The ability to transport net nitrate was conferred upon transformant cells of the non-nitrate-assimilating yeast *Pichia pastoris* after the introduction of two genes, one encoding nitrate reductase and the other nitrate transport. It was observed that cells of this lower eukaryote transformed with the nitrate transporter gene alone failed to display net nitrate transport despite having the ability to produce the protein. In addition, loss-of-function nitrate reductase mutants isolated from several nitrate-assimilating fungi appeared to be unable to accumulate nitrate. Uptake assays using the tracer \(^{15}\text{NO}_3\) showed that nitrate influx is negligible in cells of a nitrate reductase null mutant. In parallel studies using a higher eukaryotic plant, *Arabidopsis thaliana*, loss-of-function nitrate reductase strains homozygous for both *NIA1* insertion and *NIA2* deletion were found to have no detectable nitrate reductase mRNA or nitrate reductase activity but retained the ability to transport nitrate. The reasons for these fundamental differences in nitrate transport into the cells of representative members of these two eukaryotic kingdoms are discussed.

Nitrate is a major source of nitrogen for most algae, bacteria, fungi, and higher plants, and it is the nutrient that most frequently limits their growth (reviewed in Refs. 1–3). The first step in the assimilation of nitrate is the influx of nitrate into cells, which is an active process, because it can occur against an electrochemical potential gradient (4) followed by the catalytic activities of nitrate reductase and nitrite reductase that sequentially produces nitrate and ammonium, the latter being converted to organic nitrogen for cellular growth. Although there is considerable biochemical, genetical, and molecular biological information about these systems and their regulation, it is still not clear whether nitrate reductase is required for nitrate transport activity or whether transport occurs quite independently and in the absence of nitrate reductase activity (Ref. 5 and references therein). Mutants impaired in nitrate reductase activity have been studied to answer this question. Plant mutants with low levels of nitrate reductase in barley possess considerable nitrate transport activity (6, 7), and nitrate reductase-defective mutants in tobacco accumulate high levels of nitrate indicative of functional uptake (8). However, these mutants still possess some nitrate reductase activity making the interpretation of the importance of an active nitrate reductase for nitrate uptake somewhat equivocal, as low nitrate reductase activity alone might be sufficient to allow substantial transport. Fungi are more amenable to an intensive genetical approach, but, nevertheless, there are conflicting reports as to whether or not nitrate reductase activity per se is required for the expression of nitrate transport in fungi. For instance, studies carried out with *Aspergillus nidulans* implied an obligatory requirement (9), whereas the results in the related ascomycetous fungus *Neurospora crassa* (10) showed complete autonomy. Recent work (11) in the nitrate-assimilating yeast *Hansenula polymorpha* shows that nitrate transport levels correlates with transporter protein levels and not nitrate reductase levels, although they did not assay nitrate uptake in a nitrate reductase-defective mutant.

Here we demonstrate that nitrate reductase activity is mandatory for nitrate accumulation in cells of the lower eukaryotes, the fungi. The evidence comes first from heterologous studies with the *A. nidulans* NrtA (and NrtB) nitrate transporter proteins in the non-assimilating yeast *Pichia pastoris* and second from null nitrate reductase mutants of *N. crassa* and other nitrate-assimilating fungi. In contrast, studies of nitrate reductase null mutants in higher eukaryotic plant cells of *Arabidopsis thaliana* show that nitrate transport has no such obligatory requirement for nitrate reductase activity.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains—** *P. pastoris* GS115, a histidine-requiring derivative of the wild-type strain, which is unable to assimilate nitrate, and recombinant strain AtNia2, which possesses *Arabidopsis* nitrate reductase activity (12–14), were used. Strains were maintained on yeast extract peptone dextrose medium (YPD) as recommended by Invitrogen.

**Filamentous Fungal Strains—** *Aspergillus fumigatus* strains used in this study were wild-type, *cnx1* and *cnx3* mutants, *Aspergillus niger* wild-type and *niaD101*, and *Aspergillus oryzae* wild-type and *niaD100* (all this study), and *N. crassa* wild-type, *nit-3* (RIP), and *nit-10* (RIP).1 Mutant *nit-3* (RIP) was found to lack nitrate reductase activity and *nit-10* (RIP) nitrate transport activity (this study). Strain *nit-1* is defective in the synthesis of the molybdenum cofactor (15) and lacks nitrate reductase activity (this study). Mutant *Aspergillus* strains lacking nitrate reductase were selected on the basis of resistance to chloride toxicity (16). All strains were maintained on *Aspergillus* complete medium (17).

**Fungal Nitrate Transport and Nitrate Reductase Assays—** Conidial suspensions from filamentous fungi were inoculated into 200 ml of minimal medium containing 5 mM urea as the sole nitrogen source in 1-liter Erlenmeyer flasks, which were incubated with orbital shaking at
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250 rpm. For A. niger, A. oryzae, and N. crassa, incubation was carried out at 25 °C for 16–20 h and for A. fumigatus and A. nidulans at 37 °C for 6.5–7.5 h. To induce the nitrate assimilation pathway, 10 mM sodium nitrate was added 5 h before harvesting cells grown at 25 °C and 100 min before harvesting the cells grown at 37 °C. Yeast cultures were grown in 150 ml of YPD in baffled 500-ml Erlenmeyer flasks shaking at 200 rpm at 30 °C overnight to reach an A540 of 1.2–1.5. Cells were collected by centrifugation for 5 min at 2200 × g at room temperature and were washed with Pichia yeast nitrogen base (Invitrogen) containing 10 mM proline as the nitrogen source. Cells were resuspended in 150 ml of the same medium in baffled 500-ml Erlenmeyer flasks, and 0.5% methanol was added to induce the expression of genes under the control of the auxA1 (alcohol oxidase) promoter. Flasks were incubated at 30 °C with agitation at 200 rpm for a total of 20 h until a further 10% methanol was added after a 12-h incubation. Filamentous fungi were harvested by filtration through a miracloth (CN Biosciences, Nottingham, UK), and yeast cells were harvested by centrifugation as described above. Following washing in nitrate-free medium, net nitrate transport assays were carried out in minimal medium containing an initial concentration of 500 μM nitrate at 30 °C for P. pastoris, A. niger, A. oryzae, and N. crassa or at 37 °C for A. fumigatus and A. nidulans by the method described for A. nidulans (9). Aliquots were taken from the uptake medium at the start of incubation and after 20 min, wherein cells were removed rapidly by filtration through Millex filters (Millipore, Molsheim, France). Results are expressed as the nanomoles of nitrate removed rapidly by filtration through Millex filters (Millipore, Mol-

Fungal Uptake Assays Using the Tracer 15NO3—The growth of A. nidulans strains and the assay of nitrate influx were performed as detailed in Unkles et al. (19). Tracer experiments were conducted at the University of British Columbia, Vancouver, British Columbia, Canada. The routine concentration range of 250 μM nitrate was used. Growth of P. pastoris is described above, and the assay of nitrate influx was as detailed in Unkles et al. (19). Values for influx are expressed as nmol of nitrate/mg of dry weight/h.

Escherichia coli Strains, Plasmids, and Media—Standard procedures were used for propagation of plasmids as well as for subcloning and maintenance of plasmids within the E. coli strain DH5α.

Fungal Molecular Methods—DNA was isolated using a Nucleon BACC2 Kit (Amersham Biosciences). Total RNA was isolated using an RNeasy plant mini kit (Qiagen, Crawley, UK). The conditions used during dot blot analysis were as described previously (20). Nucleotide sequencing was determined by automated sequence analysis as described before (20). Yeast transformations were performed as recomended by Invitrogen with selection for zeocin resistance.

Construction of Plasmid Vectors for Expression in P. pastoris—cDNA coding for A. nidulans nitrate reductase and nrtB was amplified by reverse transcriptase-PCR from total RNA of the A. nidulans wild-type strain bia1 grown on nitrate as the sole nitrogen source. Primers NRTAF (5'-AGAGAAATTCAGATGACCCTCTCAATCTAGTGTCG) and NRTAR (5'-AGAGACTGAGCTCCTCTCATTGATTTTCG) allowed the cloning of the nrtA cDNA into the EcoR1-Xhol sites, and primers NRTBP (5'-TACTGATATGGAGACTTACGACGAG) and NRTBE (5'-TACTTATGGTTCCTCGTGTTAAGACG) allowed the cloning of the nrtB cDNA into the EcoRI-SmaI sites of the expression vector pPICZ-C (Invitrogen) to give plasmids PICNRTA and PICNRTB, respectively. The complete sequence of both genes was verified using primers PICF (5'-GACTGGCTTCAATCTCAGGAG) and PICR (5'-ACC-GTTGCTTCCTCTG) of the plasmid pPICZ-C.

Peptide and Antibody Production—Three peptides were purchased from the Molecular Biology Unit, University of Newcastle, Newcastle-upon-Tyne, United Kingdom. These were IPDVEKKGTETPLEP (NrtA residues 269–283) and two from the large central loop and SWVRPVKSMIE (NrtA residues 498–509) and 501–512, respectively. The peptides were conjugated to keyhole limpet hemocyanin via cysteine residues synthesized at the N terminus of each peptide. A pool of the three conjugated peptides was used to raise antibodies in rabbits by a standard procedure of six serial subcutaneous injections.

Western Blotting—Yeast and filamentous fungi were grown as described for net nitrate transport using filamentous fungi by filtration and yeast by centrifugation. Spore germlings and yeast cells were washed with cold sterile distilled water and were used fresh (yeast) or were frozen in liquid nitrogen (filamentous fungi). Yeast cells (500-mg wet weight) were resuspended in 7 ml of extraction buffer (250 mM sucrose, 5% (v/v) glycerol, 1 mM magnesium chloride, 1 mM EDTA, 35 mM MOPS, pH 7.4 at 4 °C containing 1 mM dithiothreitol, 100 μM phenylmethylsulfonyl fluoride, and one Complete Mini protease inhibitor mixture tablet (Roche Diagnostics)/10 ml of buffer. Cold acid-washed sand (VWR International Ltd., Lutterworth, UK) (6 ml) was added, and cells were disrupted by vortexing vigorously for a total of 6 min in 30± bursts with a 1-min cooling on ice between bursts. Filamentous fungi (300–500 mg of pressed wet weight of the spore germlings) were ground in liquid nitrogen, and the powder was suspended in 10 ml of cold extraction buffer. Crude plasma membrane preparations from yeast and filamentous fungi were made first by the centrifugation of suspensions at 2000 × g for 10 min at 4 °C to remove sand and whole cells followed by the centrifugation of the supernatant at 18,000 × g for 30 min at 4 °C. The supernatant was resuspended in 150 μl of extraction buffer, and aliquots were stored at −85 °C. Protein estimations were carried out using a BCA protein assay kit (Pierce). Protein samples were denatured by boiling for 5 min in the presence of 1% SDS and 14 mM 2-mercaptoethanol, then the samples were centrifuged for 1 min, electrohoresed on 10% polyacrylamide gels (21), and transferred to nitrocellulose membrane (22). Blots were blocked by incubation overnight at 4 °C in Tris-buffered saline (137 mM sodium chloride, 20 mM Tris, pH 7.5) containing 5% (w/v) membrane-blocking agent (Amersham Biosciences). NrtA was detected by an incubation of the blot with 1:500 anti-NrtA antibody in Tris-buffered saline containing 0.5% (w/v) membrane-blocking agent for 2 h at room temperature following a washing with TBS containing 0.1% (v/v) Tween 20 with 1.0,000 horse radish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. After further washing with TBS, peroxidase visualization was carried out using ECL Plus reagents (Amersham Biosciences) and Hyperfilm ECL (Amersham Biosciences).

Arabidopsis Strains—The nitrate reductase null mutant strain was generated by crossing the nia2 deletion strain chil-5 (23) with a line containing a dissociation element insertion in NIA1 (24). F2 segregants that were homozygous for the NIA1 insertion and NIA2 deletion were identified and propagated. These plants had no detectable nitrate reductase mRNA or activity (data not shown).

Arabidopsis Growth Conditions—Plants were grown hydroponically with 20–25 ml of medium in 50-ml beakers and 24-h light with 2.5 mM ammonium succinate for 10 days, then NH4NO3 was added to the cultures to a final concentration of ~350 μM as described by Wang et al. (25).

Arabidopsis Nitrate Determinations—Nitrate concentrations in media and tissue extracts were determined chemically by the hydrazine sulfate method as described by Kamphake et al. (26). To measure nitrate accumulation, fresh tissues were ground into frozen powder in liquid nitrogen. Approximately 50 mg of the frozen powder was transferred into a 2-ml microcentrifuge tube and weighed. 1 ml of water was added to the tube and then boiled with the lid closed for 15 min. The supernatants were centrifuged at maximum speed for 5 min at 4 °C. The supernatants were removed for nitrate assay.

RESULTS

Net Nitrate Transport Activity in P. pastoris, a Nitrate Nonassimilating Yeast—Plasmids PICNRTA and PICNRTB containing the cloned high affinity nitrate transporter genes of A. nidulans, nrtA, and nrtB, respectively, under the control of the yeast AOX1 promoter were transformed individually into the recipient P. pastoris GS115, a yeast strain which does not assimilate nitrate or possess nitrate reductase activity (12–14). Transformants were selected on the basis of zeocin resistance, and the DNA from selected transformants were identified by sequencing of the DNA in each transformant harboring the nrtA or nrtB gene were grown, with the expression of the genes being induced by methanol, and net nitrate transport activity was assayed. No detectable nitrate transport activity was observed in representative transformants of GS115, such as T5 and T9, with the nrtA (Table I) or nrtB (data not shown) expression constructs. This result was supported by the phenotype of T5 or T9 strains (Fig. 1). Both transformants were found to be resistant to chlorate (a toxic analogue of nitrate),

2 The abbreviation used is: MOPS, 4-morpholinepropanesulfonic acid.
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TABLE I

| Species and mutation | Nitrate reductase activity | Net nitrate uptake |
|----------------------|---------------------------|-------------------|
| A. fumigatus         |                           |                   |
| wild-type            | 64.3                      | 10.9              |
| cnx1                 | 0                         | 0                 |
| cnx3                 | 0                         | 0                 |
| A. niger             |                           |                   |
| wild-type            | 41.0                      | 7.2               |
| niaD101              | 0                         | 0                 |
| A. oryzae            |                           |                   |
| wild-type            | 73.7                      | 6.3               |
| niaD100              | 0                         | 0                 |
| N. crassa            |                           |                   |
| wild-type            | 100.2                     | 7.8               |
| nit-3 (RIP)          | 0                         | 0                 |
| nit-1                | 0                         | 0                 |
| nit-10 (RIP)         | 11.4                      | 0                 |

Values ± S.E. are the result of at least three independent assays. Where no net nitrate uptake was detected (0) at least five transformant strains of each class were assayed once with the same result, and those shown are representatives (i.e., T5 and T9). Net nitrate transport is expressed as nmol of nitrate depleted/min/mg of dry weight.

**TABLE II**

Net nitrate uptake in nitrate reductase defective filamentous fungal mutants

Nitrate reductase activity is expressed as nmol of nitrite formed/min/mg of protein, and nitrate transport is expressed as nmol of nitrate depleted/min/mg of dry weight. Assays were carried out in triplicate using cells derived from a single culture. A value of 0 denotes undetectable activity.

**Protein Analysis**—Western blot analysis was carried out using anti-NrtA antibodies to determine whether the NrtA protein was present in crude membrane preparations from yeast or filamentous fungal cells with and without nitrate reductase activity. A major immunoreactive polypeptide of ~50 kDa was observed in a representative transformed P. pastoris strain, T9, a transformant of GS115 that lacks nitrate reductase (Fig. 2), and also in transformants T8 and T15 (data not shown). This 50-kDa polypeptide was absent from the recipient strain GS115 but was present in the P. pastoris transformant T51. However, the protein was not observed in the corresponding nitrate reductase-containing recipient strain AtNia2 or a in zeocin-resistant transformant of AtNia2, T52, which had no detectable nitrate uptake (data not shown). Western blots of crude membrane preparations from nitrate-induced A. nidulans nitrate reductase-defective strains also showed a faint
immunoreactive polypeptide of 50 kDa similar to the wild-type band. It is noteworthy that confirmation that the 50-kDa polypeptide is indeed the NrtA protein came from studies of an A. nidulans strain generated by disruption of both nitrate reductase, nrtA747 nrtB101, and the corresponding untransformed recipient strain GS115, which do contain nitrate reductase, were subjected to Western blot analysis using anti-NrtA antibodies. The position of molecular mass markers is shown, and the 50-kDa NrtA band indicated with the arrow.

Fungal Uptake Assays Using the Tracer $^{13}$NO$_3$—To circumvent the possibility that in nitrate reductase null mutants, nitrate influx was normal but then a rapid efflux occurred (an unlikely but possible explanation for observed net flux in net nitrate uptake results), nitrate influx was undertaken using the very sensitive short lived tracer $^{13}$NO$_3$ on a few selected strains. Unlike the depletion method of measuring net nitrate uptake by measuring the disappearance of nitrate from the uptake solution, the tracer method measures tracer contained within samples of the fungus after a rinsing with non-nitrate solution. A. nidulans mutant niaD171 lacked nitrate reductase activity (i.e. no measurable nitrite was formed in nitrate reductase assays of niaD171 cell-free extracts grown under inducing conditions, whereas the wild-type extract formed 36 mmol of nitrite/min/mg), and net nitrate influx was undetectable with niaD171 (>0.06 nmol/min/mg compared with 13.6 nmol/min/mg for wild-type). The results presented in Fig. 3 showed that the A. nidulans mutant niaD171 likewise displayed negligible $^{15}$NO$_3$ influx values that were approximately similar to the double mutant strain nrtA747 nrtB110.

Nitrate Depletion in a Nitrate Reductase Loss-of-function A. thaliana Strain—Studies over a period of 24 h indicated that the net nitrate uptake of a nitrate reductase null mutant (see “Experimental Procedures”), as judged by nitrate depletion assays, was similar to the wild-type (Table III). Furthermore, nitrate accumulated to a significantly higher level in the roots and shoots of the nitrate reductase null mutant compared with the wild-type levels, presumably because of the lack of nitrate reductase activity (Table IV).

**DISCUSSION**

This study was undertaken to determine whether nitrate reductase is required for nitrate uptake activity in the cells of fungi and plants, because certain previous studies were equivocal, possibly due to mutant strains retaining some nitrate reductase activity. Our goals therefore were to generate cells for both lower and higher eukaryotic representatives devoid of nitrate reductase activity and to study their nitrate-transport profiles.

Initially, studies were carried out using the non-nitrate-assimilating yeast *P. pastoris*, which is a lower eukaryote. Net nitrate transport activity was determined in cells transformed with the *A. nidulans* nitrate transporter gene nrtA in the absence and presence of a recombinant *A. thaliana* nitrate reductase gene NIA2. Net nitrate transport was clearly observed in nrtA transformant cells with, but not without, NIA2. Subsequently, nitrate reductase-defective mutants were generated in several different filamentous fungi. Nitrate reductase loss-of-function mutants were identified, and all lacked net nitrate transport, including *N. crassa* for which mutants were originally reported to possess significant nitrate transport activity (10). Finally, results from experiments using the short lived tracer $^{13}$NO$_3$ confirm that there is a loss of nitrate accumulation in fungal strains lacking nitrate reductase activity. It is clear therefore that yeast and filamentous fungi fail to accumulate nitrate in the complete absence of nitrate reductase. In contrast, in the higher eukaryotic plant, *A. thaliana*, nitrate transport activity is completely independent of nitrate reductase activity as judged by the retention of transport and the accumulation of nitrate in a loss-of-function nitrate reductase mutant, a strain generated by disruption of both nitrate reductase structural genes, NIA1 and NIA2.

Lack of nitrate uptake is not because of a down-regulation of

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**Table III**

| Incubation time | Wild-type | Nitrate reductase null |
|-----------------|-----------|-----------------------|
| 1 h             | 337.4 ± 41.3 | 342.4 ± 45.4         |
| 2 h             | 322.4 ± 34.7 | 313.1 ± 22.3         |
| 3 h             | 307.5 ± 6.5  | 294.8 ± 14.6         |
| 4 h             | 281.5 ± 7.5  | 270.3 ± 14.1         |
| 5 h             | 173.5 ± 7.0  | 205.1 ± 14.0         |
| 6 h             | 96.5 ± 94.0  | 158.4 ± 49.5         |
| 24 h            | 30.3 ± 15.7  | 48.7 ± 49.5          |

**Table IV**

| Tissue              | Nitrate accumulation (µmol of tissue) |
|---------------------|---------------------------------------|
| Wild-type root      | 9.37 ± 3.42                           |
| Wild-type shoot     | 2.00 ± 1.28                           |
| Nitrate reductase null root | 21.35 ± 0.31                     |
| Nitrate reductase null shoot  | 6.97 ± 0.27                           |
transporter gene expression or a lack of translation in fungi. It has been shown previously that nitrate reductase activity was not required for the transcription of \textit{nrtA} (the \textit{A. nidulans} nitrate transporter coding gene), and indeed, the \textit{nrtA} transcript abundance in nitrate reductase loss-of-function mutants was elevated compared with wild-type levels because of the continued presence of the inducer (19, 27). This conclusion was supported in this study of the nitrate non-assimilating yeast, \textit{P. pastoris}, in which regulation by nitrate-mediated induction was replaced by alcohol. The resulting methanol-induced \textit{nrtA} transcript abundance was demonstrated in strains lacking nitrate reductase. Moreover, it would appear from Western blot experiments that the loss of nitrate transport activity in fungal nitrate reductase minus strains is not because of a failure to translate the transporter protein, as NrtA protein expression was observed. We concluded that the lack of nitrate reductase activity appears to be the reason for the lack of nitrate uptake, rather than altered regulation, and nitrate reductase from the same species is not required to permit nitrate transport. Nitrate transport in yeast could be elicited in the presence of a plant nitrate reductase, and this indicates that there is no absolute requirement for fungal nitrate reductase \textit{per se} to be present for transport activity.

These results raise the obvious question what is the basis for the fundamental difference in nitrate reductase requirements for nitrate influx between these two eukaryotic groups? The first possibility is that there is a physical/mechanistic interaction or cross-talk between nitrate reductase and the transporter protein in fungal but not in plant cells. In this regard, nitrate, as well as being a nutrient, plays a role in morphogenic processes acting as a signal for instance in lateral root formation and stomatal closure in plants (28, 29). For these additional functions, plants may have evolved a mechanism(s) to bypass the need for nitrate reductase. This proposition, however, may appear less probable, because plant nitrate reductase can substitute adequately for the fungal enzyme in yeast.

A second possibility is that nitrate reductase is required to maintain a gradient for nitrate entry into cells. Therefore if nitrate is not removed by nitrate reductase to nitrite (i.e. biochemical sequestration of nitrate), nitrate influx ceases. If this is indeed the situation, why can plants apparently concentrate nitrate against a large gradient in the absence of nitrate reductase activity, whereas fungi cannot? Estimates suggest that a lower limit for plant root nitrate concentration may be \(~5\) mm (30). The answer may lie in the storage of nitrate. Although the lower eukaryotes are incapable of nitrate storage, the higher eukaryote may reserve large quantities of nitrate for example within the vacuole of root cells. Indeed, given free access to nitrate plant root cells may accumulate up to \(80 \mu\text{mol/g of fresh weight, equivalent to ~80}\) mm nitrate (4). In addition, many higher plants conduct most of the absorbed nitrate to the shoot for assimilation. The maintenance of a gradient due to nitrate removal is attractive, because the plant possesses no less than seven high affinity nitrate transporter paralogues (compared with just one gene observed in the genome of most fungi sequenced so far), and the function of several of these plant genes could be involved in storage processes. Therefore, in the absence of nitrate reductase, the nitrate concentration at the plasma membrane of plant cells may be maintained at a relatively low concentration by sequestration into the vacuole or by transfer to the shoots. In other words, such biophysical removal of nitrate in plant cells would maintain an appropriate gradient necessary to sustain nitrate transport, even in the absence of nitrate reductase activity. In fungal cells, the nitrate concentration localized at the membrane could rise rapidly in the absence of nitrate reductase activity to a concentration which would preclude further transport. Notwithstanding, it is something puzzling that no significant fungal \(^{13}\text{NO}_3\) accumulation is observed in the absence of nitrate reductase activity, because some accumulation into the cell might be expected, at least until the free energy gradient of nitrate was dissipated and the pump was stalled. Further experimentation should shed more light into these processes.

Acknowledgments—We thank Dr. G. Marzluf, Ohio State University, for kindly providing Rip mutants in the nitrate assimilation pathway of \textit{N. crassa}. We thank Drs. W. Campbell, Michigan Technological University, and D. Lowe, John Innes Centre, for sending us recombinant strains of \textit{P. pastoris}. We thank the University of British Columbia Tri University Meson Facility for provision of \(^{13}\text{N}\).

Note Added in Proof—Details of the Rip-generated nitrate assimilation defective mutants in \textit{N. crassa} have been published recently, Gao-Rubinelli, F., and Marzluf, G. A. (2004) Biochem. Genet. \textbf{42}, 21–34.

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