, nitrate is probably the most ephemeral of inorganic nutrients, ranging in concentration across orders of magnitude

(Vidmar *et al.*, 2000). In addition, nitrate is readily leached from soils, contributing to eutrophication of natural water systems and simultaneously lowering nitrogen availability to land plants.

In the soil saprophytic fungus, *Aspergillus nidulans*, we observe two paralogous genes encoding nitrate transporters. The first eukaryotic nitrate transporter gene described, *nrtA* (formerly designated *crnA*), has acted as a paradigm for plant high-affinity transport systems (Unkles *et al.*, 1991), and provided a springboard to the cloning and identity of orthologous *Nrt2* genes from a large number of species (Quesada *et al.*, 1994; Trueman *et al.*, 1996; Pérez *et al.*, 1997; Quesada *et al.*, 1997; Amarasinghe *et al.*, 1998; Filleur and Daniel-Vedele, 1999; Zhou *et al.*, 1999). Indeed, a major problem confronting plant biologists now is to explain the unique roles of as many as 11 different nitrate transporter genes belonging to the *Nrt1* and *Nrt2* families in *Arabidopsis thaliana*. In contrast, *nrtA* and *nrtB* appear to be the only genes encoding nitrate transporters in *A. nidulans*. Both genes are induced rapidly by exposure to nitrate, are under identical regulatory control, appear to be of equivalent abundance and show the same temporal expression patterns. The cloning and sequencing of the *nrtB* gene and generation of mutant strains provided the opportunity to investigate the kinetic properties and relative contributions of each of the nitrate transporters, using the short-lived tracer $^{13}\text{NO}_3^-$.

Results

The *nrtB* gene structure and predicted protein

An open reading frame (ORF) of 1491 nucleotides interrupted by four putative introns is evident from the genomic DNA sequence and the inferred NrtB protein (Figure 1A). Introns 1, 2 and 4 occupy the same position as those of *nrtA*, with intron 3 being unique. In the *nrtB* 5' region, there are potential binding sites for the NirA and AreA regulatory proteins, that mediate nitrate induction and nitrogen metabolite repression, respectively (Strauss *et al.*, 1998; Morozov *et al.*, 2000 and references therein). One potential NirA-binding motif (CCGCGGAG) is observed at nucleotide position –455 as well as 10 possible AreA-binding motifs (GATA). Alignment of the 497 residue NrtB (53.7 kDa) with the 507 NrtA (57 kDa) shows 76% similarity (61% identity) overall and up to 90% similarity (76% identity) over the proximal 230 residues (Figure 1B). As with NrtA, there are 12 putative transmembrane α -helical segments (Figure 1B, boxed).

Phenotypic analysis of mutants

Growth tests with nitrate as the sole nitrogen source showed that mutant strain *nrtB110*, generation of which is described in Materials and methods, grew similarly to the

Table I. Growth responses of wild-type and mutant strains

Strain	Nitrate	Nitrate + caesium	Nitrite	Nitrite + caesium	Proline + chlorate
Wild type	4	3	4	3	0
<i>nrtA747</i>	4	0/1	4	3	3
<i>nrtB110</i>	4	3	4	3	0
<i>nrtA747 nrtB110</i>	0	0/1	4	3	3
<i>niaD171</i>	0	0	4	3	3

All tests were carried out on minimal medium, supplemented with the substances stated above as well as vitamins (to satisfy vitamin auxotrophic requirements). For growth tests, the concentration of the nitrogen source was 10 mM. The concentration of caesium chloride was 20 mM, the concentration used in a previous study (Unkles *et al.*, 1991), and the concentration of sodium chlorate was 150 mM. For discussion of chlorate concentrations and toxicity, see Cove (1976). A score of 0 represents ungerminated conidiospores or faint scraggly growth (i.e. none or very scant growth), and 4 a mature and conidiating colony, while 1, 2 and 3 indicate intermediate levels. Strains are discussed in the Materials and methods.

The 1.6 kb *nrtB* transcript was observed in total RNA from young wild-type germlings grown with nitrate, nitrite, or nitrate plus proline, but not from those grown with ammonium plus nitrate, ammonium plus proline (Figure 2B) or ammonium alone (our unpublished data). A barely detectable *nrtB* transcript was observed with proline (Figure 2B). In regulatory gene mutants, under inducing conditions, the *nrtB* transcript is absent in the *nirA1* loss-of-function mutant strain (Figure 2C). Furthermore, under non-inducing conditions, the *nirA^c1* constitutive mutant strain produces the *nrtB* transcript, in contrast to the wild type. The loss-of-function *ΔareA* (Figure 2C) and *areA217* (our unpublished data) mutants lack *nrtB* transcripts under inducing conditions and, conversely, the *nrtB* message is observed in cells of the ammonium-derepressed mutant, *xprD1*, grown on ammonium (Figure 2C). The *niaD* transcript was present in the conditions and mutants expected from previous studies, which confirmed the employment of correct growth regimes.

The availability of both genes provided an opportunity to investigate temporal forms of regulation, at least at the level of transcript accumulation. Cells were grown for periods of up to 16 h at 37°C with 10 mM nitrate as the nitrogen source and aliquots harvested at hourly intervals. Total RNA was prepared and hybridized with *nrtA*- or *nrtB*-specific probes. Whereas no *nrtA* or *nrtB* signals were observed in conidiospores harvested from complete solid medium (Figure 3A, lane CM), even after long autoradiographic exposure, strong signals were observed after only 1 h incubation of conidiospores in liquid minimal medium containing nitrate as the sole source of nitrogen. Much less intense but clearly visible signals were seen thereafter with decreasing intensity, until ~6–8 h when the conidiospores germinated. Beyond this period, only weak signals could be observed. The signal intensity after 1 h seemed to be higher for *nrtA* than *nrtB* relative to the respective 2–8 h samples. The *niaD* signal also showed the highest intensity after 1 h, was clearly visible until 6 h, but was barely detectable thereafter. The level of nitrate remaining in the medium was measured over the growth period of 16 h at

Fig. 2. Analysis of transcriptional regulation in cells grown under different nitrogen conditions. (A) Gene-specific probes for *nrtA* and *nrtB*. The mutant $\Delta 506$, known to have a deletion of the entire nitrate assimilation cluster including *niaD*, *niiA* and *nrtA*, was grown at 37°C in minimal medium for 5.5 h and transferred to medium containing 10 mM nitrate for 30 min. Total RNA was isolated, a northern blot was prepared and hybridized sequentially with the ^{32}P -labelled, PCR-generated probes for *nrtA* and *nrtB* described in the text, followed by the ^{32}P -labelled probes for *niaD* (a 2.4 kb *XbaI* fragment of pSTA8; Johnstone *et al.*, 1990) and *actA* (a 0.85 kb *KpnI*–*NcoI* fragment of pSFR5; Fidel *et al.*, 1988). (B) Regulation of *nrtA*. The wild-type strain, *biA1*, was grown at 25°C for a total of 13–14 h in minimal medium containing 10 mM sodium nitrite (lane 1), 10 mM sodium nitrate (lane 2), 10 mM proline plus 5 mM ammonium tartrate (lane 3), 10 mM proline plus 10 mM sodium nitrate (lane 4), 10 mM proline (lane 5) or 10 mM sodium nitrate plus 5 mM ammonium tartrate (lane 6). A northern blot of total RNA was hybridized sequentially using the probes described in (A). (C) Influence of regulatory genes. The wild type and regulatory mutants were grown at 25°C for a total of 13–14 h in minimal medium containing the following nitrogen sources: wild type (lane 1) and *nirA1* loss-of-function (lane 2) with 10 mM proline and transferred to 10 mM sodium nitrate 100 min before harvesting; wild type (lane 3) and *nirA^c1* constitutive mutant (lane 4) with 10 mM proline; wild type (lane 5) and *ΔareA* a deletion (lane 6) with 5 mM ammonium tartrate and transferred to 10 mM sodium nitrate 100 min before harvesting; wild type (lane 7) and *xprD1* a derepressed *areA* allele (lane 8) with 5 mM ammonium tartrate. A northern blot of total RNA was hybridized sequentially using the probes described in (A).

37°C. The results presented in Figure 3A (bottom panel) show that nitrate utilization followed a sigmoidal curve, a sharp decrease in nitrate concentration being observed after 5 h when the conidiospores began to germinate. After 10 h, nitrate levels were below the limit of detection.

To circumvent the possibility that the decreasing levels of both *nrtA* and *nrtB* transcripts simply reflect the decreasing availability of nitrate, mycelium was grown for different periods on proline and transferred to nitrate for 30 min prior to harvesting. High levels of both *nrtA* and *nrtB* mRNA accumulated upon induction with nitrate after 6 and 16 h incubation (Figure 3B).

The above results suggest that the transcripts are not present in dormant conidiospores harvested from complete medium. However, it was unclear if conidiospores formed on agar minimal medium with inducing nitrogen sources, such as nitrate, contained pre-formed *nrtA* and *nrtB* transcripts. Figure 4A shows that there is a similar level of transcript, albeit barely detectable, of both genes present in conidiospores produced from mycelium grown on

Fig. 3. Time course of *nrtA* and *nrtB* expression. (A) Conidiospores, filtered twice through miracloth to remove mycelial fragments, were examined for *nrtA* and *nrtB* transcripts from the inoculum grown on solid complete medium (CM) and transferred to liquid minimal media with 10 mM nitrate as the nitrogen source. Samples (100 ml) were taken at the time intervals shown above (hours) up to 16 h at 37°C. A northern blot prepared from total RNA of inoculum and each time sample was probed sequentially with the 32 P-labelled probes indicated on the right and described in the legend to Figure 2A. The level of nitrate remaining in the medium at each time point is shown in the bottom panel. (B) Induction of transcripts in young germlings and older mycelial cells. Cultures were grown for a total of 6 or 16 h at 37°C with 10 mM proline (lanes 1, uninduced cells) or transferred to 10 mM nitrate 30 min prior to harvesting (lanes 2, nitrate-induced cells). A northern blot of total RNA was hybridized sequentially with the 32 P-labelled probes indicated on the right and described in the legend to Figure 2A.

minimal medium with proline or nitrate as nitrogen source, and even with ammonium. As before (Figure 3A), no transcripts were detectable in RNA isolated from conidiospores harvested from complete medium. A time course experiment (Figure 4B) demonstrated that the transcripts were abundant only after 60 min growth of conidia in minimal medium containing nitrate as sole nitrogen source. Since the actin control seemed not to be constitutive in these conidiospores just exposed to medium, i.e. increased in abundance up to 60 min, the rRNA bands were included to show approximate equality of RNA loading. A similar phenomenon for *A.nidulans* actin expression was observed during the transition from

Fig. 4. Transcript levels in conidiospores. (A) The wild-type strain, *biA1*, was grown on agar minimal medium containing 10 mM proline (lane 1), 10 mM sodium nitrate (lane 2) or 5 mM ammonium tartrate (lane 3) as sole nitrogen source, or on agar complete medium (lane 4). Conidiospores, filtered twice through miracloth to remove mycelial fragments, were pelleted by centrifugation. A northern blot of total RNA was hybridized sequentially with the 32 P-labelled probes indicated on the right and described in the legend to Figure 2A. (B) Conidiospores from agar complete medium, harvested, filtered and pelleted, were grown at 37°C in liquid minimal medium containing 10 mM sodium nitrate as sole nitrogen source for up to 60 min. Numbers at the top refer to minutes incubation following inoculation of the medium. Conidiospore samples (100 ml) following incubation were harvested by rapid filtration through a 0.45 μ m cellulose acetate filter. A northern blot of total RNA was hybridized with the probes shown on the right described in the legend to Figure 2A. A photograph showing the rRNA bands (B, bottom panel) was included to demonstrate parity of RNA loading since the *actA* transcript itself was not constitutive.

dormancy to growth by Tazebay *et al.* (1997) There is an apparent discrepancy between the results in Figures 3B and 4B in terms of the time required for induction. This probably reflected a period of latency in freshly harvested conidiospores compared with germlings or mycelium grown for 6 and 16 h, respectively.

The levels of *nrtA* and *nrtB* transcripts were assayed in loss-of-function mutations of nitrate assimilation genes: *niiA17*, lacking nitrite reductase, *niaD24*, lacking nitrate reductase and *cnxH4* (Cove, 1979; and references therein), which lacks nitrate reductase due to abolition of synthesis of the molybdenum cofactor (Unkles *et al.*, 1999). Figure 5 shows that the *nrtA* and *nrtB* mRNAs, and the *niaD* transcript, were much higher in the induced cells of all three mutants than in wild type.

Characterization of nitrate uptake using tracer $^{13}\text{NO}_3^-$

Nitrate influx was measured using the short-lived isotope ^{13}N ($t_{0.5} = 10$ min). Figure 6A shows influx values for germlings of the wild type expressing NrtA and NrtB, the *nrtA747* mutant which expresses only NrtB, the *nrtB110* mutant expressing only NrtA and the *nrtA747 nrtB110* double mutant expressing neither gene. $^{13}\text{NO}_3^-$ influx usually was measured at concentrations of NO_3^- up to 500 μM , although measurements of $^{13}\text{NO}_3^-$ influx at higher NO_3^- concentrations up to 5 mM gave no evidence

Fig. 5. Nitrate-induced transcript levels in wild type and structural gene mutants. Strains were grown in minimal medium at 25°C for a total of 14 h, first with 10 mM proline and then transferred to minimal medium containing 10 mM sodium nitrate for 100 min prior to harvesting. Strains were *cnxH4* (lane 1), wild type (lane 2), *niaD24* (lane 3), *niaD42* (lane 4) and *niiA17* (lane 5). Total RNA in northern blots was hybridized with the probes shown to the right and described in the legend to Figure 2A.

for low-affinity transporters of the sort defined in higher plants (Crawford, 1995). In young cells grown for 6 h, wild-type and *nrtB110* strains exhibited K_m values of $\sim 100 \mu\text{M}$, while the *nrtA747* mutant had values of $\sim 10 \mu\text{M}$. $V_{\max}$ values were ~ 900 , 600 and $150 \text{ nmol/mg dry weight (DW)/h}$ for wild-type, *nrtB110* and *nrtA747* strains, respectively (Table II), while the double mutant failed to accumulate detectable amounts of $^{13}\text{NO}_3^-$ (Figure 6A). Therefore, the NrtA and NrtB transporters have K_m values of ~ 100 and $10 \mu\text{M}$, respectively. The $V_{\max}$ value for the *nrtA747* mutant was $\sim 17\%$ that of the wild type, a result roughly in accord with the net nitrate uptake rates recorded by Brownlee and Arst (1983). As expected from the work of Brownlee and Arst (1983), no measurable uptake of $^{13}\text{NO}_3^-$ was observed in a nitrate reductase loss-of-function mutant, *niaD171*, although this experiment was performed only once and so is not included in Table II. Net nitrate uptake studies using *niaD171* (our unpublished data) also confirm the finding that active nitrate reductase is required for nitrate uptake.

The results of $^{13}\text{NO}_3^-$ uptake in mature cells are also given in Table II. When mycelium was grown for a total of 16 h, there was a clear decrease in $^{13}\text{NO}_3^-$ influx in wild-type mycelia at 16 h compared with values obtained in 6 h mycelia ($V_{\max}$ values of 896 ± 81 and $382 \pm 33 \text{ nmol/mg DW/h}$ at 6 and 16 h, respectively). In contrast to earlier findings using *nrtA1* (Brownlee and Arst, 1983), also a loss-of-function mutant (our unpublished data), $^{13}\text{NO}_3^-$ influx in loss-of-function *nrtA747* mycelia appeared to be relatively unchanged ($V_{\max}$ values of 140.9 ± 5.8 and $161.7 \pm 25.7 \text{ nmol/mg DW/h}$ at 6 and 16 h, respectively). More importantly, the data in Table II demonstrate that the K_m values for $^{13}\text{NO}_3^-$ influx at 16 h remain the same as those evident at 6 h, namely $\sim 10 \mu\text{M}$ for the *nrtA* mutant and $\sim 100 \mu\text{M}$ for the wild-type strain. Thus, rather than an increase in $^{13}\text{NO}_3^-$ influx in the *nrtA* mutant in older mycelia (Brownlee and Arst, 1983), the present data instead indicate a decrease in $^{13}\text{NO}_3^-$ influx in wild-type mycelia at 16 h, bringing $V_{\max}$ values closer to those of the mutant. The *nrtB* mutant strain also retained a K_m value close to that at 6 h and also revealed a reduction of $V_{\max}$ from 564 ± 67 to $300 \pm 71 \text{ nmol/mg DW/h}$.

Fig. 6. (A) $^{13}\text{NO}_3^-$ influx values for *A. nidulans* wild type (squares), mutant *nrtA747*, expressing only NrtB protein (circles), mutant *nrtB110*, expressing only NrtA protein (upright triangles), and the double mutant *nrtB110 nrtA747*, expressing neither protein (inverted triangles), measured at various concentrations up to $500 \mu\text{M}$. (B) $^{13}\text{NO}_3^-$ influx values for *A. nidulans* wild type, presented as a Hoffstee plot ($^{13}\text{NO}_3^-$ influx against $^{13}\text{NO}_3^-$ influx/external NO_3^- concentration). The y-intercepts of regression lines generate estimates of $V_{\max}$ (762 ± 138 and $100 \pm 8 \text{ nmol/mg DW/h}$, respectively) and slopes provide K_m estimates (99 ± 28 and $6 \pm 0.6 \mu\text{M}$, respectively) of the two transporters. (C) $^{13}\text{NO}_3^-$ influx values for *nrtA* and *nrtB* mutants computed from the K_m and $V_{\max}$ values at low external nitrate concentration for 6 h germlings. Mutant *nrtA747*, expressing only NrtB protein (circles), and mutant *nrtB110*, expressing only NrtA protein (triangles).

Clearly, in young (6 h) wild-type cells, activity of the NrtA transporter is a major component of nitrate uptake since influx in the *nrtA747* mutant was substantially

Table II. Kinetic constants for $^{13}\text{NO}_3^-$ influx by wild-type, NrtB (*nrtA747* mutant) and NrtA (*nrtB110* mutant) transport systems

Strain	Transporter	K_m		$V_{\max}$	
		6 h	16 h	6 h	16 h
Wild type	NrtB, NrtA	107.5 $\pm$ 20.3	82.6 $\pm$ 19.6	896.9 $\pm$ 81.3	382.0 $\pm$ 32.9
<i>nrtA747</i>	NrtB	11.1 $\pm$ 2.7	14.4 $\pm$ 10.0	140.9 $\pm$ 5.8	161.7 $\pm$ 25.7
<i>nrtB110</i>	NrtA	96.3 $\pm$ 29.7	75.4 $\pm$ 38.0	564.4 $\pm$ 66.5	300.1 $\pm$ 71.3

Strains were grown for 6 or 16 h in media containing 2.5 mM urea as sole nitrogen source and exposed to 10 mM NO_3^- for 100 min prior to influx measurements. K_m (μM) and $V_{\max}$ (nmol/mg DW/h) values were determined by computer, using direct fits of data to rectangular hyperbolae.

reduced, and there appeared to be a lack of compensation by the NrtB transporter. In order to investigate the participation (if any) of the NrtB transporter in wild-type kinetics, Hoffstee plots were generated and examined for bimodality. Figure 6B presents a Hoffstee plot of the $^{13}\text{NO}_3^-$ influx data for 6 h wild-type mycelium after 100 min exposure to nitrate. Bimodality was always observed in experiments that included influx measurements at lower concentrations of nitrate. The slopes of the regression lines in this Hoffstee plot (equivalent to K_m values) revealed contributions of a transporter with a K_m value of $\sim 100 \mu\text{M}$ and another with a K_m value of $\sim 10 \mu\text{M}$, as was observed in the mutants. $V_{\max}$ values (y-intercepts) also corresponded closely (762 and 100 nmol/mg DW/h, respectively) to values obtained in the mutants where only one transporter operated.

Induction of nitrate assimilation genes

The question of whether nitrate assimilation systems are still induced in a strain that lacks capacity to transport nitrate was addressed by comparing young (6 h) double mutant *nrtA747 nrtB110* with wild-type cells. The northern blot presented in Figure 7 clearly shows that all three genes (*niaD*, *niiA* and *nrtA*) were induced by the addition of nitrate to the growth medium of both the wild type and the double mutant (*nrtB* was not tested, since the double mutant has a disrupted *nrtB* gene). Quantitative measurements indicated that the double mutant had reduced levels of both *niaD* and *niiA* transcripts relative to the wild type (41 and 45%, respectively). However, the abundance of *nrtA* transcript was roughly the same in the double mutant as in the wild type. The enzyme activity was assayed in the case of nitrate reductase and this was in approximate accord with transcript levels. This induction of the nitrate assimilation pathway is not due to contaminating nitrite in the nitrate solution, since no induction of nitrate reductase activity was obtained following treatment with a final concentration of 20 nM nitrite (our unpublished data). Our stock sodium nitrate solution used for induction would have provided 10–15 nM nitrite in each flask.

Discussion

The data establish that *A.nidulans* possesses only two functional NO_3^- transporters, *nrtA* and *nrtB*, in contrast to the 11 NO_3^- transporter genes in *A.thaliana* (*Arabidopsis* genomic sequence <http://www.arabidopsis.org/home.html>). *nrtA* and *nrtB* may appear to be redundant genes since their cognate proteins (i) are clearly homologues with only short regions of amino acid differences and (ii) are involved in

Fig. 7. Induction of *nrtA*, *niaD* and *niiA* transcripts in wild type (WT) and the double mutant *nrtA747 nrtB110*. Total RNA was isolated from mycelium grown at 37°C in minimal medium for 6 h with 10 mM proline as sole nitrogen source (lanes 1) or for 5.5 h with 10 mM proline and 10 mM sodium nitrate added for 30 min prior to harvesting (lanes 2). A northern blot was hybridized sequentially with the probes shown to the right and detailed in the legend to Figure 2A. The *niiA* probe was a 1.1 kb *EcoRI* fragment of pSTA3 (Johnstone *et al.*, 1990). To the right of the respective probes are histograms showing the quantitative data obtained from the northern blot. The open bar represents values obtained for lanes 1 and the closed bar values for lanes 2. The scale shows arbitrary units determined by the actual signal values for each probe as a percentage of those signal values for the internal control *actA* probe in the same lane.

the same function, i.e. high-affinity nitrate transport. Moreover, deletion mutants in either gene have no detectable phenotype, *vis-à-vis* growth on 1–10 mM nitrate, and both genes are regulated identically under an extensive range of conditions and in different genetic backgrounds. Nevertheless, the proteins have different K_m and $V_{\max}$ values for nitrate. We hypothesize that at high ambient nitrate, the NrtA transporter makes the major contribution to influx, but when ambient nitrate declines to $<10 \mu\text{M}$, as shown in Figure 6C, the contribution to influx of the NrtB transporter is actually double that of the NrtA counterpart. In environments with rapidly fluctuating nitrate concentrations, therefore, a mutant in either gene would be disadvantaged.

The kinetic characterization of individual nitrate transporters has been hampered by the lack of a convenient tracer to measure nitrate influx rather than net $^{14}\text{NO}_3^-$

uptake, the balance between influx and efflux. Using measurements of net nitrate uptake by wild-type *A.nidulans*, Brownlee and Arst (1983) recognized that the kinetics of uptake were more complex than could be explained by a single nitrate transporter, but were unable to resolve the individual transporter contributions contained in their data. In *Chlamydomonas reinhardtii*, Galvan *et al.* (1996) used net nitrate uptake measurements to document K_m values of 1.6 and 11 μM , respectively, for strains expressing *Nrt2;1* and *Nrt2;2*. In higher plant systems, e.g. in barley roots, even when $^{13}\text{NO}_3^-$ was employed to characterize influx (Siddiqi *et al.*, 1989), the reported kinetics might result from the combined activities of several high-affinity transporters. In *A.thaliana*, Filleur *et al.* (2001) have measured the kinetics of $^{15}\text{NO}_3^-$ influx in a T-DNA insertional mutant; however, this strain was compromised in both *Nrt2.1* and *Nrt2.2* genes. Thus, up to now, only with the *C.reinhardtii* data (and with the caveat that net uptake was determined) has it been possible to characterize individual nitrate transporters in *planta*. Alternatively, several studies have employed *Xenopus* oocytes to attempt electrical characterization of the *A.nidulans crnA* gene and the *C.reinhardtii Nar2* and *Nrt2;1* genes (Zhou *et al.*, 2000a,b). These and other studies employing heterologous expression systems may provide valuable qualitative information, but, given that these proteins are expressed in foreign membrane systems, quantitative data obtained by such methods should be interpreted with some caution.

It is instructive to note that in both *C.reinhardtii* and *A.nidulans* the two transporters show a 10-fold difference in K_m values, allowing a greatly expanded range of nitrate acquisition. It is also noteworthy that in wild-type mycelium, expressing two transporter systems with markedly different K_m values, the kinetics conform to a simple (Michaelis–Menten) hyperbola. Thus, conformity to Michaelis–Menten kinetics cannot be taken as evidence for the exclusive operation of but a single transport/enzyme activity.

Whereas *nrtA* and *nrtB* single mutants grow on nitrate, the *nrtA nrtB* double mutant is unable to grow even if the nitrate concentration is increased to 200 mM. The inability to grow on nitrate is similar to *niaD* loss-of-function mutants (lacking nitrate reductase). The failure to detect further homologues from the *A.nidulans* genome sequencing project (V.Gavrias, personal communication) supports our conclusion that NrtA and NrtB are the only nitrate transporters in this species. In young cells, as judged by V_{max} values, a loss-of-function *nrtA* mutant has ~17% of the nitrate uptake rate of the wild type, which is presumably due to NrtB activity. NrtA involvement is, therefore, required in order to achieve wild-type levels of uptake. In contrast, the *nrtB* mutant displays a close to wild-type uptake rate in young cells, suggesting that a functional NrtA can almost completely compensate for the loss of NrtB activity. In mature mycelial cells, the maximum rate of nitrate uptake in the wild type falls relative to young cells to a level only around twice that of a *nrtA* mutant. The fact that uptake rates in the wild-type and *nrtB110* strains are similar, and in *nrtA747* are only around half the wild-type value, indicates that in older cells NrtB can compensate more effectively for the lack of functional NrtA. Whether the reduction in V_{max} in the wild-type and

nrtB110 strains is due to an intrinsic change in the NrtA protein or to a reduction in NrtA protein per unit biomass is unclear. However, the reduction may indicate a degree of developmental control at the protein level. Furthermore, in both net nitrate (our unpublished data) and $[^{13}\text{N}]$ nitrate experiments, the wild-type uptake rate is less than the sum of the rates observed for *nrtA747* and *nrtB110*. This suggests that there may be some form of cross-regulation in older wild-type cells, such as has been described for mammalian Rh proteins (Ridgewell *et al.*, 1994) and yeast ammonium transporters (Marini *et al.*, 2000). The reduction in nitrate uptake rate between young and older cells is in contrast to the results of Brownlee and Arst (1983) who found that the wild-type rate was maintained between young and old cells, and that the value for the *nrtA* mutant increased to a level similar to wild type in mature mycelium. The reason for this discrepancy is not clear but, in this study, a reduction in uptake rate of wild type in the longer incubation period was observed reproducibly in both net nitrate (our unpublished data) and $[^{13}\text{N}]$ nitrate experiments. A similar pattern has also been observed repeatedly in higher plant root studies (Siddiqi *et al.*, 1989; Zhuo *et al.*, 1999).

Although both *nrtA* and *nrtB* mutants grow as wild type on nitrate as sole nitrogen source, unlike *nrtA*, *nrtB* mutants are able to grow normally on nitrate in the presence of caesium. Previously, it was postulated that caesium inhibited the growth of the *nrtA1* mutant on nitrate because it inhibited the other, at that time uncharacterized, nitrate uptake system (Unkles *et al.*, 1991). This suggestion, however, appears to be incorrect since net nitrate uptake by the wild type, *nrtA747* mutant or *nrtB110* mutant is unaffected in the presence of caesium (our unpublished data). Secondly, the *nrtB* mutant differs from that of *nrtA* in that *nrtB* is not resistant to chlorate toxicity at the standard concentrations used to monitor chlorate resistance. This suggests that while NrtA actively takes up chlorate, resulting in sensitivity to chlorate toxicity in the wild-type strain, NrtB does not. Resistance to toxicity is therefore the result of mutations within *nrtA*. The *nrtB* mutant strain remains sensitive since it still possesses a wild-type NrtA. In this regard, recent characterization of *nrtA* expressed in *Xenopus* oocytes has cast doubt on toxicity being due to chlorate. Instead, it has been suggested that contaminating chlorite is the agent responsible for toxicity since it appears in this heterologous expression system that *nrtA* transports chlorite but not chlorate (Zhou *et al.*, 2000b). However, two factors require consideration in interpretation of these results. First, commercially available chlorite itself contains a high proportion (~20%) of uncharacterized contaminants, which could elicit a response in electrical conductivity experiments. Secondly, preliminary growth tests of *nrtA* and *nrtB* mutants on medium containing chlorite (our unpublished data) show that the mutants do not conform to the recognized growth phenotype obtained with chlorate, and so the results of Zhou *et al.* (2000b) remain enigmatic. Finally, with regard to the phenotypes of *nrtA* and *nrtB* mutants, the growth of the double mutant is similar to wild type on nitrite (in the presence or absence of caesium) as the sole source of nitrogen, suggesting that neither NrtB nor NrtA is required for nitrite transport. It was reported previously that caesium reduced growth of *nrtA1* when

nitrite was the sole nitrogen source (Unkles *et al.*, 1991). However, strain *nrtA747* and other newly isolated *nrtA* mutants (our unpublished data) grow normally on nitrite plus caesium, suggesting that the original phenotype may have been caused by a secondary mutation.

The regulation of *nrtB* appears to be identical to that of *nrtA* as judged by the abundance of transcript levels in northern blot experiments. Clearly, *nrtB* is under the control of the nitrate assimilation pathway-specific control gene for induction, *nirA*, and *areA*, the global control gene for nitrogen metabolite repression. In nitrate assimilation structural gene mutants, both *nrtA* and *nrtB* transcripts are elevated upon nitrate induction, probably reflecting the fact that these mutants are unable to utilize nitrate. Hence there is no reduction of the inducer, nitrate, or production of repressor(s), ammonium or glutamine. The question was addressed as to whether the two nitrate transporter genes might be expressed differentially in young germinating conidiospores and older mycelial cells. Cells grown on nitrate as the sole nitrogen source for up to 16 h show maximum levels of both *nrtA* and *nrtB* transcripts (and also the *nirA* transcript) after only 1 h incubation (in liquid medium), falling significantly thereafter to barely detectable levels at 7 h. Unlike the transcript of the *A.nidulans* proline transporter, *prnB*, which has been shown to accumulate in conidiospores even in the absence of proline induction (Tazebay *et al.*, 1997), the rapid increase in *nrtA* and *nrtB* transcript abundance in conidiospores requires induction by nitrate (our unpublished data). The fall in transcript abundance occurs after 1 h despite a high concentration of nitrate remaining in the external medium. This suggests that there may be some repression of transcription arising from increasing intracellular pools of ammonium or glutamine, which are known to be the co-repressors mediating AreA function (Morozov *et al.*, 2000; and references therein). Reduction of transcripts to extremely low levels after 8 h additionally may be the result of consumption of nitrate with the resultant loss of induction. However, our northern blot analysis suggested that there was no differential expression of the *nrtA* and *nrtB* mRNAs, at least at the level of RNA accumulation. This was confirmed by the demonstration that both of these transcripts can be highly expressed in young cells as well as in mature mycelium after a short induction period by nitrate. Therefore, it would seem that transcription of the two genes is not under the influence of developmental control, rather being subject to control by metabolic signals of nitrate induction and ammonium (nitrogen metabolite) repression only.

A surprising result obtained in the course of this study was the demonstration of inducibility of nitrate assimilation genes in the *nrtA747 nrtB110* double mutant, a mutant devoid of measureable nitrate transport. That transcription of the nitrate reductase and nitrite reductase genes is induced to 40–45% of the wild-type level in the absence of measurable nitrate transport, with the level of the *nrtA* gene transcript being similar to that in the wild type, leads us to the conclusion that nitrate may not need to enter the cell to induce the pathway. It is possible that very low levels of nitrate (sufficient for gene induction but not for mycelial growth) enter via another transport system. However, given that the double mutant (i) fails to grow on 200 mM nitrate whereas the wild type exhibits tangible

growth at 1 mM nitrate, and (ii) we failed to detect $^{13}\text{NO}_3^-$ influx in the double mutant despite the great sensitivity of this methodology, a hypothesis involving external nitrate sensing remains a distinct possibility.

Materials and methods

Aspergillus nidulans strains and media

The standard wild-type (with regard to nitrogen regulation) strain used in this study was *biA1*. Nitrate assimilation-defective mutants (*nirA171*, *nirA24*, *nirA17* and *cnxH4*) are discussed by Cove (1979; and references therein). Strain *A506* is a deletion in the nitrate assimilation pathway and lacks *nirA*, *nirA* and *nrtA* genes (Tomsett and Cove, 1979). Strain *ΔareA* is deleted in a substantial portion of the coding region (M.A.Davis, unpublished data) and *nrtA747* is a single base deletion in transmembrane domain 2 which places a stop codon immediately distal to the deletion (our unpublished data). Routine *Aspergillus* growth media and handling techniques were as described before (Clutterbuck, 1974). Shake flask cultures for nitrate reductase and nitrate uptake assays as well as for northern blots were grown in liquid minimal medium (Cove, 1966) containing the sole nitrogen sources as stated in the text or figure legends. Harvesting of mycelium was by filtration through 0.45 μm cellulose acetate filters (conidiospores) or miracloth (mycelia) (CN Biosciences, Nottingham, UK); samples were frozen in liquid nitrogen and stored at -80°C .

Genetic transformation

This was carried out as described before (Riach and Kinghorn, 1995; and references therein) using initially the riboflavin auxotrophic mutant *yA2 pabaA1 prnΔORF rboB2* (strain number CS2290) and, later, mutant strain *yA2 rboB2 nrtA747* (strain JK2001).

Escherichia coli strains, plasmids and media

Standard procedures were used for propagation of cosmids and bacteriophage, as well as for subcloning and propagation of plasmids in *Escherichia coli* strain DH5α.

Molecular methods

DNA was isolated using a Nucleon BACC2 Kit (Amersham Pharmacia Biotech, Little Chalfont, UK) and RNA using an RNeasy Plant Mini Kit (Qiagen, Crawley, UK). The conditions used during Southern and northern blot analysis were as described previously (Unkles *et al.*, 1999). The nucleotide sequence of the *A.nidulans* wild-type *nrtB* gene was determined in both strands using the Sequenase version 2 DNA sequencing kit according to the manufacturer's instructions (Amersham Pharmacia Biotech) and by automated sequence analysis as described before (Unkles *et al.*, 1999). Hybridization signals in northern blots were quantified using an Instant Imager (Packard, Meriden, USA).

Isolation of the *nrtB* gene and creation of a gene disruption

An *A.nidulans* cosmid library was generated using a pWEB Cosmid Cloning Kit (Epicentre Technologies, Madison, WI) and hybridized under conditions of low stringency with a probe derived by PCR amplification and encompassing the *nrtA* coding region. Twelve positively hybridizing clones were obtained and these subsequently were hybridized with a probe derived from the *nirA* gene for nitrite reductase, which is contiguous with *nrtA* in the nitrate assimilation gene cluster on chromosome 8 (Johnstone *et al.*, 1990). One clone failed to hybridize to *nirA*, i.e. was not contiguous with *nirA*, and was characterized further by Southern blotting with the *nrtA* gene probe, identifying a 6 kb *HindIII* fragment convenient for subcloning into pUC19 to give pMON13. A 2.5 kb blunt-ended fragment containing the wild-type *riboB* gene (Oakley *et al.*, 1987) was used to replace a 780 bp *NcoI* fragment of the *nrtB* coding region (also blunt ended) (nucleotides 441–1220 in Figure 1A) to give pMON15. This was transformed into a double mutant strain *riboB2 nrtA747* (strain JK2001). Several riboflavin prototrophic transformants failed to grow on 10 mM nitrate. Southern analysis showed that the *nrtB* gene had been disrupted in all nitrate non-utilizing transformants examined. One of these transformants, T110, containing the disrupted *nrtB* mutation, was designated allele 110. To obtain the single *nrtB110* mutant, transformant strain T110 was outcrossed to the *yA2 pabaA2 argB2* strain. Four progeny showing the wild-type phenotype were examined by Southern blot. One of these four, progeny 41, possessed the disrupted *nrtB* bands. Finally, progeny 41 (*nrtB110*) was crossed to the single mutant strain *nrtA747* and progeny

unable to grow on nitrate were recovered, thus confirming that progeny strain 41 did indeed harbour the *nrtB110* mutation (our unpublished data).

Nitrate reductase assays

The growth of conidiospores, young germings and mycelium was at 37°C with orbital shaking at 250 r.p.m.. Nitrate reductase assays were performed at 25°C according to the method of Cove (1966).

Uptake assays using the tracer $^{13}\text{NO}_3^-$

The generation and purification of $^{13}\text{NO}_3^-$ was undertaken at the University of British Columbia TRIUMF cyclotron as detailed in Kronzucker *et al.* (1995). Approximately 5×10^9 viable conidiospores were inoculated into two 400 ml amounts of minimal medium containing appropriate vitamin supplements and 2.5 mM urea as nitrogen source, and incubated at 37°C with orbital shaking at 250 r.p.m. After induction with 5 mM sodium nitrate for 100 min, strains were filter-harvested through miracloth, thoroughly washed with warm nitrate-free medium and held in minimal medium containing vitamin supplements and 10 μM sodium nitrate at 37°C. Three 30 ml samples were filtered and dried to estimate dry weights and 50 ml samples aliquoted for uptake of $^{13}\text{NO}_3^-$ at 37°C with reciprocal shaking at 100 r.p.m. After 2 or 10 min incubation, 5×5 ml aliquots were filtered rapidly through 25 mm glass fibre filters, type GF/C (Whatman, Maidstone, UK). These were washed with 200 ml of non-labelled medium at 37°C containing 200 μM sodium nitrate to eliminate $^{13}\text{NO}_3^-$ adhering to cell wall material. Samples taken after 5 s incubations were used to confirm that this washing protocol was effective. Filters were counted as described in Kronzucker *et al.* (1995). Michaelis-Menten curves were generated by non-linear regression using Microcal Origin software.

Growth conditions for transcript analysis

Incubations were carried out at 25°C for 13–14 h with orbital shaking at 250 r.p.m., conditions that give young germings at a growth state similar to that achieved by incubation at 37°C for 6–7 h. The standard inoculum was conidiospores scraped from half of a fully covered 9 cm Petri dish containing *Aspergillus* complete agar medium and suspended by vigorous vortexing in sterile 0.9% (w/v) sodium chloride, 0.1% (w/v) Tween-80. This was inoculated into 200 ml of minimal medium containing appropriate vitamin supplements and the nitrogen sources indicated. Exceptions to this growth regime were experiments in which samples were taken at timed intervals during incubation of conidiospores or mycelium. Conidiospores from 11 and four Petri dishes for a 16 h and a 60 min time course, respectively, suspended in sterile 0.9% (w/v) sodium chloride, 0.1% (w/v) Tween-80, were filtered twice through miracloth to remove mycelial fragments and pelleted by centrifugation. The conidiospores were inoculated into 1600 or 500 ml for the 16 h and 60 min time course, respectively. In these experiments, and those for comparison of induction of nitrate assimilation genes in the wild type and *nrtA nrtB* double mutant, strains were grown at 37°C with orbital shaking at 250 r.p.m.. Nitrate concentrations in the growth medium were estimated using the procedure of Cataldo *et al.* (1975). The nitrite concentration of the 1 M stock solution of sodium nitrate was determined colorimetrically as described by Cove (1966).

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